Unraveling Piglet Gut Microbiota Dynamics in Response to Feed Additives

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Abstract

The gastrointestinal (GI) tract of pigs is colonized by a dense and metabolically active microbiota, comprising mainly bacteria, that have not only a commensal but also a symbiotic (beneficial for both) relationship with the host. These bacteria are important for host health, as they provide the pig with essential products, form a key barrier against pathogens and are involved in development and homeostasis of morphology, digestion, and host immunity. The fact that the GI tract microbiota plays such an important role in pig health, allows to influence host health by modifying microbiota composition and activity. Supplementing pigs with diet ingredients that can have an effect on the GI tract microbiota is a common strategy to affect pig's health, especially around the time of weaning. During weaning, piglets suffer a dramatic imbalance in composition and activity of the GI tract microbial community that, during the last decades, has been treated with antibiotics to control harmful micro-organisms. This practice has raised concerns in the last years due to the cross-resistance of pathogens to antibiotics used in humans, causing a ban on their use in the European Union from January 2006 onwards. This triggered an increased interest in finding alternatives for in-feed antibiotics, such as prebiotics, probiotics, organic acids and other plant-derived bioactive compounds. This thesis presents the results of various *in vitro* and *in vivo* studies addressing the response of piglet GI tract microbiota to the supplementation with different feed additives. To this end, we developed the Pig Gastrointestinal Tract Chip (PITChip), a semi-quantitative and high-throughput diagnostic tool that allows us to gain novel insight in composition and dynamics of porcine GI tract microbiota at high taxonomic and spatiotemporal resolution. Furthermore, a specific and sensitive real-time PCR assay was developed for quantification of the pathogen *Streptococcus suis*. This revealed that abundance of *S. suis* in the piglet intestine increased after weaning, whereas that of *Lactobacillus* populations decreased. The application of the PITChip, together with other molecular tools, allowed us to follow microbial changes in time and in response to a range of different dietary supplements. Addition of sodium butyrate (SB) and different blends of organic acids had a significant effect on the microbial profiles in different sections of the GI tract, reinforcing previous reports on the antimicrobial effect of organic acids on piglet GI tract microbiota. SB administration most strongly affected populations within the *Clostridium* cluster XIVa and the *Bacteroidetes*. Results from different studies proved as well the antimicrobial effect of essential oils, and more specifically oregano oil (carvacrol), both *in vivo* and *in vitro*. The essential oil lowered overall microbiota diversity and relative abundance of members of *Clostridium* clusters IV and XIVa, and several *Streptococcus*- and *Bacteroides*-like species. In conclusion, the development and application of complementary molecular tools described in this thesis provided important information towards sound design of sustainable dietary strategies aiming at the replacement of in-feed antibiotics.

Keywords: GI tract, microbiota, pig, PITChip, weaning

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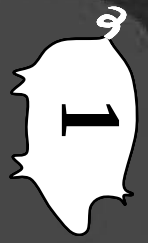
Chapter



General Introduction and Outline

Introduction

The widespread use of in-feed antibiotics in pig production to control harmful microorganisms has raised concerns in the last years about the cross-resistance of pathogens to antibiotics in humans [1, 2], causing a ban on their use in the European Union from January 2006. Because antibiotics are considered responsible for reducing post-weaning disorders such as diarrhea, it had been anticipated that their ban would have a negative effect on pigs and therefore important losses in the pig industry [3]. Hence an increased interest was raised in alternatives for in-feed antibiotics, including prebiotics, probiotics, organic acids and plant extracts to enhance pig health and performance. In order to identify the best alternative strategies it is necessary to have an improved understanding of the complex relationship between the indigenous intestinal microbiota and the host immune system. The work described in this thesis and outlined at the end of this chapter aimed at improving our knowledge on the effect of several natural alternatives on piglet gastrointestinal (GI) tract microbiota. For that purpose, we used molecular techniques targeting the 16S ribosomal RNA gene (rRNA) that allowed the comprehensive detection of microbial species most of which are still recalcitrant to cultivation [4, 5].

Gut microbiota and weaning

From birth to death, piglets are colonized by a vast, complex, and dynamic consortium of microorganisms [6]. During birth, the sterile GI tract of piglets experiences a first fast colonization by a very diverse microbial community originating from the sow and the environment [7, 8]. In this colonization step the main bacteria are aerobes and facultative anaerobes that make up to 80% of the total bacteria. After 12h from birth, total bacterial numbers in the distal colon are already in the order of 10^9 CFU/g of colonic content [9]. Due to this high number of aerobic bacteria, consuming oxygen and reducing the redox potential, 48h after birth 90% of the total bacteria are strict anaerobes, including mainly lactobacilli and streptococci [9]. After the initial colonization of the piglet GI tract, the microbiota remains quite stable during suckling, although qualitative changes may occur [10, 11]. Some of the dominant bacterial genera found in this period are *Clostridium*, *Bacteroides* and *Bifidobacterium* [9]. The introduction of solid food causes important qualitative and quantitative alterations in the microbiota, and can increase the vulnerability of young pigs to the presence of potentially pathogenic microorganisms [12]. This problem is aggravated in a production environment where piglets experience a very early and sudden weaning. Diet changes together with separation from the mother and other social alterations (i.e. new litter mates) cause a lot of stress to the piglets resulting in important changes in the gut microbiota [12] and growth stasis [13, 14]. More specifically, weaning-associated starvation causes a reduction in fermentable substrates in the GI tract provoking shifts in microbial communities that become unstable and less diverse [15]. One of the important changes is an increase in strict anaerobes such as *Bacteroides*, that will remain as one of the most predominant groups, and a decrease of facultative organisms [16, 17]. Another important change is a decrease in lactobacilli species at the same time as enterobacteria increase in abundance. All these alterations result in an increased susceptibility to pathogenic bacteria [18].

Two to three weeks after weaning the gut microbiota develops and becomes stable and diverse [6, 11, 12, 19]. This resident microbiota confers many benefits to the intestinal physiology of the host and can, therefore, be seen as an example of a symbiotic relationship [20, 21]. Some of these benefits include the metabolism of nutrients and organic substrates, and the contribution to the phenomenon of colonization resistance which is the ability of this community to resist invasion by exogenous microorganisms [22, 23].

It is well established that bacterial numbers differ greatly between the small and the large intestine due to the different conditions in these two sections of the GI tract. With respect to cultivable bacteria, the lower gastrointestinal tract, caecum and colon, harbors around 10^{11}-10^{12} CFU/g digesta [7], mainly gram-positive anaerobes: streptococci, lactobacilli, eubacteria, clostridia and peptostreptococci and only 10% of gram-negative bacteria, including mostly *Bacteroides* and *Prevotella* [24]. In contrast, the upper GI tract, stomach and duodenum, and jejunum and ileum, harbors only up to around 10^{0}-10^{4} and 10^{7}-10^{9} CFU/g fresh matter, respectively [25], mainly lactobacilli and streptococci [26], due to the acidic conditions that characterize this part of the gut, the rapid flow of digesta and the concomitant rate of microbial washout.

Even though the composition of the GI tract microbiota remains quite stable during life, it is now well established that changes in the diet can cause changes in microbial activity and composition [27-29]. For example it was shown that feeding benzoic acid to weaning pigs improved piglets' performance and was associated with a greater ileal microbiota biodiversity [30]. Hence, manipulation of the diet is a viable strategy towards the identification of alternatives to the use of in-feed antibiotics.

Plant extracts and other natural substances (PENS) as alternatives

Plants have been recognized as rich and diverse sources of a broad range of different bioactive ingredients of potential use in animal production, including prebiotic dietary fibers and oligosaccharides, as well as a broad range of additional bioactive compounds that can be extracted from plant biomass [31] (Table 1).

Dietary fibers

Dietary fiber refers to indigestible carbohydrates of plant origin. This category of plant-derived dietary ingredients mainly constitutes non-starch polysaccharides (such as arabinoxylans, cellulose, waxes, chitins, pectins and beta-glucans), resistant starch (RS) and lignin [66]. Pigs do not have endogenous enzymes that can degrade non-starch polysaccharides (NSP). As a result, NSP and RS can reach the lower part of the GI tract being available for fermentation by bacteria [67]. Short chain fatty acids (SCFA) are the principal end products of dietary fibers fermentation and have several physiological and clinical properties, such as enhancement of water and electrolyte absorption, modulation of intestinal mucosal growth and relief of symptoms in patients with ulcerative colitis. Furthermore, butyrate is the major energy source for colonocytes [68, 69]. However, microbial fermentation differs among different types of dietary fibers, depending on solubility, degree of lignification, technological processing of the

feed (ingredients) and inclusion levels in the diet. For example, fibers fermented at a slow rate have beneficial effects such as increase in stool output, dilution of colonic contents and production of distal colonic SCFA [70].

Table 1. Some of the most frequently studied PENS to use as alternatives to in-feed antibiotics.

		Effects	References
Dietary fibres	Laminaran	Anti-inflammatory, modulation of microbial activity	[32, 33]
	Mannuronic block	Anti-inflammatory, modulation of microbial activity	[34-36]
	Oligo-laminaran	Anti-inflammatory, modulation of microbial activity	[33]
	Oligo-xylan	Anti-inflammatory, modulation of microbial activity	[37]
	Xylan	Anti-inflammatory, modulation of microbial activity	[33]
Prebiotics	Carobpulp	Antibacterial	[38, 39]
	Fructo-oligosaccharide (FOS)	Modulation of the microbiota	[40-42]
	Galacto-oligosaccharide (GOS)	Modulation of the microbiota	[40-42]
	Guargum	Modulation of microbial activity	[38, 43]
	Inulin	Promotes bifidobacteria and lactobacilli	[42, 44, 45]
	Lactulose	Bifidogenic	[42]
	Sanguinarine	Antibacterial, antioxidant, anti-inflammatory	[46]
	Transgalacto-oligosaccharides (TOS)	Bifidogenic	[42, 47]
Organic acids	Acetic acid	Antimicrobial, acidifier	[48, 49]
	Butyric acid	Antimicrobial, anti-inflammatory, acidifier	[48-50]
	Citric acid	Antimicrobial, acidifier	[49]
	Formic acid	Antimicrobial, acidifier	[49]
	Lactic acid	Antimicrobial, acidifier	[48, 49]
	Propionic acid	Antimicrobial, acidifier	[48, 49]
Plant Extracts	Allicin	Antibacterial	[51, 52]
	Capsaicin	Antimicrobial	[53]
	Carvacrol	Antimicrobial	[54]
	Cinnamaldehyde	Antimicrobial, fungicide	[53, 54]
	Daidzein	Antimicrobial, antioxidant, phytoestrogenic	[55, 56]
	Essentail oils	Antimicrobial	[57-59]
	Eugenol	Antimicrobial	[53, 54]
	Genistein	Antioxidant, phytoestrogenic	[55, 60]
	Naringin	Antioxidant, anticancer	[61, 62]
	Saponin	Antibacterial, fungicide	[63, 64]
	Thymol	Antimicrobial	[54, 65]

The potential of these PENS, as well as of organic acids, as alternatives to in feed-antibiotics is outlined in the present chapter, providing an overview of the state of the art of their application in animal nutrition.

Prebiotics

Prebiotics have been defined as “selectively fermented ingredient that allows specific changes, both in the composition and/or activity in the gastrointestinal microbiota that confers benefits upon host well-being and health” [71]. Thus the function of prebiotics is to provide a substrate to the gut beneficial microbiota.

Not all dietary carbohydrates are prebiotics. In order to classify a food ingredient as a prebiotic it has to fit the following criteria [42]: a prebiotic has to be 1) resistant to gastric acidity, to hydrolysis by mammalian enzymes, and to gastrointestinal absorption; 2) fermented by intestinal microbiota; and 3) selectively stimulating the growth and/or activity of those intestinal bacteria that contribute to health and well-being. The fact that a prebiotic has to be resistant to host digestion does not imply that the carbohydrate has to be completely indigestible but at least a significant amount should be available in the intestine to be used as a fermentation substrate by the microbiota.

Usually short-chain prebiotics (e.g. oligofructose, containing 2-8 links per saccharide molecule) are usually fermented faster and already in the ascending colon, whereas long-chain prebiotics (e.g. inulin, containing 9-64 links per saccharide molecule) tend to be fermented slower, and in the descending colon [6, 72].

Early studies reported that in human diets prebiotics have mainly a bifidogenic effect, shifting microbial metabolism from being proteolytic to being saccharolytic, concomitant with reduced production of toxic products of protein fermentation [73]. However, later studies indicated that prebiotics stimulate, as well, strains of *Lactobacillus sp*., *Bacteroides sp*., *Clostridium sp*., *Eubacterium sp*. and *Escherichia coli* [42]. In studies focusing on piglets, oligosaccharides have been shown to be a potential alternative to in-feed antibiotics [74] due to their effect on the gut microbiota by providing a substrate for beneficial microorganisms. Therefore, some prebiotic feed additives could be applied to control post-weaning diarrhea. In the last years, several studies investigated the effect of prebiotics on the GI tract microbiota in piglets, especially focusing on the period around weaning. Konstantinov and co-workers [75, 76] showed that the composition of the microbiota in the gut of weaning piglets was affected by the dietary addition of a mix of sugarbeet pulp, inulin, lactulose and wheat starch. Moreover, they showed evidence of fermentable carbohydrates being able to enhance colonic microbial stability and diversity at the same time as enhancing the growth of *Lactobacillus sobrius* [76]. *Lactobacillus sobrius* has recently been reported to belong to the *Lactobacillus amylovorus* group, that has been found to specifically colonize the intestinal tract of pigs being abundant in the ileum, where it exerts probiotic activity, preventing epithelial damage by enteropathogenic *E. coli* as well as increasing daily weight gain in weaning piglets [77-81].

Organic acids

During many years organic acids supplementation in piglet feed has been a common practice for enhancing animal performance and to prevent digestive problems, especially in the post-weaning period, when intestinal problems are more frequent. Several studies indicated that organic acids and their salts are among the best alternatives to the use of growth promoting antibiotics in pig production [49, 82, 83]. Moreover, it is known that SCFA and their salts, independently of their origin (bacterial fermentation product or supplemented with the diet), have important antibacterial effects [26, 84]. Some of the most used organic acidifiers include formic, acetic, propionic, butyric, lactic, sorbic, fumaric, malic, and citric acid, Ca-formate, Ca-lactate, Ca-propionate, K-diformate, Ca-butyrate, Mg-citrate and Na-lactate [85].

The addition of organic acids generally lowers the pH and buffering capacity of the diet, increases gastrointestinal acidity, proteolysis and nutrient digestibility, promotes beneficial

bacteria that decrease pathogens and modulates several physiological processes after absorption. These effects can confer a series of benefits to the host; improved health and resistance to disease, faster growth, better diet utilization and better carcass quality [85].

The mode of action of organic acids is not yet fully understood, but a consensus has been reached on several hypotheses. Their antimicrobial effect in the diet itself is due to a decreased pH, acting as a preservative and inhibiting the growth of many bacterial species, yeasts and moulds on the feed before its consumption [86, 87]. Their antimicrobial effect in the pig GI tract is most probably explained by the ability of acids to change from the undissociated to the dissociated form depending on the environmental pH. Undissociated forms are lipophilic and can diffuse across cell membranes of pathogens [82], destroying their cytoplasm or inhibiting growth through the inactivation of bacterial decarboxylases and catalases. The antimicrobial activity is mainly against bacteria belonging to the *Enterobacteriaceae* family and against yeasts [88].

Plant extracts

Natural plant or herbal extracts are another potential viable alternative to traditional antimicrobials [89]. Several *in vitro* studies have proved antimicrobial activity of different plant extracts: the addition of daidzein to the diet caused an increase in the relative abundance of lactobacilli in *in vitro* fermentation assays [90]; a mixture of carvacrol, cinnamaldehyde and capsicum oleoresin, given to piglets at increasing doses, linearly increased intestinal lactobacilli as well as lactobacilli:enterobacteria [91]; dietary addition of essential oils and antibiotics into diets for weanling pigs improved growth performance, IgG concentration and nitrogen digestibility and decreased noxious gas concentration [92].

Molecular techniques for the study of intestinal microbiota

During many years, the intestinal microbiota has been studied using traditional methods of culture, microscopy and identification based on phenotypic characterization, but this is laborious, time consuming and it filters our view of the biodiversity of microbial life as only 10-40% of all bacteria present in the mammalian intestine are cultivable [93, 94]. As a result, molecular cultivation-independent techniques are now being used increasingly as routine tools for the analysis of the complex intestinal ecosystem, contributing to a better understanding of the interaction between host and microbes in the GI tract [94].

The application of molecular techniques, mainly targeting 16S rRNA and the corresponding gene, allows researchers to bypass cultivation. These ribosomal sequences are present in all cellular life forms and are highly conserved (due to the fact that their essential function in all organisms translates into a very slow genetic evolution), but also contain variable regions, the latter of which provide the means to discriminate among bacterial phylotypes [95]. These characteristics make 16S rRNA sequences very useful in the identification of bacterial species by aligning the sequences with those stored in publicly available databases, and analysis of bacterial communities. In the following, molecular approaches used in this PhD thesis are being described in more detail.

Denaturing Gradient Gel Electrophoresis

Denaturing Gradient Gel Electrophoresis (DGGE) is a powerful technique to study community behavior in response to environmental changes and to compare different communities [96]. 16S rRNA gene fragments of no more than 500 bp are amplified by polymerase chain reaction (PCR) from environmental DNA samples and are subsequently separated in a polyacrylamide gel where a gradient of denaturing chemicals has been applied. Because of the variation in the 16S rRNA gene sequences of different bacterial species, chemical stability is also different, causing double-stranded 16S rRNA gene fragments to migrate through the gel until the point in the gradient where partial denaturation occurs (melting point of the sequence). At this point the migration almost stops allowing the separation of sequences of the same size but different base-pair composition [97]. Complete denaturation of the fragments is prevented by addition of a GC clamp that is introduced with one of the primers during the amplification step.

DNA bands in DGGE are visualized using ethidium bromide, SYBR Green or silver staining [98]. Resulting banding patterns represent the diversity of the rRNA gene amplicons present in a given sample. Band intensities can be used as a semi quantitative measure for the relative abundance of this sequence in the community. Furthermore, DNA fragments can be cut from the gel and re-amplified for sequencing and thus, identification.

Another possibility is the analysis of 16S rRNA fragments reverse transcribed and PCR-amplified from RNA samples, providing information on the 16S rRNA sequences of those bacteria that are most metabolically active in the ecosystem, based on the assumption that for the majority of bacteria, activity is related to the cellular concentration of ribosomes.

Because DGGE bands patterns can be quite complex, advanced statistical analysis is often used to discern their diversity as well as similarities between patterns obtained from different samples, using software package such as Bionumerics (Applied Maths, St-Martens-Latem, Belgium) for the analysis. Denaturing Gradient Gel Electrophoresis has been applied successfully to study community changes in many different environments, including the GI tract of pigs [65, 76, 77, 99-101].

Real time PCR

Real time PCR or quantitative PCR (qPCR) is a quantitative method where the data is collected after each cycle of the reaction (real time) instead of at the end of the procedure as for conventional end-point PCR. The mode of action of the qPCR is based on the detection and quantification of a fluorescent reporter [102]. In this thesis, SYBR Green dye is used as a fluorescent DNA intercalating dye, which has hardly any fluorescence in solution but emits a strong signal when it is bound to double-stranded DNA [103].

Quantification of amplicons takes place during the exponential phase of the reaction, and the first significant increase in PCR product, defined as the threshold cycle (C_T), corresponds to the initial amount of the target sequence in the sample. To be able to quantify the amount of DNA it is necessary to interpolate in a standard curve that is created with dilutions of a known amount of the target gene. To this end, it has to be kept in mind that bacterial species differ in genome sizes and numbers of 16S rRNA gene copies per genome, making extrapolation of the data to cell numbers inherently dependent on this bias [104, 105].

There are several reports of application of qPCR for the quantification of GI tract microbiota in pigs [106-108]. Table 2 provides a list of the recent development of qPCR assays for the quantification of different microbial groups in the pig.

Table 2. Quantitative PCR assays developed for microbial groups in the pig.

Target microbe	Target regions	Reference
Streptococcus suis	V2-V3 region of the 16S rRNA gene	[109]
Brachyspira hyodysenteriae *Brachyspira pilosicoli* *Brachyspira intermedia*	198 bp of the NADH oxidase gene	[110]
Desulfobulbus sp. *Desulfovibrio sp.*	*dsrA* gene	[111]
Lactobacillus sobrius	V2-V3 region of the 16S rRNA gene	[112]
Lactobacillus sobrius 001^{T}	Type-strain-specific genomic fragment	[112]
Eperythrozoon suis	V1-V9 region of the 16S rRNA gene	[113]
Bacteroidetes *Firmicutes*	16S rRNA gene	[114]
Mycoplasma suis	*Msg*1 gene	[115]
Campylobacter jejuni *Campylobacter coli* *Campylobacter lari* *Campylobacter upsaliensis*	*glyA* gene	[116]
Mycoplasma hyopneumoniae	Repeated element MHYP1-03-950	[117]

Phylogenetic Microarrays

In order to make statistically relevant links between the bacterial composition and GI tract disorders high-throughput techniques that allow for the comprehensive and fast analysis of microbiota profiles at sufficient spatiotemporal resolution, are necessary. Phylogenetic microarrays are currently among the most used high-throughput tools and are mainly based on the small subunit ribosomal RNA (SSU rRNA) gene [118].

Phylogenetic microarrays are glass surfaces spotted with thousands of covalently linked DNA or RNA probes. These microarrays have a very successful application in diversity analysis [119, 120]. In the last years, specific phylogenetic microarrays have been designed focusing on

Table 3. List of phylogenetic microarrays based on the SSU rRNA targeting different specific ecosystems (mod. from Zoetendal et al. [94].

Target microbes	Number of target organisms	SSU rRNA database	Number of probes	Reference
Human commensal isolates	40 bacterial species	GenBank	120	[121]
Sulfate-reducing bacteria	All recognized lineages	ARB[1]	132	[125]
All microbes	842 prokaryotic subfamilies	Greengenes, 15 March 2002 version[2]	297851	[120]
Human oral cavity microbes	NA	NA	NA	[123]
Known GI tract commensals and medically relevant microbes	1590 bacterial and 39 archaeal species	prokMSA SSU rDNA sequence database, 2004 version[3]	10500	[122]
Human GI tract microbiota	1140 bacterial species	Human GI tract microbiota database, 2006 version[4]	4809	[124]
Mouse GI tract microbiota	1885 bacterial species	Mouse GI tract microbiota database 2008 version	3580	Derrien et al. in preparation
Pig GI tract microbiota	627 bacterial species	Pig GI tract microbiota database 2008 version	3762	This thesis

[1]Described by Ludwig et al. [126]
[2]Described by DeSantis et al. [127]
[3]Described by DeSantis et al. [128]
[4]Described by Rajilić-Stojanivić et al. [129]
NA, not available

microbial communities of specific ecosystems, including the human oral cavity as well as the human intestinal tract [121-124] (Table 3). In this thesis, the development of a phylogenetic microarray specific for pig gut microbiota is being described.

It should be noted that while phylogenetic microarrays represent powerful diagnostic tools [120], the fact that these analyses are dependent on the isolation of nucleic acids and PCR amplification of SSU rRNA genes makes them vulnerable to the same technical biases as other approaches described before, such as sequence artifacts and unequal amplification.

454 pyrosequencing

In the last decades, molecular techniques like PCR, qPCR, DGGE and cloning and sequencing of the 16S rRNA gene have been very successful to study microbial communities. However, conventional clone library analysis by Sanger sequencing is costly and time-consuming, preventing its application for the analysis of a larger number of samples in order to provide information of microbial composition at sufficient spatiotemporal resolution. Recently, microarrays appeared as an addition to the already existent molecular techniques, allowing us to fill this apparent gap as described above. Still, microarrays target only known sequences, their design being dependent on the publicly available sequence space. The newest approach to improve our knowledge on complex microbial studies is the next-generation sequencing technologies that are able to sequence genomes faster and cheaper than with Sanger sequencing. At the moment there are several high-throughput sequencing technologies available, such as the 454 GS20 pyrosequencing platform, GS FLX platform, Solexa GA or SOLID platform. The Roche GS20 and FLX systems developed by 454 Life Sciences (454 pyrosequencing) represent emerging sequencing techniques, able to generate around one million DNA sequence reads of up to 450 base pairs when using the latest Titanium chemistry using a parallel sequencing-by-synthesis approach. There are already several studies describing the use of 454 pyrosequencing in complex communities and benchmarking the results with previous techniques (this thesis) [130-132]. It should be noted, however, that also pyrosequencing-based analysis of microbial diversity based on rRNA genes is subject to generally acknowledged biases introduced by PCR, such as the generation of chimera [133]. Furthermore, pyrosequencing is specifically sensitive to homonucleotide stretches.

Statistical analyses in microbial ecology

Advances in molecular techniques are increasing the amount of biological information and environmental variables that have to be analyzed to get a clear picture of the behavior of a given target ecosystem. When generating microbial profiles with techniques such as DGGE or microarrays, multivariate statistical analyses are needed to be able to explain the effects of the environmental variables on the distribution of species and the relation among samples taken along spatial and temporal gradients. DGGE statistical analyses, for example, usually include principal component analysis, multidimensional scaling and hierarchical cluster analysis [134-138] and measurement of community diversity using different indexes like Shannon's H' and Simpson's reciprocal [139, 140].

Multivariate statistical analyses are techniques with a great potential for microbial ecology because they allow us to test different ecological hypotheses including several environmental variables that otherwise would be out of the picture. Some of these tools are cluster analysis, principal component analysis (PCA), canonical correspondence analysis (CCA) and redundancy analysis (RDA) (Table 4).

Table 4. Multivariate statistical methods more common in the analysis of microbial profiles [134].

Exploratory analysis		
Cluster	**PCA**	**MDS**
(hierarchical clustering)	(principal component analysis)	(multidimensional scaling)
Groups objects based on their similarities or differences	Calculates new variables that are a linear combinations of the original ones and explain their variation	Calculates new variables that best represent the pairwise distances between species

Hypothesis-driven analysis				
CCA	**RDA**	**MANOVA**	**Mantel**	**ANOSIM**
(canonical correspondence analysis)	(redundancy analysis)	(multivariate analysis of variance)	(Mantel test)	(analysis of similarities)
Determines the best model to model species response to environmental variables	Determines which environmental variables are the most important explaining the variation in species	The observed variance is divided into components due to the multiple environmental variables	Calculates the correlation between 2 matrices	Determines significant differences between two or more groups

Outline of this Thesis

The work presented in this thesis, embedded in a larger EU-FP6 funded project, Feed for Pig Health, aimed to provide an improved knowledge of the diversity and population dynamics of the pig GI tract microbiota, in response to a broad range of different in-feed additives explored as an alternative to antibiotics.

In **Chapter 1**, an overview is provided on the current state of the art in molecular approaches towards microbial community analysis, specifically focusing on the mammalian GI tract. Furthermore, dietary additives of plant origin, including those investigated in more detail in the framework of this thesis, are described, including indigestible fibers, prebiotic oligosaccharides, plant extracts as well as organic acidifiers.

In **Chapter 2** the effect of weaning on *Lactobacillus* spp. and *Streptococcus suis* is presented, together with the development of a specific and sensitive real-time PCR assay for the quantification of *S. suis*. This assay showed *S. suis* to predominate in the stomach, jejunum and ileum samples of weaned piglets whereas it was not detected before weaning. In the following chapters, the effect of different additives used to overcome problems such as the one presented in Chapter 2, are described. In addition, **Chapter 3** describes the development of a diagnostic microarray specific for pig intestinal microbiota, the PITChip (Pig Intestinal Tract Chip). In order to benchmark the performance of this new phylogenetic microarray, the microbiota composition generated by the PITChip was compared to that obtained by pyrosequencing of PCR-amplified 16S rRNA gene fragments as well as to the quantification of specific groups by 16S rRNA gene-targeted quantitative PCR. Furthermore, this chapter describes the application of the PITChip as a novel high-throughput tool for the comprehensive analysis of pig intestinal microbial diversity in samples obtained during an animal experiment focusing on the effect of different blends of organic acids.

In order to allow for the efficient and simultaneous screening of a large number of potential dietary additives (PENS: plant extracts and natural substances) that could have a positive effect on piglets' intestinal microbiota, a combination of *in vitro* fermentation and molecular fingerprinting techniques was applied, as described in **Chapter 4.** The additives studied here belonged to the categories of dietary fibers, organic acids, plant extracts and prebiotics. Analysis of the data revealed a possible lack of effect due to the fact that additives were tested at inclusion levels that are frequently used in animal nutrition practice, and which are relatively low in comparison to those normally used in *in vitro* experiments.

The next chapters summarize the use of some of these additives in different animal experiments, designed to gain insight into the effect of these PENS *in vivo*. Essential oils are plant extracts, often with an antimicrobial effect. Carvacrol is the active compound in oregano oil and is such an essential oil with antimicrobial activity. As described in **Chapter 5**, the administration of carvacrol to piglets at weaning resulted in more stable microbial profiles in time. In this study, a T-cannula was fitted into the piglets to be able to follow the same animal in time, reducing the impact of inter-individual variation often impairing significance of observed effects.

In **Chapter 6** the effect of sodium butyrate treatment from birth until weaning on the gut microbiota of piglets is evaluated. The application of molecular techniques such as denaturing

gradient gel electrophoresis, real time PCR and PITChip analysis revealed that the microbiota was affected predominantly before weaning, whereas the animals' intestinal microbiota only differed in a limited number of microbial groups in the ileum after the butyrate treatment was terminated after weaning.

Chapter 7 compares the effect that two different antimicrobials such as an antibiotic (oxytetracyclin) and a blend of organic acids, had on the performance and microbial dynamics of piglets weaned at around 4 weeks of age. These feeding strategies decreased the incidence of diarrhea in the animals and caused a change in GI tract microbiota composition, without having a strong effect in any particular microbial group.

Finally, the general discussion (**Chapter 8**) summarizes and discusses the results of the work described in this thesis, relating them with the latest findings in the field, with an emphasis on the different directions towards which studies of gut microbiota composition and functionality are evolving in response to the need to find effective and sustainable alternatives to antibiotic growth promoters.

References

1. **Cromwell, G. L.** (2002) Why and how antibiotics are used in swine production. *Anim. Biotechnol.* 13: 7-27.
2. **Hillman, K.** (2001) Bacteriological aspects of the use of antibiotics and their alternatives in the feed of non-ruminant animals, in *Recent Advances in Animal Nutrition*. Garnsworthy, P. C. and Wiseman, J. (ed.). Notthingam University Press, Notthingam.
3. **Casewell, M., Friis, C., Marco, E., McMullin, P., and Phillips, I.** (2003) The European ban on growth-promoting antibiotics and emerging consequences for human and animal health. *J. Antimicrob. Chemother.* 52: 159-61.
4. **Vaughan, E. E., Schut, F., Heilig, H. G., Zoetendal, E. G., de Vos, W. M., and Akkermans, A. D.** (2000) A molecular view of the intestinal ecosystem. *Curr. Issues Intest. Microbiol.* 1(1): 1-12.
5. **Tannock, G. W.** (2001) Molecular assessment of intestinal microflora. *Am. J. Clin. Nutr.* 73: 410S-414.
6. **Bauer, E., Williams, B. A., Smidt, H., Mosenthin, R., and Verstegen, M. W. A.** (2006) Influence of dietary components on development of the microbiota in single-stomached species. *Nutr. Res. Rev.* 19: 63-78.
7. **Ewing, W. and Cole, D.** (1994) The Living Gut. An Introduction to Micro-organisms in Nutrition. p. 45-65 Context Publication, Ireland, UK.
8. **Katouli, M., Lund, A., Wallgren, P., Kuhn, I., Soderlind, O., and Mollby, R.** (1997) Metabolic fingerprinting and fermentative capacity of the intestinal flora of pigs during pre- and post-weaning periods. *J. Appl. Microbiol.* 83: 147-54.
9. **Swords, W. E., Wu, C. C., Champlin, F. R., and Buddington, R. K.** (1993) Postnatal changes in selected bacterial groups of the pig colonic microflora. *Biol. Neonate*. 63(3): 191-200.
10. **Drasar, B. S.** (1989) The bacterial flora of the stomach and small intestine. *Gastroenterol. Clin. Biol.* 13: 18B-20B.
11. **Inoue, R., Tsukahara, T., Nakanishi, N., and Ushida, K.** (2005) Development of the intestinal microbiota in the piglet. *J. Gen. Appl. Microbiol.* 51: 257-265.
12. **Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8: 1191-1199.
13. **Lallès, J. P., Boudry, G., Favier, C., Le Floc'h, N., Lurona, I., Montagne, L., Oswald, I. P., Pie, S., Piel, C., and Seve, B.** (2004) Gut function and dysfunction in young pigs: physiology. *Anim. Res.* 53: 301-316.
14. **Montagne, L., Boudry, G., Favier, C., Le Huerou-Luron, I., Lalles, J. P., and Seve, B.** (2007) Main intestinal markers associated with the changes in gut architecture and function in piglets after weaning. *Br. J. Nutr.* 97: 45-57.
15. **Wallgren, P. and Melin, L.** (2001) Weaning systems in relation to disease, in *The Weaner Pig*. Varley, M. A. and Wiseman, J. (ed.). CABI Publishing, UK.
16. **Savage, D. C.** (1977) Microbial ecology of the gastrointestinal tract. *Annu. Rev. Microbiol.* 31: 107-133.
17. **Mallet, A. K. and Rowland, I. R.** (1998) Factors affecting the gut microflora. , in *Role of the Gut Flora in Toxicity and Cancer*. Rowland, I. (ed.). p. 347-382. Academic Press, London.
18. **Pluske, J. R., Le Dividich, J., and Verstegen, M. W. A.** (2003) Weaning the Pig: Concepts and Consequences. Wageningen Academic Publishers, Wageningen, The Netherlands.
19. **Zoetendal, E. G., Cheng, B., Koike, S., and Mackie, R. I.** (2004) Molecular microbial ecology of the gastrointestinal tract: from phylogeny to function. *Curr. Issues Intest. Microbiol.* 5: 31-47.
20. **Ewing, W. and Cole, D.** (1994) The Living Gut: An Introduction to Micro-organisms in Nutrition. p. 70. Context Publication, Ireland, UK.
21. **Hooper, L. V. and Gordon, J. I.** (2001) Commensal host-bacterial relationships in the gut. *Science*. 292: 1115-1118.
22. **van der Waaij, D., Berghuis-de Vries, J. M., and Lekkerkerk, L.-v.** (1971) Colonization resistance of

the digestive tract in conventional and antibiotic-treated mice. *J. Hyg. (Lond).* 69: 405-11.

23. **Berg, R. D.** (1996) The indigenous gastrointestinal microflora. *Trends Microbiol.* 4): 430-435.
24. **Moore, W. E. C., Moore, L. V. H., Cato, E. P., Wilkins, T. D., and Kornegay, E. T.** (1987) Effect of high-fiber and high-oil diets on the faecal flora of swine. *Appl. Environ. Microbiol.* 53: 1638-1644.
25. **Jensen, B. B. and Jorgensen, H.** (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl. Environ. Microbiol.* 60: 1897-904.
26. **Jensen, B. B.** (2001) Possible ways of modifying type and amount of products from microbial fermentation in the gut, in *Gut Environment of Pigs*. Piva, A., Bach Knudsen, K. E., and Lindberg, J. E. (ed.). Nottingham University Press, UK.
27. **Houdijk, J. G., Hartemink, R., Verstegen, M. W., and Bosch, M. W.** (2002) Effects of dietary non-digestible oligosaccharides on microbial characteristics of ileal chyme and faeces in weaner pigs. *Arch. Tierernahr.* 56: 297-307.
28. **Bikker, P., Dirkzwager, A., Fledderus, J., Trevisi, P., le Huerou-Luron, I., Lalles, J. P., and Awati, A.** (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J. Anim. Sci.* 84: 3337-45.
29. **Awati, A., Konstantinov, S. R., Williams, B. A., Akkermans, A. D. L., Bosch, M. W., Smidt, H., and Verstegen, M. W. A.** (2005) Effect of substrate adaptation on the microbial fermentation and microbial composition of faecal microbiota of weaning piglets studied in vitro. *J. Sci. Food. Agric.* 85: 1765-1772.
30. **Torrallardona, D., Badiola, I., and Broz, J.** (2007) Effects of benzoic acid on performance and ecology of gastrointestinal microbiota in weanling piglets. *Livest. Sci.* 108: 210-213.
31. **Greathead, H.** (2003) Plants and plant extracts for improving animal productivity. *Proc. Nutr. Soc.* 62: 279-290.
32. **Kuda, T.** (2005) Inhibitory effects of laminaran and low molecular alginate against the putrefactive compounds produced by intestinal microflora in vitro and in rats. *Food Chem.* 91: 745-749.
33. **Michel, C. and Macfarlane, G. T.** (1996) Digestive fates of soluble polysaccharides from marine macroalgae: involvement of the colonic microflora and physiological consequences for the host. *J. Appl. Bacteriol.* 80: 349-69.
34. **Fujihara, M. and Nagumo, T.** (1992) The effect of the content of D-mannuronic acid and L-guluronic acid blocks in alginates on antitumor activity. *Carbohydr. Res.* 224: 343-7.
35. **Roselli, M., Britti, M. S., Le Huerou-Luron, I., Marfaing, H., Zhu, W. Y., and Mengheri, E.** (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. *Toxicol. In Vitro*. 21: 224-9.
36. **Roussel, M., Benvegnu, T., Lognoné, V., Le Deit, H., Soutrel, I., Laurent, I., and Plusquellec, D.** (2005) Synthesis and Physico-Chemical Properties of Novel Biocompatible Alkyl D-Mannopyranosiduronate Surfactants Derived from Alginate. *Eur. J. Org. Chem.* 14: 3085-3094.
37. **Campbell, J. M., Fahey, G. C., Jr., and Wolf, B. W.** (1997) Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *J. Nutr.* 127: 130-6.
38. **Van Nevel, C. J., Decuypere, J. A., Dierick, N. A., and Molly, K.** (2005) Incorporation of galactomannans in the diet of newly weaned piglets: effect on bacteriological and some morphological characteristics of the small intestine. *Arch. Anim. Nutr.* 59: 123-38.
39. **Andrés-Elias, N., Pujols, J., Badiola, I., and Torrallardona, D.** (2007) Effect of nucleotides and carob pulp on gut health and performance of weanling piglets. *Livest. Sci.* 108: 280-283.
40. **Fanaro, S., Boehm, G., Garssen, J., Knol, J., Mosca, F., Stahl, B., and Vigi, V.** (2007) Galacto-oligosaccharides and long-chain fructo-oligosaccharides as prebiotics in infant formulas: A review. *Acta Paediatr.* 94: 22-26.
41. **Mikkelsen, L. L., Jakobsen, M., and Jensen, B. B.** (2003) Effects of dietary oligosaccharides on microbial diversity and fructo-oligosaccharide degrading bacteria in faeces of piglets post-weaning. *Anim. Feed Sci. Tech.* 109: 133-150.
42. **Gibson, G. R., Probert, H. M., Van Loo, J., Rastall, R. A., and Roberfroid, M. B.** (2004) Dietary modulation of the human colonic microbiota: updating the concept of prebiotics. *Nutr. Res. Rev.* 17: 259-

275.

43. **Bauer, E., Williams, B. A., Voigt, C., Mosenthin, R., and Verstegen, M. W. A.** (2001) Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Anim Sci*. 73: 313-322.
44. **Gibson, G. R. and Wang, X.** (1994) Bifidogenic Properties of Different Types of Fructo-Oligosaccharides. *Food Microbiol.* 11: 491-498.
45. **Kaplan, H. and Hutkins, R. W.** (2000) Fermentation of fructooligosaccharides by lactic acid bacteria and bifidobacteria. *Appl. Environ. Microbiol.* 66: 2682-2684.
46. **Kosina, P., Walterova, D., Ulrichova, J., Lichnovsky, V., Stiborova, M., Rydlova, H., Vicar, J., Krecman, V., Brabec, M. J., and Simanek, V.** (2004) Sanguinarine and chelerythrine: assessment of safety on pigs in ninety days feeding experiment. *Food Chem. Toxicol.* 42: 85-91.
47. **Mikkelsen, L. L. and Jensen, B. B.** (2004) Effect of fructo-oligosaccharides and transgalacto-oligosaccharides on microbial populations and microbial activity in the gastrointestinal tract of piglets post-weaning. *Anim. Feed Sci. Tech.* 117: 107-119.
48. **Van Deun, K., Haesebrouck, F., Van Immerseel, F., Ducatelle, R., and Pasmans, F.** (2008) Short-chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. *Avian Pathol.* 37: 379-383.
49. **Partanen, K. H. and Mroz, Z.** (1999) Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12: 117-145.
50. **Mazzoni, M., Le Gall, M., De Filippi, S., Minieri, L., Trevisi, P., Wolinski, J., Lalatta-Costerbosa, G., Lalles, J. P., Guilloteau, P., and Bosi, P.** (2008) Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. *J. Nutr.* 138: 1426-31.
51. **Ankri, S. and Mirelman, D.** (1999) Antimicrobial properties of allicin from garlic. *Microbes Infect.* 1: 125-129.
52. **Fujisawa, H., Watanabe, K., Suma, K., Origuchi, K., Matsufuji, H., Seki, T., and Ariga, T.** (2009) Antibacterial Potential of Garlic-Derived Allicin and Its Cancellation by Sulfhydryl Compounds. *Biosci. Biotech. Bioch.* 73: 1948-1955.
53. **Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., and Ferret, A.** (2007) Essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* 90: 2580-2595.
54. **Michiels, J., Missotten, J., Fremaut, D., De Smet, S., and Dierick, N.** (2007) *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. *Livest. Sci.* 109: 157-160.
55. **Xiao, C. W.** (2008) Health effects of soy protein and isoflavones in humans. *J. Nutr.* 138: 1244S-1249S.
56. **Han, Z. K., Wang, G. J., Yao, W., and Zhu, W. Y.** (2006) Isoflavonic phytoestrogens - new prebiotics for farm animals: A Review on research in China. *Curr. Issues Intest. Microbiol.* 7: 53-60.
57. **Suhr, K. I. and Nielsen, P. V.** (2003) Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol.* 94: 665-74.
58. **Burt, S.** (2004) Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* 94: 223-53.
59. **Janczyk, P., Pieper, R., Urubschurov, V., Wendler, K. R., and Souffrant, W. B.** (2009) Investigations on the Effects of Dietary Essential Oils and Different Husbandry Conditions on the Gut Ecology in Piglets after Weaning. *Int. J. Microbiol.*
60. **Sakai, T. and Kogiso, M.** (2008) Soy isoflavones and immunity. *J. Med. Invest.* 55: 167-173.
61. **Ueng, Y.-F., Chang, Y.-L., Oda, Y., Park, S. S., Liao, J.-F., Lin, M.-F., and Chen, C.-F.** (1999) *In vitro* and *in vivo* effects of Naringin on cytochrome P450-dependent monooxygenase in mouse liver. *Life Sci.* 65: 2591-2602.
62. **Benavente-Garcia, O. and Castillo, J.** (2008) Update on uses and properties of Citrus flavonolds: New findings in anticancer, cardiovascular, and anti-inflammatory activity. *J. Agric. Food Chem.* 56: 6185-6205.
63. **Killeen, G. F., Madigan, C. A., Connolly, C. R., Walsh, G. A., Clark, C., Hynes, M. J., Timmins, B.**

F., James, P., Headon, D. R., and Power, R. F. (1998) Antimicrobial Saponins of *Yucca schidigera* and the Implications of Their *in Vitro* Properties for Their *in Vivo* Impact. *J. Agric. Food Chem.* 46: 3178-3186.

64. **Saleem, M., Nazir, M., Ali, M. S., Hussain, H., Lee, Y. S., Riaz, N., and Jabbar, A.** (2009) Antimicrobial natural products: an update on future antibiotic drug candidates. *Nat. Prod. Rep.* 27: 238-254.
65. **Janczyk, P., Trevisi, P., Souffrant, W. B., and Bosi, P.** (2008) Effect of thymol on microbial diversity in the porcine jejunum. *Int. J. Food Microbiol.* 126: 258-261.
66. **Conway, P. L.** (1994) Function and Regulation of the Gastrointestinal Microbiota of the Pig, in *Vth International Symposium on Digestive Physiology in Pigs, Proceedings, Vols 1 and 2*. Souffrant, W. B. and Hagemeister, H. (ed.). p. 231-240.
67. **Montagne, L., Pluske, J. R., and Hampson, D. J.** (2003) A review of interactions between dietary fibre and the intestinal mucosa, and their consequences on digestive health in young non-ruminant animals. *Anim. Feed Sci. Tech.* 108: 95-117.
68. **Topping, D. L. and Clifton, P. M.** (2001) Short-chain fatty acids and human colonic function: Roles of resistant starch and nonstarch polysaccharides. *Physiol. Rev.* 81: 1031-1064.
69. **Cummings, J. H.** (1995) Short chain fatty acids, in *Human Colonic Bacteria. Role in Nutrition, Physiology and Pathology*. Gibson, G. R. and Macfarlanne, G. T. (ed.). p. 101-130. CRC Press, Boca Raton, USA.
70. **Edwards, C. A.** (1995) Dietary fibre, fermentation and the colon, in *Dietary Fibre - Mechanisms of Action in Human Physiology and Metabolism*. Cherbut, C., Barry, J. L., Lairon, D., and Durand, M. (ed.). p. 51-60.
71. **Roberfroid, M.** (2007) Prebiotics: The concept revisited. *J. Nutr.* 137: 830S-837S.
72. **Williams, B. A., Verstegen, M. W. A., and Tamminga, S.** (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutr. Res. Rev.* 14: 207-227.
73. **Gibson, G. R. and Roberfroid, M. B.** (1995) Dietary modulation of the human colonic microbiota: introducing the concept of prebiotics. *J. Nutr.* 125: 1401-1412.
74. **Hartemink, R.** (1997) Non Digestible Oligosaccharides: Healthy Food for the Colon, in *Proceedings Symposium Wageningen*. Wageningen, The Netherlands.
75. **Konstantinov, S. R., Zhu, W.-Y., Williams, B. A., Tamminga, S., de Vos, W. M., and Akkermans, A. D. L.** (2003) Effect of fermentable carbohydrates on faecal bacterial communities as revealed by DGGE analysis of 16S rDNA. *FEMS Microbiol. Ecol.* 43: 225-235.
76. **Konstantinov, S. R., Awati, A., Smidt, H., Williams, B. A., Akkermans, A. D. L., and de Vos, W. M.** (2004) Specific response of a novel and abundant *Lactobacillus amylovorus*-like phylotype to dietary prebiotics in the ileum and colon of weaning piglets. *Appl. Environ. Microbiol.* 70: 3821-3830.
77. **Su, Y., Yao, W., Perez-Gutierrez, O., Smidt, H., and Zhu, W. Y.** (2008) 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of *Lactobacillus sobrius* S1. *Anaerobe*. 14: 78-86.
78. **Roselli, M., Finamore, A., Britti, M. S., Konstantinov, S. R., Smidt, H., de Vos, W. M., and Mengheri, E.** (2007) The novel porcine *Lactobacillus sobrius* strain protects intestinal cells from enterotoxigenic *Escherichia coli* K88 infection and prevents membrane barrier damage. *J. Nutr.* 137: 2709-2716.
79. **Konstantinov, S. R., Smidt, H., Akkermans, A. D. L., Casini, L., Trevisi, P., Mazzoni, M., De Filippi, S., Bosi, P., and de Vos, W. M.** (2008) Feeding of *Lactobacillus sobrius* reduces Escherichia coli F4 levels in the gut and promotes growth of infected piglets. *FEMS Microbiol. Ecol.* 66: 599-607.
80. **Konstantinov, S. R., Poznanski, E., Fuentes, S., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) *Lactobacillus sobrius* sp. nov., a novel isolate abundant in the intestine of weaning piglets. *Int. J. Syst. Evol. Micr.* 56: 29-32.
81. **Lähteinen, T., Malinen, E., Koort, J. M., Mertaniemi-Hannus, U., Hankimo, T., Karikoski, N., Pakkanen, S., Laine, H., Sillanpää, H., Söderholm, H., and Palva, A.** (2009) Probiotic properties of Lactobacillus isolates originating from porcine intestine and feces. *Anaerobe*. [Epub ahead of print] (PMID: 19695336).
82. **Partanen, K. H.** (2001) Organic acids - Their efficacy and modes of action in pigs, in *Gut Environment of*

Pigs. Piva, A., Bach Knudsen, K. E., and Lindberg, J. E. (ed.). p. 201-217. Nottingham University Press, Nottingham.

83. **Witte, W., Jorsal, S. E., Roth, F. X., M., K., Göransson, L., Lange, S., and Pedersen, K. B.** (2000) Future strategies with regard to the use of feed without antibiotic additives in pig production. *Scientific Report, Pig News and Information*. 21: 27N-32N.
84. **Canibe, N., Steien, S. H., Overland, M., and Jensen, B. B.** (2001) Effect of K-diformate in starter diets on acidity, microbiota, and the amount of organic acids in the digestive tract of piglets, and on gastric alterations. *J. Anim. Sci.* 79: 2123-2133.
85. **Mroz, Z.** (2005) Organic Acids as Potential Alternatives to Antibiotic Growth Promoters for Pigs. *Adv. Pork. Prod.* 16: 169-182.
86. **Selwet, M.** (2008) Effect of organic acids on numbers of yeasts and mould fungi and aerobic stability in the silage of corn. *Pol. J. Vet. Sci.* 11: 119-123.
87. **Brul, S. and Coote, P.** (1999) Preservative agents in foods - Mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol*. 50: 1-17.
88. **Frank, K.** (1994) Measures to preserve food and feeds from bacterial damage. *Übersicht. Tierernähr*. 22: 149-163.
89. **Roselli, M., Britti, M. S., Le Huerou-Luron, I., Marfaing, H., Zhu, W. Y., and Mengheri, E.** (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. *Toxicol. In Vitro*. 21: 224-229.
90. **Yao, W., Zhu, W. Y., Han, Z. K., Williams, B. A., Tamminga, S., and Akkermans, A. D. L.** (2004) Daidzein increased the density but not composition of *Lactobacillus* community in piglet digesta during *in vitro* fermentation as revealed by DGGE and dilution PCR. *Reprod. Nutr. Dev.* 44: Suppl., S17.
91. **Manzanilla, E. G., Perez, J. F., Martin, M., Kamel, C., Baucells, F., and Gasa, J.** (2004) Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* 82: 3210-8.
92. **Cho, J. H., Chen, Y. J., Min, B. J., Kim, H. J., Kwon, O. S., Shon, K. S., Kim, I. H., Kim, S. J., and Asamer, A.** (2006) Effects of essential oils supplementation on growth performance, IgG concentration and fecal noxious gas concentration of weaned pigs. *Asian Austral. J. Anim.* 19: 80-85.
93. **Zoetendal, E. G., Vaughan, E. E., and de Vos, W. M.** (2006) A microbial world within us. *Mol. Microbiol*. 59: 1639-1650.
94. **Zoetendal, E. G., Rajilić-Stojanović, M., and de Vos, W. M.** (2008) High throughput diversity und functionality analysis of the gastrointestinal tract microbiota. *Gut*. 57: 1605-1615.
95. **Stackebrandt, E. and Rainey, F. A.** (1995) Partial and complete 16S rDNA sequences, their use in generation of 16S phylogenetic trees and their implications in molecular ecological studies, in *Molecular Microbial Ecology Manual*. Akkermans, A. D. L., Van Elsas, J. D., and de Bruijn, F. J. (ed.). p. 1-17. Kluwer Academic Publisher, Dordrecht, The Netherlands.
96. **Muyzer, G., de Waal, E. C., and Uitterlinden, G. A.** (1993) Profiling of complex populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695-700.
97. **Muyzer, G. and Smalla, K.** (1998) Application of denaturing gradient gel electrophoresis (DGGE) and temperature gradient gel electrophoresis (TGGE) in microbial ecology. *Antonie Van Leeuwenhoek*. 73: 127-141.
98. **Felske, A., Engelen, B., Nubel, U., and Backhaus, H.** (1996) Direct ribosome isolation from soil to extract bacterial rRNA for community analysis. *Appl. Environ. Microbiol.* 62: 4162-4167.
99. **Wang, H. F., Zhu, W. Y., Yao, W., and Liu, J. X.** (2007) DGGE and 16S rDNA sequencing analysis of bacterial communities in colon content and feces of pigs fed whole crop rice. *Anaerobe*. 13: 127-133.
100. **Petersson, A., Domig, K. J., Nagel, P., Zollitsch, W., Hagmuller, W., and Kneifel, W.** (2009) Denaturing gradient gel electrophoresis (DGGE)-based monitoring of intestinal lactobacilli and bifidobacteria of pigs during a feeding trial. *Arch. Anim. Nutr.* 63: 112-126.
101. **Janczyk, P., Pieper, R., Smidt, H., and Souffrant, W. B.** (2007) Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing

gradient gel electrophoresis. *FEMS Microbiol. Ecol.* 61: 132-140.

102. **Lee, L. G., Connell, C. R., and Bloch, W.** (1993) Allelic Discrimination by Nick-Translation Pcr with Fluorogenic Probes. *Nucleic Acids Res.* 21: 3761-3766.
103. **Morrison, T. B., Weis, J. J., and Wittwer, C. T.** (1998) Quantification of low-copy transcripts by continuous SYBR (R) green I monitoring during amplification. *Biotechniques*. 24: 954-+.
104. **Smith, C. J. and Osborn, A. M.** (2009) Advantages and limitations of quantitative PCR (Q-PCR)-based approaches in microbial ecology. *FEMS Microbiol. Ecol.* 67: 6-20.
105. **Klappenbach, J. A., Dunbar, J. M., and Schmidt, T. M.** (2000) RRNA operon copy number reflects ecological strategies of bacteria. *Appl. Environ. Microbiol.* 66: 1328-1333.
106. **Castillo, M., Martin-Orue, S. M., Manzanilla, E. G., Badiola, I., Martin, M., and Gasa, J.** (2006) Quantification of total bacteria, enterobacteria and lactobacilli populations in pig digesta by real-time PCR. *Vet. Microbiol.* 114: 165-70.
107. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
108. **Hein, I., Lehner, A., Rieck, P., Klein, K., Brandl, E., and Wagner, M.** (2001) Comparison of different approaches to quantify *Staphylococcus aureus* cells by real-time quantitative PCR and application of this technique for examination of cheese. *Appl. Environ. Microbiol.* 67: 3122-6.
109. **Su, Y., Yao, W., Perez-Gutierrez, O. N., Smidt, H., and Zhu, W. Y.** (2008) Changes in abundance of Lactobacillus spp. and *Streptococcus suis* in the stomach, jejunum and ileum of piglets after weaning. *FEMS Microbiol. Ecol.* 66: 546-555.
110. **Song, Y. and Hampson, D. J.** (2009) Development of a multiplex qPCR for detection and quantitation of pathogenic intestinal spirochaetes in the faeces of pigs and chickens. *Vet. Microbiol.* 137: 129-136.
111. **Spence, C., Whitehead, T. R., and Cotta, M. A.** (2008) Development and comparison of SYBR Green quantitative real-time PCR assays for detection and enumeration of sulfate-reducing bacteria in stored swine manure. *J. Appl. Microbiol.* 105: 2143-2152.
112. **Konstantinov, S. R., Smidt, H., and de Vos, W. M.** (2005) Representative difference analysis and real-time PCR for strain-specific quantification of *Lactobacillus sobrius* sp. nov. *Appl. Environ. Microbiol.* 71: 7578-7581.
113. **Messick, J. B., Cooper, S. K., and Huntley, M.** (1999) Development and evaluation of a polymerase chain reaction assay using the 16S rRNA gene for detection of *Eperythrozoon suis* infection. *J. Vet. Diagn. Invest.* 11: 229-236.
114. **Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., and Wang, K.** (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* 47: 367-373.
115. **Hoelzle, L. E., Helbling, M., Hoelzle, K., Ritzmann, M., Heinritzi, K., and Wittenbrink, M. M.** (2007) First LightCycler real-time PCR assay for the quantitative detection of *Mycoplasma suis* in clinical samples. *J. Microbiol. Methods*. 70: 346-354.
116. **Jensen, A. N., Andersen, M. T., Dalsgaard, A., Baggesen, D. L., and Nielsen, E. M.** (2005) Development of real-time PCR and hybridization methods for detection and identification of thermophilic Campylobacter spp. in pig faecal samples. *J. Appl. Microbiol.* 99: 292-300.
117. **Dubosson, C. R., Conzelmann, C., Miserez, R., Boerlin, P., Frey, J., Zimmermann, W., Hani, H., and Kuhnert, P.** (2004) Development of two real-time PCR assays for the detection of *Mycoplasma hyopneumoniae* in clinical samples. *Vet. Microbiol.* 102: 55-65.
118. **Bodrossy, L. and Sessitsch, A.** (2004) Oligonucleotide microarrays in microbial diagnostics. *Curr. Opin. Microbiol.* 7: 245-54.
119. **Guschin, D. Y., Mobarry, B. K., Proudnikov, D., Stahl, D. A., Rittmann, B. E., and Mirzabekov, A. D.** (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Appl. Environ. Microbiol.* 63: 2397-2402.
120. **DeSantis, T. Z., Brodie, E. L., Moberg, J. P., Zubieta, I. X., Piceno, Y. M., and Andersen, G. L.**

(2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb. Ecol.* 53: 371-383.

121. **Wang, R. F., Beggs, M. L., Robertson, L. H., and Cerniglia, C. E.** (2002) Design and evaluation of oligonucleotide-microarray method for the detection of human intestinal bacteria in fecal samples. *FEMS Microbiol. Lett.* 213: 175-82.
122. **Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O.** (2007) Development of the human infant intestinal microbiota. *PLoS Biolology*. 5: e177.
123. **Smoot, L. M., Smoot, J. C., Smidt, H., Noble, P. A., Konneke, M., McMurry, Z. A., and Stahl, D. A.** (2005) DNA microarrays as salivary diagnostic tools for characterizing the oral cavity's microbial community. *Adv. Dent. Res.* 18: 6-11.
124. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
125. **Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H., and Wagner, M.** (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* 68: 5064-5081.
126. **Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.-H.** (2004) ARB: a software environment for sequence data. *Nucleic Acids Res.* 32: 1363-1371.
127. **DeSantis, T. Z., Hugenholtz, P., Larsen, N., Rojas, M., Brodie, E. L., Keller, K., Huber, T., Dalevi, D., Hu, P., and Andersen, G. L.** (2006) Greengenes, a chimera-checked 16S rRNA gene database and workbench compatible with ARB. *Appl. Environ. Microbiol.* 72: 5069-5072.
128. **DeSantis, T. Z., Dubosarskiy, I., Murray, S. R., and Andersen, G. L.** (2003) Comprehensive aligned sequence construction for automated design of effective probes (CASCADE-P) using 16S rDNA. *Bioinformatics*. 19: 1461-8.
129. **Rajilić-Stojanović, M., Smidt, H., and de Vos, W. M.** (2007) Diversity of the human gastrointestinal tract microbiota revisited. *Environ. Microbiol*. 9: 2125-2136.
130. **Quince, C., Lanzen, A., Curtis, T. P., Davenport, R. J., Hall, N., Head, I. M., Read, L. F., and Sloan, W. T.** (2009) Accurate determination of microbial diversity from 454 pyrosequencing data. *Nature Methods*. 6: 639-U27.
131. **Andersson, A. F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P., and Engstrand, L.** (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE*. 3: e2836.
132. **Claesson, M. J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J. R., Smidt, H., de Vos, W. M., Ross, R. P., and O'Toole, P. W.** (2009) Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. *PLoS ONE*. 4:e6669.
133. **MacLean, D., Jones, J. D. G., and Studholme, D. J.** (2009) Application of 'next-generation' sequencing technologies to microbial genetics. *Nat. Rev. Microbiol.* 7: 287-296.
134. **Ramette, A.** (2007) Multivariate analyses in microbial ecology. *FEMS Microbiol. Ecol.* 62: 142-160.
135. **Ogino, A., Koshikawa, H., Nakahara, T., and Uchiyama, H.** (2001) Succession of microbial communities during a biostimulation process as evaluated by DGGE and clone library analyses. *J. Appl. Microbiol.* 91: 625-635.
136. **Boon, N., De Windt, W., Verstraete, W., and Top, E. M.** (2002) Evaluation of nested PCR-DGGE (denaturing gradient gel electrophoresis) with group-specific 16S rRNA primers for the analysis of bacterial communities from different wastewater treatment plants. *FEMS Microbiol. Ecol.* 39: 101-112.
137. **Iwamoto, T., Tani, K., Nakamura, K., Suzuki, Y., Kitagawa, M., Eguchi, M., and Nasu, M.** (2000) Monitoring impact of in situ biostimulation treatment on groundwater bacterial community by DGGE.

FEMS Microbiol. Ecol. 32: 129-141.

138. **Gafan, G. P., Lucas, V. S., Roberts, G. J., Petrie, A., Wilson, M., and Spratt, D. A.** (2005) Statistical analyses of complex denaturing gradient gel electrophoresis profiles. *J. Clin. Microbiol.* 43: 3971-3978.
139. **Hill, M. O.** (1973) Diversity and Evenness - Unifying Notation and Its Consequences. *Ecology*. 54: 427-432.
140. **Peet, R. K.** (1975) The measurement of species diversity. *Annu. Rev. Ecol. Syst.* 5: 285-307.

Chapter 2

Changes in Abundance of *Lactobacillus* spp. and *Streptococcus suis* in Stomach, Jejunum and Ileum of Piglets after Weaning

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Abstract

Weaning is a crucial period for piglets as they can experience many stresses due to the change of diet and environment. The stress can result in an imbalance of the gastrointestinal (GI) microbiota, which allows opportunistic pathogens to multiply and cause GI disorders. The present study investigated the changes in bacterial community composition, with emphasis on *Lactobacillus* spp. and *Streptococcus suis* populations as potentially beneficial and harmful groups, in stomach, jejunum and ileum of piglets after weaning (21 day postpartum) by 16S ribosomal RNA gene-based molecular methods. Denaturing gradient gel electrophoresis (DGGE) profiles of bacterial communities in the stomach, jejunum and ileum of piglets showed that, after weaning, predominant bands related to *Lactobacillus* spp. disappeared and were replaced by potential pathogenic species, such as *Peptostreptococcus anaerobius*, *Moraxella cuniculi*, *S. suis* and *Porphyromonas catoniae*. Quantitative real-time PCR revealed that the abundances of lactobacilli and *Lactobacillus sobrius* as a proportion of total bacterial abundance were significantly lower in foregut samples of weaned piglets than in 21-day-old piglets. A specific and sensitive real-time PCR assay was developed for quantification of the important pathogen *S. suis* in complex gastrointestinal microbiota. This assay showed that *S. suis* predominated in stomach samples of weaned piglets with population levels of up to 10^7 copies/g digesta, although this species was not detected in the stomach before weaning. *S. suis* was dominant in jejunum and ileum digesta before weaning, and became more dominant after weaning, with population levels of up to 10^7 copies/g digesta. The results for the first time demonstrated the dominance of *S. suis*, a potentially harmful species, in the piglet intestine and that the abundance of this species increased after the piglets were weaned. Our study may also suggest that the defensive barrier of the stomach can be impaired as *S. suis* became most dominant while the proportion of *Lactobacillus* populations decreased after weaning, which may further result in an increase of *S. suis* abundance in the intestine. This finding may imply that practical cares should be taken to ensure that piglets have a smooth transition of weaning.

Introduction

Weaning is a particularly crucial period for piglets since they can experience many stresses including separation from the sow, the end of lactational immunity and changes in their environment and diet. Such periods of stress can result in an imbalance of the gastrointestinal (GI) microbiota, which allows opportunistic pathogens to multiply and cause GI disorders. Qualitative and quantitative analysis of the predominant microbiota around weaning is therefore essential for an understanding of the patterns of porcine GI tract bacterial colonization. Moreover, greater insight is required into the role of specific bacteria in the maintenance of a healthy porcine microbiota. Over the past decade, 16S ribosomal RNA gene-targeted PCR, in combination with denaturing gradient gel electrophoresis (DGGE), and cloning and sequence analysis have been used widely to study GI microbiota [1-3]. Recently introduced real-time PCR assays allow quantitative analysis of specific microbial groups and species in complex ecosystems such as the porcine GI tract [4, 5].

The mammalian intestine harbors a highly diverse microbial community, including potentially beneficial, neutral and harmful bacteria, the latter often constituting transitory bacterial populations [4]. Within the microbial community, *Lactobacillus* spp. are considered to be beneficial for the host due to their potential effect on gut function and health [5, 6]. In particular, *Lactobacillus sobrius* sp. nov. has been identified as an abundant member of the intestinal microbiota of piglets in different regions [7, 8]. Our present knowledge of the bacterial diversity associated with the GI tract of piglets is based mainly on analysis of fecal samples or representative intestinal segments such as the ileum and colon. In contrast, only limited information is available on bacterial colonization of different foregut segments, particularly the stomach, or sites in piglets. Because of their importance to the health of piglets, further studies on the changes in microbial communities from the stomach to the small intestine are still needed, especially around the weaning period.

While numerous studies have been focused on microbial community and potential beneficial bacterial populations such as *Lactobacillus* spp. and *Bifidobacterium* spp. in the gut, little information is available on changes in potentially harmful bacteria, which may be present particularly when animals experience stress. *Streptococcus suis* is an important cause of a wide variety of infections in pigs, including meningitis, pneumonia, septicemia and arthritis [9, 10]. In addition, *S. suis* has been described as a human pathogen [11, 12]. To date, 35 serotypes of *S. suis* have been described [15, 16], of which *S. suis* serotype 2 is the most prevalent type isolated from diseased pigs. This serotype was identified as the causative agent of more than 10 human deaths for the first time in China (Jiangsu province) in 1998 [13] and, subsequently, of an outbreak of 30 human deaths in Sichuan province of China in 2005 [14]. This has led to extensive research on pathogenesis and etiopathogenesis but no information is available on the existence and distribution of *S. suis* in the GI tract of piglets.

During investigations into the development of bacterial communities in the GI tract of weaning piglets, in which dominant bacterial species were characterized by analysis of 16S rRNA gene sequencing, we surprisingly found, for the first time, dominance by sequences related to *S. suis*. The aim of this study was to determine changes in the bacterial community composition in stomach, jejunum and ileum of piglets during the weaning transition using

16S rRNA gene-based techniques, and in particular, to compare changes in populations of *Lactobacillus*, a potential beneficial species, and *S. suis*, a species related to human pathogens.

Materials and methods

Piglets and Sampling

Three litters (L1-3), comprising triplicates, of neonatal piglets (10-11 piglets in each litter) from a commercial maternal line herd (Landrace-Yorkshire-Duroc) were investigated. All 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, to avoid the stress caused by the environment change. Piglets were fed *ad libitum* with free access to water. The diet did not contain any antibiotics and the composition is presented in Table 1. On days 7, 14, 21, 24 and 35 (age), one piglet from each litter was euthanized. Fresh digesta from stomach, jejunum and ileum (approximate middle segments) were collected and stored at -20°C for molecular analysis. To analyze the microbiota intimately attached to jejunum mucosa, middle segments of jejunum (3 - 4 cm) were excised, immediately snap frozen in liquid nitrogen, and stored at -20°C. Mucosal samples were collected from the frozen tissue after defrosting by scraping the luminal surface with a sterile glass slide after draining the luminal fluid. All surgical and animal care procedures throughout the study followed protocols approved by Experimental Animal Care and Use guidelines (Chinese Science and Technology Committee, 1998).

Table 1. Composition of the diet used for weaned piglets.

Ingredients	g/kg
Corn	525
Soybean meal	304
Wheat middlings	50
Fish meal (70.6% crude protein)	35
Whey powder	30
Soybean oil	15
Limestone	11.7
Dicalcium phosphate	15
Mineral and vitamin premix[a]	10
Salt	2.5
L-Lysine	1.8

[a]The mineral and vitamin premix (1%) consisted of (per kg diet): 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 (pyridoxin) 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.

DNA extraction and PCR amplification

Total DNA was extracted from digestive and mucosal samples by bead-beating for 3 min using a mini-bead beater (Biospec Products, Bartlesville, OK, USA), followed by phenol-chloroform extraction [15]. DNA was then precipitated with ethanol and pellets were re-suspended in 50 μl of TE.

Primers U968-GC and L1401 were used to amplify the V6-V8 variable regions of the bacterial 16S rRNA gene [16] (see Table 2 for all primers used in this study). PCR amplification was performed with the *Taq* DNA polymerase kit from Promega (Madison, WI, USA) in a T1 Whatman Biometra Thermocycler (Göttingen, Germany) using the following program: 94°C for 5 min, and 35 cycles of 94°C for 30 sec, 56°C for 20 sec, 68°C for 40 sec, and 68°C for 7 min final extension. Aliquots of 5 µl were analyzed by electrophoresis on a 1.2% agarose gel (w/v) containing ethidium bromide to determine the size and concentration of the PCR products.

Table 2. DNA primers used in this study.

Primer	Sequence 5´-3´	Reference
U968-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACG-GGGGGAACGCGAAGAACCTTAC	[16]
L1401	CGGTGTGTACAAGACCC	[16]
8f	CACGGATCCAGAGTTTGAT(C/T)(A/C)TGGCTCAG	[17]
1510r	GTGAAGCTTACGGCTACCTTGTTACGACTT	[17]
T7	TAATACGACTCACTATAGG	Promega
Sp6	GATTTAGGTGACACTATAG	Promega
Lab0677	CACCGCTACACATGGAG	[18]
Lab0159	CGGTATTAGCACCTGTTTCC	[18]
LAC1	AGCAGTAGGGAATCTTCCA	[19]
L-*-OTU171-0077-a-S-2	ACTTCGGTAATGACGTTG	[8]
Bact1369	CGGTGAATACGTTCYCGG	[20]
Prok1492	GGWTACCTTGTTACGACTT	[20]
16S-195	CAGTATTTACCGCATGGTAGATAT	[21]
16S-489	GTAAGATACCGTCAAGTGAGAA	[21]

DGGE analysis

PCR amplicons obtained from V6-V8 regions of 16S rRNA genes were separated by DGGE according to the specifications of Muyzer et al. (1993) [22], using a Dcode TM system (Bio-Rad, Hercules, CA, USA). DGGE was performed in 8% polyacrylamide gels containing 37.5:1 acrylamide-bisacrylamide and a denaturing gradient of 38-51% of urea and 40% (v/v) formamide. Electrophoresis was initiated by pre-running for 10 min at 200 V and subsequently continued at a fixed voltage of 85 V for 12 h at 60°C. Gels were stained with $AgNO_3$ after completion of electrophoresis [23]. DGGE gels were scanned using a GS-800 Calibrated Densitometer (Bio-Rad) and analyzed using Molecular Analyst 1.61 software (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 [24, 25].

Cloning and sequencing of PCR amplified products

Bacterial full-length 16S rRNA genes from stomach samples of 24-day piglets in L1 and L2 were amplified by PCR with primers 8f and 1510r [17]. PCR products were purified using the PCR product purification kit (Invitrogen, Shanghai, P. R. China) and cloned in *E. coli* JM109 using the pGEM-T vector system (Invitrogen). After overnight growth, single colonies of ampicillin-resistant transformants were picked, transferred to Luria Broth medium and incubated at 37°C overnight. One hundred microliters of the above cultures were centrifuged and the pellets were resuspended in 100 μl TE buffer. Suspensions were boiled for 5 min to lyse cells, and were then used as templates to perform PCR amplification using pGEM-T-specific primers Sp6 and T7 (Promega) to check the size of the cloned inserts. Plasmids containing an insert of approximately 1.6 kb were used to amplify the V6-V8 regions of 16S rRNA gene as described above. The amplicons were then compared with the bands derived from the same, original samples by DGGE profiling. Amplicons corresponding to distinct bands on DGGE profiles were selected and then sequenced commercially (Invitrogen, Shanghai, China). Similarity searches of the GenBank DNA database were performed with the BLAST Search tool [26].

Real-time PCR quantification of total bacteria, lactobacilli, L. sobrius and S. suis

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 by Konstantinov et al. [8]. The 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. Quantification of 16S rRNA gene copies in each sample was performed in triplicate, and the mean values were calculated. Standard curves were generated using the seriate 10-fold diluted 16S rRNA gene amplified from the respective target strains. *L. sobrius* was also used as reference strain in assays for quantification of total bacteria and lactobacilli. Universal primers, Bact1369 and Prok1492 [20], were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample, and PCR amplification was performed with an initial denaturation step of 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, 56°C for 30 sec and 72°C for 30 sec. Species-specific primer L-*-OTU171-0077-a-S-2 [8] and the primer Lab0159 [18] were used for the quantification of *L. sobrius*. The PCR conditions were: An initial DNA denaturation step at 95°C for 3 min, followed by 40 cycles of denaturation at 95°C for 15 sec, and primer annealing and extension at 62.5°C for 45 sec. Total lactobacilli were quantified using the combination of forward, LAC1 [19], and reverse primer, Lab0677 [18], in a cycling program, after which the initial denaturation conditions of 95°C for 3 min, 40 cycles were applied at 95°C for 30 sec, with binding and extension at 60°C for 1 min.

Species-specific primers 16S-195 and 16S-489, initially designed for analysis of *S. suis* in tonsil samples [21], were optimized to determine the abundance of *S. suis* in complex microbial samples of pig digesta. The 16S rRNA gene amplified from a *S. suis*-related single clone was used as standard template for the real-time PCR assay. Gradient PCR was initially used to

determine the optimal annealing temperature, with amplification efficiency, range of linearity and lowest detectable concentration as criteria. Efficiency of amplification was calculated using the formula Eff *(n)* = [10 $^{(-1/slope)}$ -1]. Amplification conditions included an initial step at 95°C for 3 min, followed by 40 cycles of 95°C for 15 sec, annealing temperatures from 53°C to 60°C for 30 sec and 72°C for 30 sec. Optimal amplification was observed at an annealing temperature of 57°C, which was subsequently used for all analyses (see results for details on optimization). To confirm the predicted specificity of the assay, real-time PCR products obtained from one porcine stomach sample (L1, D 24) was purified, cloned as described before, and twenty single clonal colonies were then sequenced commercially (Invitrogen, Shanghai, China).

Nucleotide sequence accession numbers

Sequences of stomach bacterial 16S rRNA gene clones have been deposited in the GenBank database under accession numbers: DQ256403, DQ256404, DQ256406, DQ318876, DQ318877, DQ318879 and DQ318880. To identify sequences corresponding to dominant bands in DGGE profiles from samples of pre-weaning piglets, three *Lactobacillus*-like clones obtained from caecum of piglets in the same litter were used as markers and their 16S rRNA gene sequences have also been deposited in GenBank (accession number DQ238616, DQ487214 and DQ487215). Fourteen 320 bp sequences of the 16S rRNA gene fragments obtained using *S. suis* specific primers were deposited under GenBank accession numbers EF431898 to EF431911.

Statistical analysis

Statistical analysis involved the use of the One-Way ANOVA program in the statistical software package SPSS10.0. All values are expressed as means of three replicates (three litters), treating one piglet from one litter as a single replicate for each day. Significant differences were declared when $p < 0.05$.

Results

Shift of bacterial community composition in stomach, jejunum and ileum of piglets after weaning

Bacterial DGGE profiles of the contents of stomach, jejunum and ileum of piglets on day 21 and 24 revealed that bands migrating to lower positions in DGGE profiles (Fig.1A, Frame I) constituted the predominant bacterial community in stomach, jejunum and ileum of piglets at the time of weaning (day 21). Using cloned 16S rRNA gene fragments of *Lactobacillus*-like organisms as markers, three of the dominant bands were identified to be related to *L. reuteri*, *L. johnsonii* and *L. sobrius* (Table 3). On day 24, however, the relative intensity of these bands had decreased significantly, and in some cases below the level of detection, and bands 6, 7, 8, 9 and 10 increased in relative intensity and became dominant (Fig. 1A, Frame II).

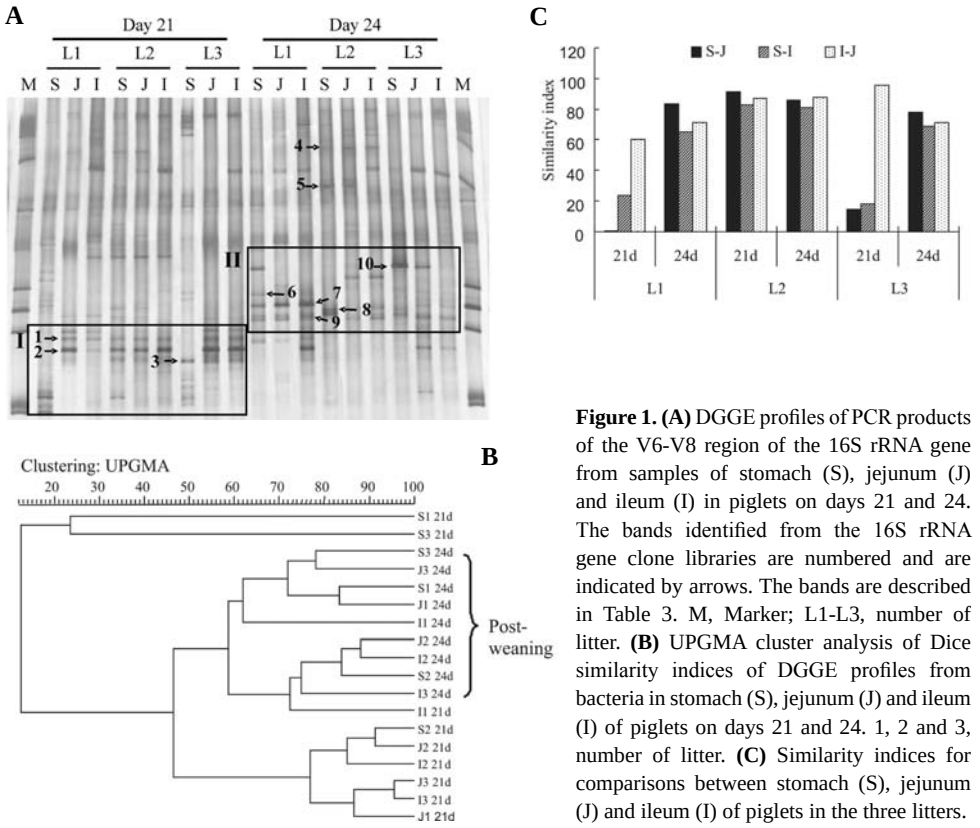

Figure 1. (A) DGGE profiles of PCR products of the V6-V8 region of the 16S rRNA gene from samples of stomach (S), jejunum (J) and ileum (I) in piglets on days 21 and 24. The bands identified from the 16S rRNA gene clone libraries are numbered and are indicated by arrows. The bands are described in Table 3. M, Marker; L1-L3, number of litter. **(B)** UPGMA cluster analysis of Dice similarity indices of DGGE profiles from bacteria in stomach (S), jejunum (J) and ileum (I) of piglets on days 21 and 24. 1, 2 and 3, number of litter. **(C)** Similarity indices for comparisons between stomach (S), jejunum (J) and ileum (I) of piglets in the three litters.

Some of the newly appearing bands from bacteria in the stomach of weaned piglets were also found in the upper intestinal tract (i. e. jejunum and ileum; bands indicated with arrows in Fig. 1A). Cloning and sequencing of the corresponding 16S rRNA genes indicated that, seven of these bands corresponded to organisms closely related to *Peptostreptococcus anaerobius* (band 4), *Moraxella cuniculi* (bands 5 and 10), S. suis (bands 6, 7 and 8) and *Porphyromonas catoniae* (band 9) (Table 3). In particular, the bands related to *S. suis* were observed in all three litters (band 6 in L1, bands 7 and 8 in L2 and L3).

Objective analysis of the DGGE profiles of the pre- and post-weaning piglets was obtained by numerical analysis based on the Dice similarity coefficient, followed by cluster analysis using the unweighed pair group method with averaging algorithm (UPGMA) (Fig. 1B). Cluster analysis revealed that all nine samples from post-weaning piglets formed a coherent cluster with similarity indices above 55%. The low similarities between pre- and post-weaning samples confirmed visual differences in the corresponding DGGE profiles (Fig. 1A). To determine whether bacterial communities in stomach, jejunum and ileum were significantly different in single individuals, similarity indices of the DGGE profiles were calculated (Fig. 1C). Before weaning, for litters L1 and L3, very low similarities (< 25%) were found between

stomach and small intestinal microbiota, which indicated that the microbial community patterns in the stomach was different to those in the jejunum and ileum. This phenomenon was not observed in L2, where similarity indices between stomach and small intestinal samples were greater than 80%. However, after weaning, stomach and small intestinal microbiota showed relatively high similarity indices (> 65%). In addition, similarity indices between samples of jejunum and ileum in the three litters remained at high levels throughout the weaning transition.

Table 3. Identification of bacteria from the stomach, jejunum and ileum in weaned piglets.

Band No.*	Closest cultured relative	Length (bp)	Acc. number	Similarity (%)
1	*Lactobacillus reuteri*	1525	DQ487215	99%
2	*Lactobacillus sobrius*	1528	DQ487214	99%
3	*Lactobacillus johnsonii*	1538	DQ238616	99%
4	*Peptostreptococcus anaerobius*	1390	DQ318876	98%
5	*Moraxella cuniculi*	1447	DQ318877	98%
6	*Streptococcus suis*	1517	DQ256406	98%
7	*Streptococcus suis*	1517	DQ256403	98%
8	*Streptococcus suis*	1519	DQ256404	96%
9	*Porphyromonas catoniae*	781	DQ318880	94%
10	*Moraxella cuniculi*	1069	DQ318879	97%

*Band numbers are indicated by arrows in Fig. 1A.

Quantitative real-time PCR analysis of total bacteria, lactobacilli, L. sobrius and S. suis in stomach, jejunum and ileum samples of piglets around weaning

Quantitative real-time PCR was used to determine the abundance of total bacteria, total lactobacilli, *L. sobrius* and *S. suis* in stomach, jejunum and ileum samples of piglets on D 21 and 24 (Table 4). For *L. sobrius*-specific assays, all 16S rRNA genes of *Lactobacillus*-related clones obtained from samples of piglets were also tested, confirming previous results that only *L. sobrius*-like sequences can be detected (data not shown). Optimization of *S. suis*-specific primers for real-time PCR was conducted using a gradient of annealing temperatures and 57°C was identified as optimal with respect to efficiency and range of linearity of amplification (data not shown), and was used as the annealing temperature throughout this study. Sequence analysis of 20 cloned amplicons obtained from a sample of piglet stomach digesta (L1, D 24) confirmed the specificity of the assay, as all sequences were most closely related to the 16S rRNA gene of *S. suis*.

The concentrations of lactobacilli and *L. sobrius* 16S rRNA gene copies were significantly higher in stomach samples of piglets on D 24 than those of pre-weaning piglets, but percentages relative to total bacteria decreased to less than 10% of those of pre-weaning piglets (Table 4). This change in lactobacilli was associated with a dramatic increase in abundance of *S. suis*, with 16S rRNA gene copies 3 orders of magnitude higher than in pre-weaning piglets. In contrast, jejunal and ileal numbers of total lactobacilli and *L. sobrius* as well as their percentage relative

to total bacteria, declined significantly after weaning, while the percentage of *S. suis* relative to total bacteria increased more than ten-fold, despite no significant change in numbers of *S. suis*.

Table 4. Quantitative real-time PCR analysis of total bacteria, lactobacilli, *L. sobrius* and *S. suis* in digesta samples of porcine stomach, jejunum and ileum.

Species	Stomach		Jejunum		Ileum	
	Day 21	Day 24	Day 21	Day 24	Day 21	Day 24
Bacteria	5.40 ± 0.07	7.95 ± 0.61*	8.99 ± 0.44	8.23 ± 0.84	9.72 ± 0.19	8.29 ± 0.77*
Lactobacilli	4.55 ± 0.13	6.04 ± 0.54*	8.11 ± 0.30	5.60 ± 0.20*	9.29 ± 0.20	6.80 ± 0.91*
Lactobacilli / bacteria (%)	14.13	1.23	13.18	0.23	37.15	3.24
L. sobrius	4.14 ± 0.25	5.64 ± 0.15*	7.12 ± 1.01	5.05 ± 0.14*	8.65 ± 0.63	5.94 ± 1.03*
L. sobrius / bacteria (%)	5.50	0.49	1.35	0.07	8.51	0.45
S. suis	< 4	7.36 ± 1.06*	6.34 ± 0.97	7.04 ± 0.61	7.24 ± 0.29	7.60 ± 0.82
S. suis / bacteria (%)	< 3.98	25.70	0.22	6.46	0.33	20.42

*Significant differences between day 21 and day 24 compared at $p < 0.05$.
Three samples were quantified for each age and counts are expressed as mean ± SD Log 10 (16S rRNA gene copies/g wet weight), n =3.

Comparison of bacterial communities in jejunal digesta and mucosa of piglets in litter 1

To gain insight into mucosa-associated microbial community in intestine, jejunal samples of piglets in L1 were also used to compare predominant digesta- and mucosa-derived microbiota by DGGE analysis (Fig. 2A) and UPGMA analysis (Fig. 2B). Cluster analysis revealed that the similarity index between digesta and mucosa samples from the same piglet was greater than 90% at day 7, while the indices decreased to below 80% from days 14 to 35. Profiles grouped into two distinct clusters: pre-weaning and post-weaning, with similarity indices below 50%, which indicated that weaning also caused significant changes in mucosa-associated bacterial communities, as well as in digestive samples in jejunum of piglets in L1. The *L. sobrius*-related band (arrow 2) was also visible in jejunal mucosa of piglets before weaning and faintly visible after weaning, while the predominant band 7, observed only in weaned piglets, did not appear in profiles generated from mucosal samples. These observations were confirmed by quantitative real-time PCR assays on samples of representatives from litter 1 (Table 5). *L. sobrius*-related populations were detected in mucosal samples until D 21, while numbers decreased below detectable levels after weaning. In contrast, *S. suis* could not be detected in jejunal mucosa of piglets from days 7 to 35.

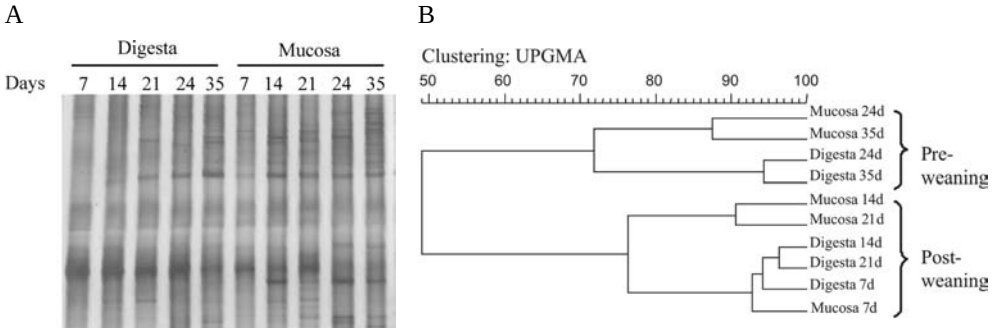

Figure 2. (**A**) DGGE analysis of amplicons generated by bacterial universal primers U968-GC and L1401, originating from samples in jejunal digesta and mucosa of piglets from L1. Bands numbered with arrows in gel were the same ones in Fig. 1A. (**B**) Similarity indices of DGGE profiles obtained from bacteria in jejunal digesta and mucosa of piglets from L1.

Table 5. Quantitative real-time PCR analysis of 16S rRNA gene copies of total bacteria, *L. sobrius* and *S. suis* in total DNA from jejunal mucosal samples of piglets in litter 1 (16S rRNA gene copies/μl DNA sample).

Species	Day 7	Day 14	Day 21	Day 24	Day 35
Bacteria	9.33×10^{3}	1.72×10^{4}	1.25×10^{5}	2.85×10^{3}	1.65×10^{3}
L. sobrius	6.49×10^{2}	1.34×10^{3}	3.00×10^{3}	$<2\times10^{1}$	$<2\times10^{1}$
S. suis	$<4\times10^{1}$	$<4\times10^{1}$	$<4\times10^{1}$	$<4\times10^{1}$	$<4\times10^{1}$
L. sobrius / bacteria (%)	6.96	7.80	2.39	< 0.70	< 1.21

Discussion

The development of intestinal microbiota of piglets from age 7 to 35 days was investigated by using 16S rRNA gene-based approaches, focusing on the stomach and small intestine. Drastic changes were observed in GI bacterial community composition of piglets during the weaning transition. This was evidenced by a significant change in the similarity indices of DGGE profiles obtained for samples from days 21 and 24. Similar results were also observed in colon and feces of piglets during weaning [24, 27, 28]. The changes were probably due to the establishment of a new bacterial community after weaning, following the dietary changes from maternal milk onto a solid feed rich in plant polysaccharides.

Previous studies on intestinal bacterial microbiota of weaned piglets have shown that *E. coli* concentrations increased while numbers of lactobacilli decreased after weaning [27, 29, 30]. In the present study, real-time PCR showed that numbers of *Lactobacillus* spp. 16S rRNA gene copies declined significantly in the jejunum and ileum of piglets after weaning, while *S. suis* tended to increase after weaning, although there were no obvious changes in the abundance of total bacteria. These changes in different bacterial populations in the intestine may be related

to the defensive barrier in the stomach. Our previous results demonstrated that the pH in the stomach of piglets increased apparently from 3.0 to 5.0 after weaning [31]. The low pH in the stomach before weaning may keep the bacterial population at a low level, especially controlling the proliferation of those potentially harmful bacteria species. The present study for the first time demonstrated bacterial community change in piglet stomach before and after weaning. Indeed, our results showed that the total bacteria population was low, with those potentially harmful bacteria species under the detection limit before weaning. After weaning, total bacteria population markedly increased, of which the percentage of lactobacilli decreased significantly, while that of *S. suis*-like populations dramatically increased. This is also evident in DGGE profiling in our study, where bands corresponding to *Lactobacillus*-like organisms decreased below detection limits, while bands corresponding to *S. suis*-like organisms became dominant after weaning. Thus, the results suggest that bacterial proliferation in the stomach, especially of potentially harmful species, may provide inocula to the proximal small intestine. Sequence analysis showed that many common bands that appeared in post-weaning piglet samples were closely related to *P. anaerobius*, *M. cuniculi*, *S. suis* and *P. catoniae*. Although phenotypic characteristics of microorganisms can not always be directly predicted from 16S rRNA gene sequences [32], all of the closest relatives have previously been reported as potential pathogens for pigs [9, 33, 34]. These results indicated that the function of the stomach as a barrier against pathogens might be impaired after weaning.

In the present study, considerable differences in the GI microbiota transition around weaning were found among different litters of piglets. This has also been observed in other studies of porcine intestinal microbiota [4, 37]. A possible reason is that different piglets from the same litter were used to study the change of microbiota patterns with time. Indeed, piglets from the same sow before weaning showed higher similarity in DGGE profiles than those from different sows, after weaning, however, all piglets developed their own characteristic DGGE profiles and no apparent cluster was observed in DGGE profiles [28]. Use of ileal cannulas may enable repeated intestinal samples from a single pig, but it would be also difficult to sample from many sites of the gut and stomach. The aim of this study was to investigate the spatial and temporal changes in the microbial community of the gut of naturally raised piglets. To overcome the limitation caused by slaughtering different piglets from the same litter, our approach used piglets from three litters as replicates. Although differences existed in the DGGE profiles among different piglets, it is evident that some dominant species before weaning, such as lactobacilli, became undetectable after weaning, while other species, such as *S. suis*-like species, appeared on DGGE gels after weaning. Further, this approach enabled identification of the gradual development of characteristic mucosa-associated microbiota in litter L1 that differed from that in digesta.

Lactobacilli related to *L. sobrius* have previously been identified as common inhabitants of the porcine intestine [35] and *L. sobrius* was therefore specifically investigated in this study, where it was detected in relatively high abundance in the intestine of piglets before weaning. Lactobacilli are known for their potential to prevent infection or colonization of pathogens by competition for nutrients and epithelial binding sites, and the production of antimicrobial factors, such as lactic acid and bacteriocins [36]. The fact that *L. sobrius* was abundant in the intestine of the suckling piglets, both in digesta and attached to small intestinal mucosa,

reinforces suggestions that this particular *Lactobacillus* species may play a crucial role in the establishment and the maintenance of the GI tract bacterial homeostasis after birth [27].

S. suis is an important cause of meningitis, septicemia, arthritis, and sudden death in young pigs [9, 10] and can infect humans [11, 12], as evidenced in the outbreaks in China in 1998 and 2005 [13]. Currently, bacteriological techniques and PCR tests are routinely applied to detect or characterize *S. suis* in pure cultures [37, 38]. Here, we developed a fast and sensitive, cultivation independent, real-time PCR assay to quantify *S. suis* in porcine intestine inhabited by a complex microbial community. This assay can quantify *S. suis* with 16S rRNA gene concentrations higher than 10 copies/ml DNA sample (data not shown). The present study using DGGE and real-time PCR demonstrated the dominance of this *S. suis*-like species in the gut of piglets, especially in the stomach and intestine of weaning piglets. However, this species was not detectable in mucosa samples of representatives from litter 1, which may suggest that *S. suis* was incapable of adhering to the small intestine or perhaps not being a regular resident in the gut. This is consistent with the generally accepted belief that the infection route of *S. suis* is through replication in the upper respiratory tract [39]. Therefore, the distribution of *S. suis* in GI tract of piglets has not received much attention. However, piglets with high concentrations of *S. suis* may serve as a source of *S. suis* transmission in pig herds if their stomach and intestine were colonized with *S. suis* at considerable abundance. This could also be a source for human infections. Indeed, with the *S. suis* human infection outbreak in 26 counties in Sichuan province in 2005, it was later speculated that *S. suis* had been present in farms for a long time and human infections outbreak when the environment favors the proliferation of the pathogen [13]. Nevertheless, further studies are needed regarding transmission of *S. suis* via intestinal carriage.

In conclusion, the present study for the first time demonstrated the dominance of *S. suis*, a potentially pathogen in the intestine of piglets. While predominant bacteria related to *Lactobacillus* spp. in stomach, jejunum and ileum of suckling piglets became less abundant after weaning, potentially pathogenic species including *S. suis* became dominant in the stomach and more abundant in the intestine after weaning. The shift in mucosa-associated microbiota in the jejunum was different from that in digesta during the porcine weaning transition, with *S. suis* being dominant in digesta while not detectable in mucosa samples. Microbiota in stomach and intestinal digesta of piglets can be adversely influenced by the weaning. Thus, practical measures should be taken to ensure that piglets have a smooth transition of weaning, especially to maintain the defensive barrier function of the stomach.

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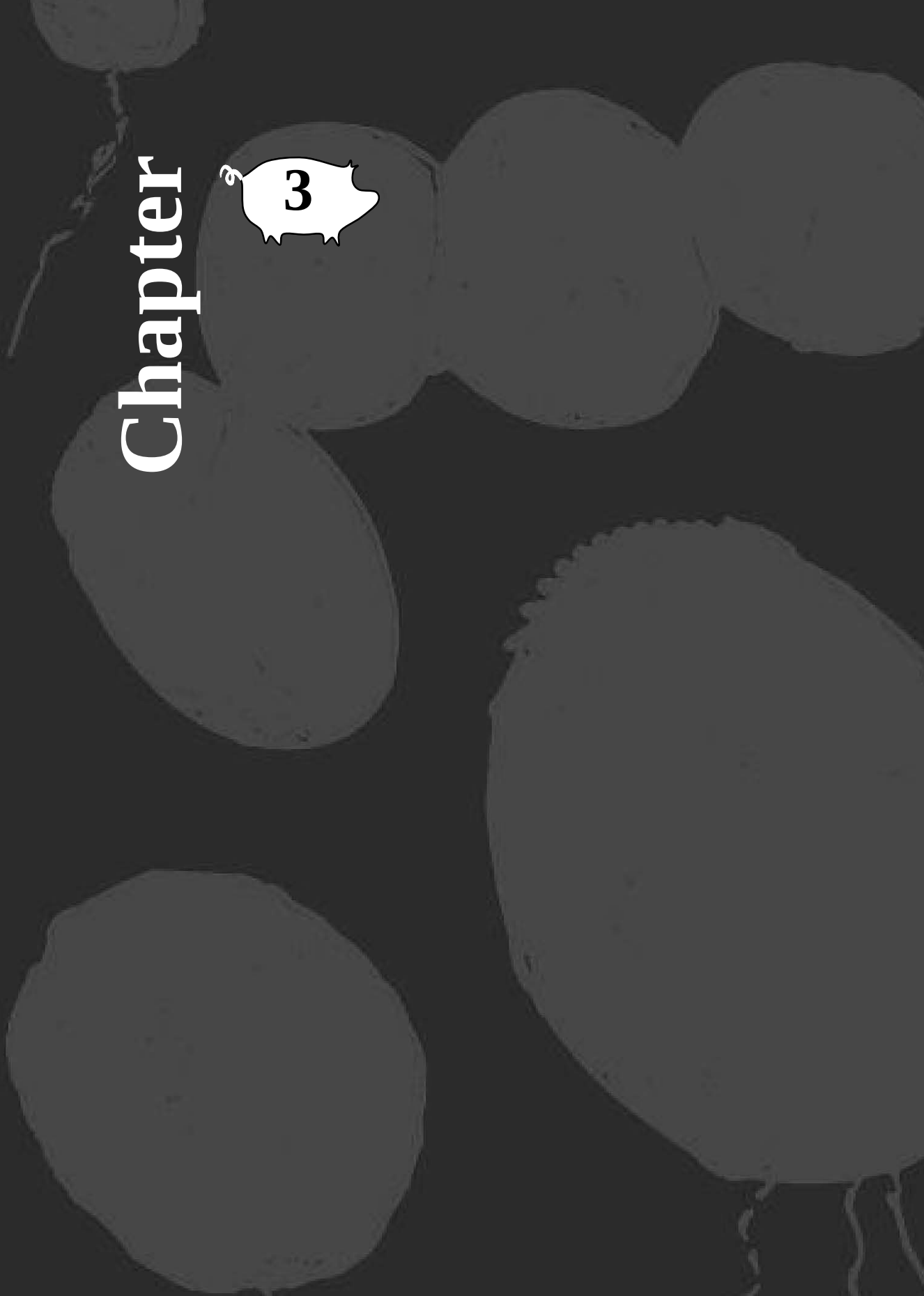
References

1. **Awati, A., Konstantinov, S. R., Williams, B. A., Akkermans, A. D. L., Bosch, M. W., Smidt, H., and Verstegen, M. W. A.** (2005) Effect of substrate adaptation on the microbial fermentation and microbial composition of faecal microbiota of weaning piglets studied in vitro. *J. Sci. Food. Agric.* 85: 1765-1772.
2. **Simpson, J. M., McCracken, V. J., White, B. A., Gaskins, H. R., and Mackie, R. I.** (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods*. 36: 167-79.
3. **Zhu, W. Y., Williams, B., and Akkermans, A. D. L.** (2000) Development of the microbial community in weaning piglets. *Reprod. Nutr. Dev.* 40: 180.
4. **Bourliox, P., Koletzko, B., Guarner, F., and Braesco, V.** (2003) The intestine and its microflora are partners for the protection of the host: report on the Danone Symposium "The Intelligent Intestine", held in Paris, June 14, 2002. *Am. J. Clin. Nutr.* 78: 675-683.
5. **Harmsen, H. J. M., Elfferich, P., Schut, F., and Welling, G. W.** (1999) A 16S rRNA -targeted probe for detection of Lactobacilli and Enterococci in faecal samples by fluorescenct in situ hybridization. *Microb. Ecol. Health Dis.* 11: 3-12.
6. **Konstantinov, S. R., Favier, C. F., Zhu, W. Y., Williams, B. A., Klüss, J., Souffrant, W. B., de Vos, W. M., Akkermans, A. D. L., and Smidt, H.** (2004) Microbial diversity study of the porcine GI tract during the weaning transition. *Anim. Res.* 54: 317-324.
7. **Janczyk, P., Pieper, R., Smidt, H., and Souffrant, W. B.** (2007) Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis. *FEMS Microbiol. Ecol.* 61: 132-140.
8. **Konstantinov, S. R., Smidt, H., and de Vos, W. M.** (2005) Representative difference analysis and real-time PCR for strain-specific quantification of *Lactobacillus sobrius* sp. nov. *Appl. Environ. Microbiol.* 71: 7578-7581.
9. **Cliftonhadley, F. A.** (1983) Zoonoses in Practice - *Streptococcus-Suis* Type-2 Infections. *Br. Vet. J.* 139: 1-5.
10. **Vecht, U., Wisselink, H. J., Vandijk, J. E., and Smith, H. E.** (1992) Virulence of *Streptococcus-Suis* Type-2 Strains in Newborn Germ-Free Pigs Depends on Phenotype. *Infect. Immun.* 60(2): 550-556.
11. **Arends, J. P. and Zanen, H. C.** (1988) Meningitis Caused by *Streptococcus-Suis* in Humans. *Rev. Infect. Dis.* 10: 131-137.
12. **Trottier, S., Higgins, R., Brochu, G., and Gottschalk, M.** (1991) A Case of Human Endocarditis Due to *Streptococcus-Suis* in North-America. *Rev. Infect. Dis.* 13: 1251-1252.
13. **Lu, C.** (2005) Swine streptococcosis and *Streptococcus suis* type 2. *Sci. Tech. Rev.* 23: 9-10.
14. **Yang, W.-z., Yu, H.-j., Jing, H.-q., Xu, J.-g., Chen, Z.-h., Zhu, X.-p., Wang, H., Liu, X.-c., Wang, S.-w., Liu, L.-g., Zu, R.-q., Luo, L.-z., Xiang, N.-j., Liu, H.-l., Zhong, W.-j., Liu, L., Meng, L., Yuan, H., Gao, Y.-j., Du, H.-m., Ou, Y.-b., Ye, C.-y., Jin, D., Lv, Q., Cui, Z.-g., Huang, Y., Zhang, S.-y., An, X.-d., Huang, T., Zhou, X.-y., Feng, L., Pang, Q.-d., Shu, Y.-l., and Wang, Y.** (2006) [An outbreak of human *Streptococcus suis* serotype 2 infections presenting with toxic shock syndrome in Sichuan, China]. *Zhonghua Liu Xing Bing Xue Za Zhi*. 27: 185-91.
15. **Zoetendal, E. G., Akkermans, A. D. L., and De Vos, W. M.** (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* 64: 3854-9.
16. **Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H.** (1996) Sequence heterogeneities of genes encoding 16S rRNA in *Paenibacillus polymixa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178: 5636-5643.
17. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
18. **Heilig, H. G. H. J., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermans, A. D. L., and de Vos, W. M.** (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human

intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68: 114-123.

19. **Walter, J., Tannock, G. W., Tilsala-Timisjarvi, A., Rodtong, S., Loach, D. M., Munro, K., and Alatossava, T.** (2000) Detection and Identification of Gastrointestinal *Lactobacillus* Species by Using Denaturing Gradient Gel Electrophoresis and Species-Specific PCR Primers. *Appl. Environ. Microbiol.* 66: 297-303.
20. **Suzuki, M. T., Taylor, L. T., and DeLong, E. F.** (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* 66: 4605-4614.
21. **Marois, C., Bougeard, S., Gottschalk, M., and Kobisch, A.** (2004) Multiplex PCR assay for detection of Streptococcus suis species and serotypes 2 and 1/2 in tonsils of live and dead pigs. *J. Clin. Microbiol.* 42: 3169-3175.
22. **Muyzer, G., de Waal, E. C., and Uitterlinden, G. A.** (1993) Profiling of complex populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695-700.
23. **Sanguinetti, C. J., Dias Neto, E., and Simpson, A. J. G.** (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques*. 17: 915-919.
24. **Konstantinov, S. R., Zhu, W.-Y., Williams, B. A., Tamminga, S., de Vos, W. M., and Akkermans, A. D. L.** (2003) Effect of fermentable carbohydrates on faecal bacterial communities as revealed by DGGE analysis of 16S rDNA. *FEMS Microbiol. Ecol.* 43: 225-235.
25. **Murray, A. E., Hollibaugh, J. T., and Orrego, C.** (1996) Phylogenetic compositions of bacterioplankton from two California estuaries compared by denaturing gradient gel electrophoresis of 16S rDNA fragments. *Appl. Environ. Microbiol.* 62: 2676-80.
26. **Altschul, S. F., Gish, W., Miller, W., Myers, E. W., and Lipman, D. J.** (1990) Basic local alignment search tool. *J. Mol. Biol.* 215: 403-410.
27. **Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8: 1191-1199.
28. **Zhu, W., Yao, W., and Mao, S.** (2003) Development of bacterial community in faeces of weaning piglets as revealed by denaturing gradient gel electrophoresis. *Acta Microbiol. Sin.* 43: 503-508.
29. **Canh, T. T., Sutton, A. L., Aarnink, A. J. A., Verstegen, M. W. A., Schrama, J. W., and Bakker, G. C. M.** (1998) Dietary carbohydrates alter the fecal composition and pH and the ammonia emission from slurry of growing pigs. *J. Anim. Sci.* 76: 1887-1895.
30. **Mathew, A. G., Sutton, A. L., Scheidt, A. B., Patterson, J. A., Kelly, D. T., and Meyerholtz, K. A.** (1993) Effect of Galactan on Selected Microbial-Populations and Ph and Volatile Fatty-Acids in the Ileum of the Weanling Pig. *J. Anim. Sci.* 71: 1503-1509.
31. **Su, Y. and Zhu, W. Y.** (2006) Shifts of total bacterial and lactobacilli community in stomach of sucking and weaned piglets. *J. Parenter. Enteral Nutr.* 13: 1-4.
32. **Jaspers, E. and Overmann, J.** (2004) Ecological significance of microdiversity: identical 16S rRNA gene sequences can be found in bacteria with highly divergent genomes and ecophysiologies. *Appl. Environ. Microbiol.* 70: 4831-9.
33. **Hill, G. B.** (1978) Enhancement of Experimental Anaerobic Infections by Blood, Hemoglobin, and Hemostatic Agents. *Infect. Immun.* 19: 443-449.
34. **Kononen, E., Vaisanen, M. L., Finegold, S. M., Heine, R., and JousimiesSomer, H.** (1996) Cellular fatty acid analysis and enzyme profiles of *Porphyromonas catoniae* - A frequent colonizer of the oral cavity in children. *Anaerobe.* 2: 329-335.
35. **Konstantinov, S. R., Poznanski, E., Fuentes, S., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) *Lactobacillus sobrius* sp. nov., a novel isolate abundant in the intestine of weaning piglets. *Int. J. Syst. Evol. Micr.* 56: 29-32.
36. **Vaughan, E. E., Mollet, B., and deVos, W. M.** (1999) Functionality of probiotics and intestinal lactobacilli: light in the intestinal tract tunnel. *Curr. Opin. Biotechnol.* 10: 505-510.

37. **Smith, H. E., Veenbergen, V., van der Velde, J., Damman, M., Wisselink, H. J., and Smits, M. A.** (1999) The cps genes of *Streptococcus suis* serotypes 1, 2, and 9: Development of rapid serotype-specific PCR assays. *J. Clin. Microbiol.* 37: 3146-3152.
38. **Wisselink, H. J., Joosten, J. J., and Smith, H. E.** (2002) Multiplex PCR assays for simultaneous detection of six major serotypes and two virulence-associated phenotypes of *Streptococcus suis* in tonsillar specimens from pigs. *J. Clin. Microbiol.* 40: 2922-2929.
39. **Madsen, L. W., Svensmark, B., Elvestad, K., Aalbaek, B., and Jensen, H. E.** (2002) *Streptococcus suis* serotype 2 infection in pigs: New diagnostic and pathogenetic aspects. *J. Comp. Pathol.* 126: 57-65.


Chapter
3

Design of a High-throughput Diagnostic Microarray for the Characterization of Pig Gastrointestinal Tract Microbiota

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Manuscript in preparation

Abstract

Microarrays are high-throughput tools ideally suited for the study of complex microbial ecosystems like the mammalian gastrointestinal (GI) tract. In this chapter we present the development and application of the PITChip, a 16S ribosomal RNA (rRNA)-targeted DNA oligonucleotide microarray for studying the pig GI tract microbiota. A total of 627 bacterial phylotypes (< 98% identity) previously reported to occur in the GI tract of pigs were identified, and the hypervariable regions V1 and V6 of their 16S rRNA gene were used to design 2985 unique probes. High technical reproducibility was observed for different hybridizations, labeling and *in vitro* transcriptions. Benchmarking of the PITChip with 454 pyrosequencing and quantitative PCR revealed comparable results, especially at higher taxonomic levels, and minor differences at lower phylogenetic ranks. Analyses of ileum and colon samples from 18 piglets that received either a control diet or one of two different organic acids blends, revealed significant differences ($p < 0.02$) between the microbiota of the piglets receiving the control diet and the ones receiving the acidified diets. PITChip analysis was instrumental in identification of phylotypes most significantly affected by the change in diet, namely members of the *Bacteroidetes* and *Proteobacteria*. The development of the PITChip as semi-quantitative and high-throughput tool now provides the necessary means to gain novel insight in composition and dynamics of porcine GI tract microbiota at high taxonomic resolution, knowledge urgently needed towards improved dietary strategies in sustainable pig production.

Introduction

The gastrointestinal (GI) tract of pigs is a complex and dynamic ecosystem colonized by a highly diverse microbiota comprising more than 400 phylotypes (microbial species-level phylogenetic types) [1, 2]. In the caecum and colon several hundred anaerobic bacterial species coexist [3, 4] with total counts of more than 10^{11}-10^{12} CFU/g digesta [5], whereas there are relatively lower numbers of bacteria (10^{7}-10^{9} CFU/g fresh matter) found in the stomach and small intestine [6]. These bacteria that inhabit the GI tract from birth, have an important impact on animal health, as they provide essential nutrients to the host from fermentation processes, form a key barrier against pathogens [7, 8] and play important roles in GI tract development, physiology and immunology [9-11].

Until recently, the majority of studies addressing the intestinal microbiota of pigs have been based on traditional methods, paying most attention to easily cultivable commensal bacteria and a few opportunistic pathogens [12, 13]. However, a large fraction of the intestinal microbiota remains difficult to cultivate and therefore, impossible to detect using conventional techniques [14, 15]. The development in the last years of high resolution molecular techniques based on *in vitro* amplification of 16S ribosomal RNA (rRNA) genes and other phylogenetic markers by polymerase chain reaction (PCR), combined with fingerprinting techniques, has highly improved our knowledge of complex microbiota such as those found in the pig gut, showing a much higher diversity than described previously by cultivation [1-3].

Phylogenetic microarrays are one of the most advanced comprehensive molecular techniques that enable high-throughput analysis of complex microbial communities and can be used to analyze the composition, structure, variance and functionality of microbial ecosystems [16-19]. To be able to detect microorganisms within complex samples, multiple DNA probes, derived from the small subunit ribosomal RNA (SSU rRNA) gene sequences, are arrayed onto solid surfaces to allow for parallel, multispecies detection [20]. Different types of phylogenetic arrays have been designed for this purpose, targeting either small or large subunit rRNA genes [21-23].

This chapter presents the development and successful application of the Pig Intestinal Tract Chip (PITChip), a new phylogenetic microarray comprehensively targeting pig GI tract microbiota, consisting of 2985 unique 16S rRNA-targeted oligonucleotide probes covering 627 intestinal microbial phylotypes. The probe design strategy applied is based on two hypervariable regions of the SSU rRNA gene, as this has previously been shown to provide sufficient resolution to discriminate human GI tract-associated phylotypes with < 98% SSU rRNA sequence identity [24]. This is a high-throughput approach that can produce probes with a similar predicted hybridization behavior and allows the easy addition of probes targeting newly discovered microbes. In order to benchmark the performance of the PITChip, the community profiles of three piglets' colon samples obtained with the phylogenetic microarray were compared to microbiota fingerprints based on 454 titanium pyrosequencing data, and the quantification of selected important groups with quantitative PCR (qPCR). In addition, the applicability of the PITChip for routine microbiota profiling in intervention trials was tested analyzing a set of samples from piglets receiving a control diet or a diet supplemented with 2 different blends of organic acids.

Materials and Methods

The design of the Pig Intestinal Tract Chip (PITChip) was done using design criteria as previously described by Rajilic-Stojanovic and collaborators, for the Human Intestinal Tract Chip (HITChip; [24]).

Phylogenetic analysis of pig intestinal microbiota and probe design

In order to design the SSU rRNA-targeted probes for the microarray, a pig-specific database was built, using the ARB software environment [25]. To this end, a total of over 1100 SSU rRNA gene sequences of pig GI tract microbiota were extracted from the SILVA database 95 release (July 2008). Only sequences of more than 1200 nucleotides were considered. Operational taxonomic units (OTU, phylotypes) were defined based on a threshold of sequence identity of 98%. For each group of similar sequences with ≥ 98% sequence identity, a representative type sequence was chosen, using sequence length, quality as well as availability of a cultured representative, as criteria. Due to the high number of sequences corresponding to yet uncultured organisms with < 98% identity to sequences of cultured strains, a customized classification was introduced based on four levels of taxonomic resolution, similar to what was previously proposed by Collins and co-workers [26]. The four levels are: Level 0 (phylum level), Level 1 (class or cluster), Level 2 (including sequences with 90% or more sequence similarity) and Level 3 (OTU's formed by unique phylotypes, for cultivated organisms, and by representatives of each group of sequences with < 98% sequence identity, for uncultured microorganisms). The phylogenetic classification of all sequences resulted in a total of 627 unique OTU's (Fig. 1, supplementary material S1).

Eight variable regions of the 16S rRNA genes were exported from ARB to test for their suitability to discriminate between individual OTU's (Table 1). Regions with lowest redundancy (V1, V6) were selected for probe design.

Table 1. Variable regions of the SSU rRNA gene from 627 unique pig phylotypes. The total number of sequences included in the analysis, as well as the total number and percentage of unique variable region sequences are given.

Variable region	Position (*E.coli*)	N° sequences	N° unique sequences	% unique sequences
V1	65-127	642	579	90.2
V2	174-235	642	595	92.7
V3	442-492	642	420	65.4
V4	705-763	642	413	64.3
V5	822-879	642	499	77.7
V6	985-1047	642	579	90.2
V7	1115-1175	639	518	81.1
V8	1253-1313	639	570	89.2

Each variable region sequence was initially divided in 3 overlapping segments of 24 nucleotides that were used to design six probes for each 16S rRNA sequence representing individual OTU's. The melting temperature of the probes was predicted using the nearest neighbor algorithm described by SantaLucia [27] with 0.5M as sodium ion concentration. The average of the Tm of all the sequences ± 2.5°C was then defined as a desired temperature range for iterative probe optimization. To maximize the number of probes with a Tm in this range their length was adjusted, starting from 24 nucleotides, and it was extended or shortened in single nucleotide steps, keeping the length of oligonucleotides between 18 and 30 to maintain similar duplex kinetics. Every time that the length was changed by one nucleotide, the predicted Tm was re-evaluated, until it fitted within a range of 60.9 ± 2.5°C. To be able to use an internal control for the hybridization, six probes targeting *Dehalococcoides ethenogenes* were designed. Microarrays were synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) in 8×15K format.

Sample preparation

DNA was extracted from 200 mg of digesta, previously defrosted, using the Fast DNA Spin Kit for Soil (Qbiogene, Inc, Carlsbad, CA, USA). Electrophoresis in agarose gels 1.2% (w/v) containing ethidium bromide was used to check the amount and quality of DNA.

PITChip analysis

Hybridization and analysis of the generated data was performed essentially using procedures as described before [24, 28].

From DNA to RNA: Near-full length 16S rRNA gene fragments were amplified from extracted DNA samples using a GS0001 thermocycler (Gene Technologies, Braintree, U.K.) with the primers *T7prom*-Bact-27-for (5´-TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGGCTCAG-3´) and Uni-1492-rev (5´-CGGCTACCTTGTTACGAC-3´) [29], using 10 ng of DNA as a template in a final volume of 50 µl. The PCR program used was: pre-denaturation of 2 min at 94°C followed by 35 cycles of 94°C during 30 sec (denaturation), 52°C during 40 sec (annealing), 72°C during 90 sec (elongation) and a final extension at 72°C for 7 min. PCR products were purified (High Pure PCR Cleanup Micro Kit, Roche Diagnostics GmbH, Mannheim, Germany) and the DNA concentration was measured using a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

The 16S rRNA genes carrying the T7-promoter were transcribed using the Riboprobe System (Promega, La Jolla, USA). The amplicon (500 ng) together with rATP, rGTP, rCTP and a 1:1 mix of rUTP and aminoallyl-rUTP (Ambion, Austin, TX, USA) were incubated at room temperature for 2h; afterwards possible DNA present was digested with the Qiagen RNAse-free DNAse kit (Qiagen, Hilden, Germany). Purification of RNA was done using the RNeasy Mini-Elute Kit (Qiagen) and concentration was quantified as mentioned above.

Labeling and hybridization: The *in vitro* transcribed RNA was coupled with CyDye using the Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK) dissolved in

DMSO. The labeling reactions containing 20 µl of CyDye, 2 µg of pure RNA and 25 mM of sodium bicarbonate buffer (pH 8.7) in a total volume of 40 µl, were incubated during 90 min in the dark and at room temperature. The reaction was stopped by adding 15 µl of 4M hydroxylamine and incubating in the dark for 15 min. RNAse-free water was added to 100 µl and the labeled RNA was quantified.

Prior to hybridization, Cy3/Cy5-labeled-target mixtures were fragmented using 10× fragmentation reagent (Ambion). Hybridization on the arrays was performed at 62.5°C for 16h in a rotation oven (Agilent). Slides were washed at room temperature in 2× SSC with 0.3% SDS (10 min) followed by 0.1× SSC + 0.3% SDS at 38°C (10 min) and 0.06× SSPE (5 min) [30].

Data extraction: Agilent Feature Extraction software, version 9.1 (http://www.agilent.com) was used to extract the data from the generated microarray images. Data normalization and further microarray analysis were performed using a set of R based scripts (http://r-project.org) in combination with a custom designed relational database, which runs under the MySQL database management system (http://www.mysql.com). The reproducibility of the hybridization was determined calculating the Pearson's linear correlation of spatially normalized samples. Reproducibility values above 0.98 were considered acceptable and the arrays that fulfilled this condition were further normalized using quantile normalization [31].

Ward's minimum variance method was used for the generation of hierarchical clustering of probe profiles by calculating a distance matrix between the samples based on the Euclidian distance, i.e. the squared difference, between each pair of profiles [32]. Similarity of the total microbiota composition based on the PITChip profiles was assessed by calculating Pearson's product moment correlation (Pearson's correlation).

Animal housing and diet

Two sets of samples were analyzed with the PITChip. Colonic digesta samples used for the comparison of the PITChip with microbiota profiling by pyrosequencing and quantitative PCR analysis of 16S rRNA gene fragments were taken from three different piglets (Piétrain x Landrace x Large-White), two males and one female (BW = 2 kg ± 0.12 kg), that during the suckling period (from day 5 after birth until weaning at day 28) received an oral saline solution of sodium butyrate (SB) supplied at a concentration of 3 g/kg of the daily milk dry matter intake (see Chapter 6 for additional details of the animal experiment). At an age of 28 days supplementation stopped and animals were weaned on a standardized starter diet (EU-reference diet). Piglets were sacrificed at the end of the weaning period (day 40) and samples from the colon were collected and kept at -20°C. The experimental procedures were carried out according to the guidelines of the French Ministry for Animal Research.

The second set of samples (18 samples) was from 9 crossbred (Yorkshire × Dutch Landrace) piglets, of both genders, that were weaned at 28 days of age at approximately 8 kg initial BW, and that were part of a larger experiment with 44 piglets. Experimental piglets were housed individually in pens (1 × 1.5 m) within a special compartment of the Experimental Unit of the Animal Sciences Group, Wageningen University and Research Centre, Lelystad, The Netherlands. The effects of 2 combinations of acidifiers, mix of organic acids 1 (OA1; formic acid, phosphoric acid, citric acid and benzoic acid) and mix of organic acids 2 (OA2; that is a

combination of OA1 plus a blend of formic acid, acetic acid, lactic acid, phosphoric acid and citric acid), in diets for young pigs were tested using a completely randomized design, in which three treatments (control, OA1 and OA2) were implemented over a period of three weeks post-weaning (Table 2).

Table 2. Ingredients of the experimental diets. Components differing between the three diets are indicated in bold.

Ingredient (g/kg feed)	Basal diet	Basal diet + 0.8% OA1[2]	Basal diet + 0.8% OA1 + 0.4% OA2[3]
	Starter (wk 1-3 post-weaning)	Starter (wk 1-3 post-weaning)	Starter (wk 1-3 post-weaning)
Barley meal	300.0	300.0	300.0
Wheat meal	297.0	297.0	297.0
Peas (44% starch)	50.0	50.0	50.0
Whey powder	80.0	80.0	80.0
Wheat bran	25.0	25.0	25.0
Soybean concentrate	40.0	40.0	40.0
Maize starch	**40.0**	**36.57**	**34.62**
Potato protein, purified	50.0	50.0	50.0
Maize gluten meal	22.0	22.0	22.0
Sunflower meal	25.0	25.0	25.0
Limestone	**10.2**	**9.8**	**6.9**
Mono Ca-phosphate	**7.80**	**5.95**	**4.80**
Trace min.-vit. premix[1]	4.0	4.0	4.0
Methionine (99%)	1.1	1.1	1.1
L-lysine-HCl (79%)	3.4	3.4	3.4
Tryptophan	0.3	0.3	0.3
Threonine (98%)	0.3	0.3	0.3
Palm oil + soybean oil	31.0	31.0	31.0
Molasses	10.1	10.1	10.1
NaCl	**2.80**	**0.48**	**0.48**
Mix OA1	**0.0**	**8.0**	**8.0**
Mix OA2	**0.0**	**0.0**	**4.0**
Total	1000.0	1000.0	1000.0

[1]This trace mineral-vitamin premix (0.4%) supplies per kg diet as follows: vit. A (retinol): 1750 IU, vit. D3 (cholecalciferol): 200 IU, vit. E (tocopherol): 11 IU, vit. K1 (phylloquinone): 0.5 mg, vit. B1 (thiamin): 1.0 mg, vit. B2 (riboflavin): 4 mg, d-pantothenic acid: 9 mg, niacin (vit. B3, nicotinic acid): 12.5 mg (available), biotin (vit. H): 50 µg, vit. B12 (cyanocobalamin): 15 µg, folic acid (folacin): 0.3 mg, vit. B6 (pyridoxin): 1.5 mg, choline: 400 mg, Fe: 80 mg, Zn: 54 mg, Mn: 30 mg, Co: 0.15 mg, I: 0.14 mg, Se: 0.25 mg, antioxidants (E310,320,321): 50 mg, and maize starch as carrier. [2]OA1: mix of organic acids 1; [3]OA2: mix of organic acids 2.

The experimental diet for the starter period (weeks 1-3 post-weaning) contained 21.8% of crude protein. The diets were allotted to particular animals and offered *ad libitum* in addition to free access to tap water from drinking-nipples, as to match typical Dutch feeding systems for pigs. The delivery rate of water was 1100 cm^3/min and Colistine sulphate (60 mg/L water) was added to the drinking water on days 0-3 post-weaning only. On days 18, 20 or 22 post-weaning, the pigs were euthanized with an intravenous injection of Nembutal and digesta samples from the distal ileum and proximal colon were collected. Handling of animals and experimental layout were reviewed and approved by the ASG-Lelystad Animal Care and Use Committee (Lelystad, The Netherlands), and were executed in accordance with Dutch legislation on the use of experimental animals.

454 Titanium pyrosequencing

V1-V6 amplification: Amplicons from the V1-V6 region of 16S rRNA genes were generated by PCR using the non-degenerated primer Bact-27-for (5´-GTTTGATCCTGGCTCAG-3´; [33]) in combination with the degenerate reverse primer 1061-rev (5´-RRCACGAGCTGACGAC-3´; [34]), for each of the three colon samples from pigs receiving SB. To facilitate 454 pyrosequencing using the titanium chemistry, each forward primer carried the 454 titanium adaptor A and an 'NNNN' barcode sequence at the 5' end, where NNNN is a tag of four nucleotides that did not start with G nor have triple successive identical bases (CCC or TTT). The reverse primer carried the 454 titanium adaptor B at the 5'end. Adaptor and barcode sequences were kindly provided by GATC Biotech (http://www.gatc-biotech.com).

PCRs were performed using the thermocycler GS0001 (Gene Technologies, Braintree, U.K.) in a final 50 µl volume containing 1x PCR buffer, 1 µl PCR grade nucleotide mix, 2.4 U of Faststart Taq DNA polymerase (Roche, Diagnostics GmbH, Mannheim, Germany), 200 nM of a forward and the reverse primer (Biolegio BV, Nijmegen, The Netherlands), and 0.2 ng/µl of template DNA. The PCR program used consisted of a 95°C pre-denaturation step during 5 min, 35 cycles of denaturation at 95°C during 5 sec, annealing step at 56°C during 40 sec and elongation at 72°C during 70 sec and a final extension step at 72°C during 10 min. The size of the PCR products was checked by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. After purification of the PCR products with the ZR-96 DNA Clean and Concentrator kit (Zymo Research, Orange, CA, USA) DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer.

Purified PCR products together with similarly prepared samples (52 amplicons in total) from additional projects (van den Bogert et al., in preparation; Derrien et al., in preparation) were equimolarly mixed to a total amount of 7500 ng with a final DNA concentration of 100 ng/µl. The pooled DNA was subsequently sent to GATC-Biotech for pyrosequencing on half a picotiterplate using a Genome Sequencer FLX in combination with the titanium chemistry. Sequencing was performed from adaptor A.

Quality filtering and taxonomic classification of sequences: To the best of our knowledge, there are no published recommendations for quality filtering of reads generated by pyrosequencing using titanium chemistry. Therefore, recommendations [35] for GS FLX pyrosequencing were applied by using an in house Perl script (van den Bogert et al., in preparation) that passed

sequences with exact matches to the forward primer, no ambiguous bases [36], and read-lengths not longer or shorter than 1 SD (standard deviation) from the average sequence length. Additionally, pyrosequencing reads were cured from primer sequences.

Taxonomic classification of sequencing reads was done using a locally installed version of the Ribosomal Database Project (RDP) Classifier [37], which produced classifications into the Bergey's taxonomy [38]. The corresponding assignments differed from those produced by PITChip analysis, which had a standard output of the contributions of the 627 phylotypes, present in the pig ARB database, at Level 0, Level 1, and/or Level 2 in the phylogeny. Therefore, the sequences present in the pig ARB database were exported with corresponding taxonomic assignments and used to train the RDP-classifier. This yielded a classifier that (in combination with a trial multiclassifier provided by the RDP staff) could classify pyrosequencing reads with the same assignments as were produced by PITChip analysis. Moreover, the multiclassifier summarized the assignments per taxon, which facilitated calculation of relative abundances and subsequent construction of microbial profiles for comparison with those that were generated by PITChip analysis.

Quantification of specific microbial groups by quantitative PCR (qPCR)

Quantification of total bacteria, *Firmicutes*, *Bacteroidetes* and *Bifidobacterium* species was done by qPCR, using an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, The Netherlands). The reaction mixture (25 µL) consisted of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 5 µL of template DNA diluted in water (10 or 100 times) to avoid PCR inhibition. All reactions were done in triplicate. The standard curves were produced using serially diluted 16S rRNA gene amplicons obtained from *Lactobacillus casei* (for total bacteria and *Firmicutes*), a *Bacteroidetes* 16S rRNA clone (for *Bacteroidetes*) and *Bifidobacterium longum (DSM 20219*) (*Bifidobacterium* quantification). Universal primers, Bact 1369 (5´-CGGTGAATACGTTCYCGG-3´) and Prok 1492 (5´-GGWTACCTTGTTACGACTT-3´) [39] were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample. For the quantification of 16S rRNA gene copy numbers of *Firmicutes*, *Bacteroidetes* or *Bifidobacterium*, primers Firm934F/Firm1060R (5´-GGAGYATGTGGTTTAATTCGAAGCA-3´, 5´-AGCTGACGACAACCATGCAC-3´) [40], Bact934F/Bact1060R (5´-GGARCATGTGGTTTAATTCGATGAT-3´, 5´-AGCTGACG ACAACCATGCAG-3´) [40], and g-Bifid-F/g-Bifid-R (5´-CTCCTGGAAACGGGTGG-3´, 5´-GGTGTTCTTCCCGATATCTACA-3´) [41] were used, respectively. Quantitative PCR reactions were performed as follows: 95ºC for 3 min, 40 cycles of 95ºC for 15 sec, 56ºC for 30 sec and 72ºC for 30 sec and a two final steps of 95ºC and 60ºC for 1 min each (for total bacteria); 50ºC for 2 min, 95ºC for 10 min and 40 cycles of 95ºC for 15 sec and 60ºC for 1 min and two final steps of 95ºC and 60ºC for 1 min each (for *Firmicutes* and *Bacteroidetes*), and 95ºC for 5 min, 40 cycles of 95ºC for 30 sec, 55ºC for 30 sec and 72ºC for 40 sec and two final steps of 72ºC for 10 min and 55ºC for 30 sec (for *Bifidobacterium*). Melting curve analysis of amplicons was done after amplification.

Statistical analysis

The number of OTUs, and total species richness estimations Chao1 [42] and Abundance-based

Coverage Estimators (ACE; [43, 44]) for the quality filtered sequences were calculated for each sample using ESPRIT [45] with default settings (without removing low quality reads) at a distance level of 0.02.

Multivariate analysis was applied for microarray data interpretation. In order to relate changes in microbial profiles to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) [46]. RDA is a multivariate direct gradient analysis method that allows ordering of samples and taxa (i.e. phylogenetic groups) considering that species have linear relationships to environmental variables [47]. The sum of hybridization signal intensities for 144 Level 2 groups, were used as predictors. Treatment classes and sampling sites were introduced as nominal environmental variables and co-variables, respectively, by introducing them as 0 or 1 (dummy variables). The redundancy analysis was performed focusing on inter-samples correlation and the Monte Carlo permutation test was applied [48] to decide whether treatment had a statistically significant influence on the microbiota composition. Since the animal experiment had a randomized design, unrestricted permutation was used that yields completely random permutations [49]. Treatment was considered to interfere significantly with microbiota composition at p-values < 0.05. Diagrams were plotted as triplots using CanoDraw. Student's t-test was used to evaluate significant differences in relative abundance of phylogenetic groups between treatments. The p-values were calculated as two tailed and were considered significant below 0.05 and highly significant below 0.01.

Diversity of microbial profiles obtained by PITChip analysis was expressed as Simpson's reciprocal index of diversity (1/D) [50]. Diversity was calculated with the equation $\lambda = 1/\Sigma Pi^2$, where Pi is the proportion of i^{th} taxon. This is the proportion of each probe signal compared to the total signal for each sample. A higher Simpson's index value indicates a higher degree of diversity. Pearson's product moment correlation (Pearson's correlation) reflects the degree of linear relationship between analyzed data sets. It was used to identify similarities of the total microbiota composition based on the PITChip profiles and similarities between different phylogenetic groups. To this end, Pearson's correlation was calculated for sets of probes corresponding to the different phylogenetic groups.

Results

Design of the PITChip

To allow for the high-throughput and comprehensive profiling of microbiota residing in the GI tract of pigs, the Pig Intestinal Tract Chip (PITChip) was developed using a generic strategy previously reported for the design of the Human Intestinal Tract Chip (HITChip) [24]. A pig GI tract-specific SSU rRNA sequence database was established, comprising approximately 1100 SSU rRNA gene sequences previously retrieved from pig intestinal samples, or corresponding to isolates obtained from this environment. These sequences were used to construct a consensus tree that was evaluated by maximum-parsimony and distance matrix analyses with the respective ARB tools ([25]; Fig. 1).

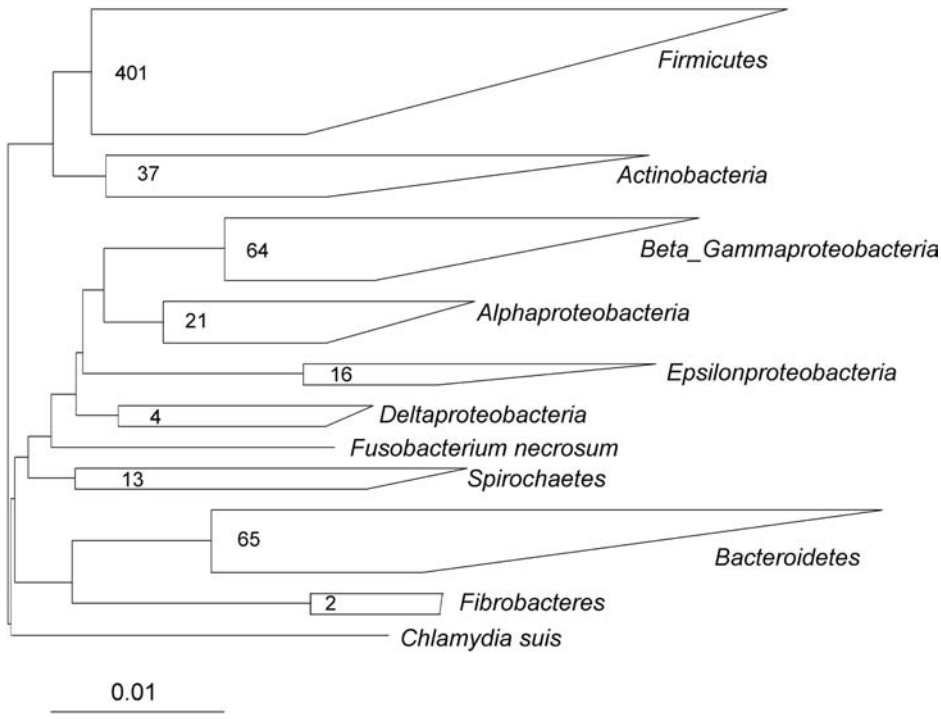

Figure 1. SSU rRNA-based phylogenetic tree of the unique phylotypes found in the pig gastrointestinal tract. Numbers of distinct phylotypes are given for each phylum.

The distance matrix analyses allowed us to group all sequences into a non-redundant set of 627 unique phylotypes based on a threshold of 98% sequence identity. A detailed analysis of the redundancy of the corresponding variable regions of the 16S rRNA gene showed that regions V1, V2 and V6 had the highest number of unique sequences (> 90%; Table 1). From these, the V1 and V6 regions were selected for the design of oligonucleotide probes. To this end, variable region sequences, which ranged from 41 to 56 nucleotides in length, were divided in 3 overlapping segments of 24 nucleotides, yielding six probes for each of the 627 OTUs. This resulted in a total of 3762 probes with melting temperatures (Tm) predicted to range between 44.2ºC and 76ºC (Fig. 2A). In order to further homogenize the hybridization behavior of the probes, their length was adjusted by addition or removal of nucleotides (maximum six) at either end to maximize the number of probes falling in a narrow Tm range of 60.9 ± 2.5°C, which is in the range of predicted error of the algorithm used for Tm prediction [51]. After this optimization, the Tm of 99% of the probes was within the desired limits (Fig. 2B).

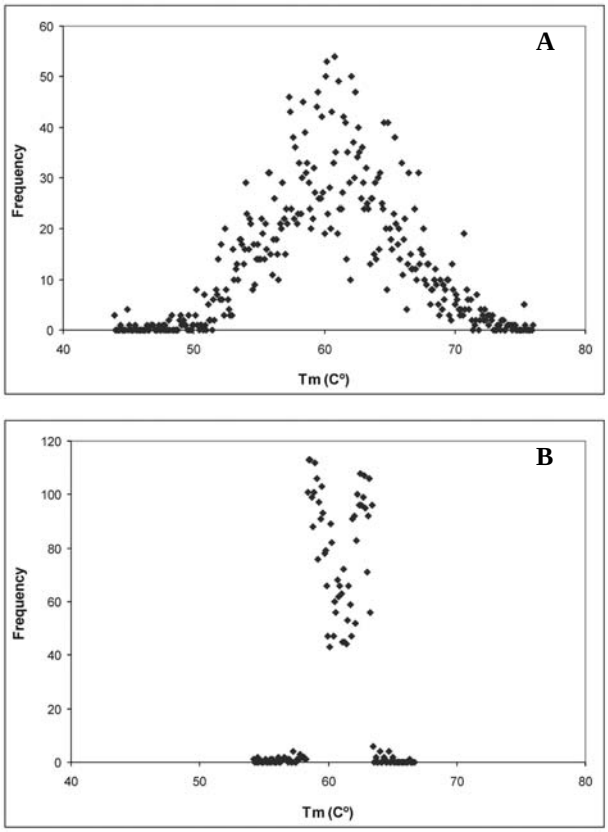

Figure 2. Distribution of melting temperatures of probes predicted using the nearest neighbor algorithm described by SantaLucia [27] with 0.5M as sodium ion concentration. **A)** Initial situation where 41.1% of the probes have a Tm in the range 58.4 to 63.4 (60.9 ± 2.5) °C. **B)** Predicted melting temperatures after iterative adjustment of probe length. 99% of the probes' melting temperatures are in the range 58.4 to 63.4 (60.9 ± 2.5) °C.

Subsequently, the complete set of 3762 probes was checked for probe redundancy, as it has been shown previously for the HITChip that closely related phylotypes share at least to some extent identical probes [24] . This resulted in a final, non-redundant, set of 2985 probes that were printed in quadruplicate on Agilent microarrays of the 8×15K custom array format.

From the 627 phylotypes, the phyla with the highest number of OTUs were the *Firmicutes* with 401 different sequences (64% of the total number), followed by the *Proteobacteria* (105 OTUs, 17%), *Bacteroidetes* (65 OTUs, 10%) and the *Actinobacteria* with 37 unique sequences (6%).

Technical reproducibility is essential for the generation of robust data, especially when applying high-throughput analysis tools such as the PITChip diagnostic microarray. Therefore, the reproducibility of PITChip experimentation with respect to microarray hybridization, *in vitro* transcription and labeling was tested. The reproducibility values obtained were 0.97 both

for different hybridizations and for different labeling reactions and 0.99 for different *in vitro* transcriptions (Fig. 3). Moreover, the median reproducibility of 147 samples analyzed was 0.992 (data not shown).

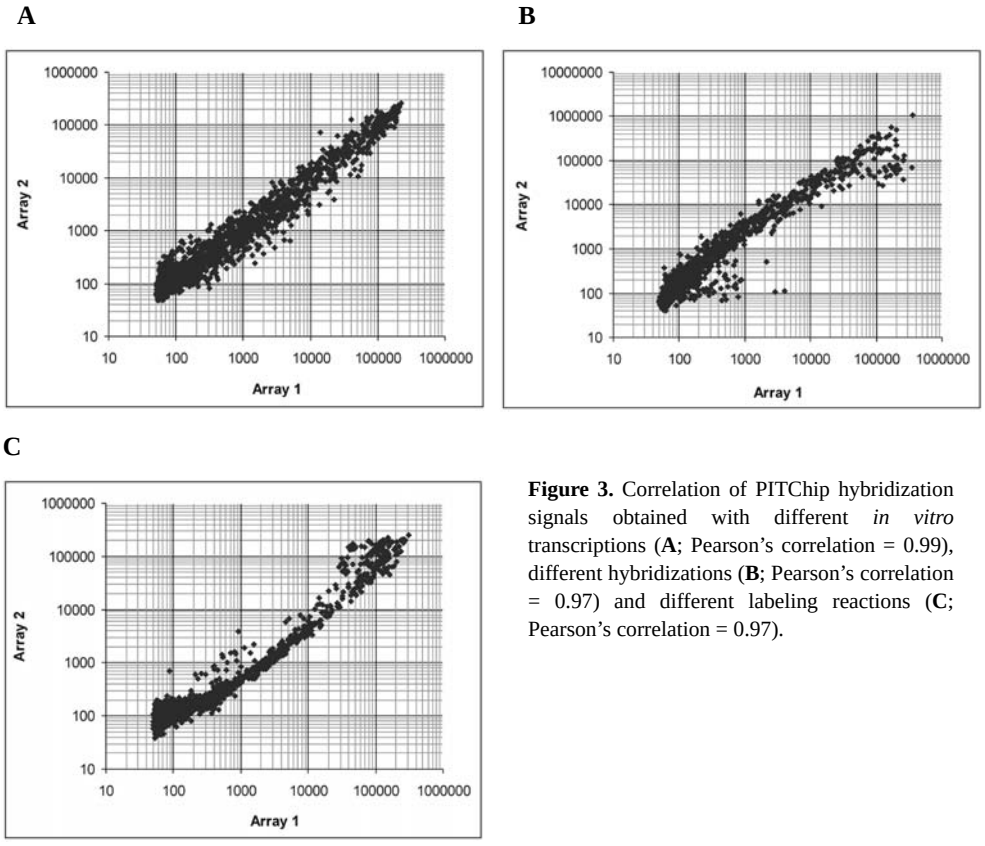

Figure 3. Correlation of PITChip hybridization signals obtained with different *in vitro* transcriptions (**A**; Pearson's correlation = 0.99), different hybridizations (**B**; Pearson's correlation = 0.97) and different labeling reactions (**C**; Pearson's correlation = 0.97).

Comparison of the PITChip with 454 pyrosequencing and quantitative PCR

In order to benchmark PITChip performance, microbiota profiles generated by array analysis were compared to those obtained by pyrosequencing of PCR-amplified 16S rRNA gene fragments as well as 16S rRNA gene-targeted qPCR of selected groups. The samples used for this comparison were colon digesta from 3 different piglets that received a sodium butyrate supplementation during the suckling period (Chapter 6).

PITChip analysis resulted in the summed hybridization signal data of 144 level 2 taxonomic groups, of 27 level 1 groups and of 10 level 0 groups, and data on the hybridization signal for each probe. Pyrosequencing analysis yielded in total 15,558 sequences with an average of 5,186 sequences per colon sample. After quality filtering approximately 50% of

the sequences remained, with an average length of 218, 221 and 235 nt in the three samples, respectively (Table 3). At 0.02 distance level, the number of observed OTUs per sample ranged from 694 to 938. The total species richness, as estimated by Chao1 and ACE, ranged from 1242 to 1499 and 1203 to 1481 OTUs, respectively.

Table 3. Characteristics of the sequences obtained by pyrosequencing, before (upper table) and after (lower table) quality filtering.

Characteristics before quality filtering				
Sample	**Nº sequences**	**Total nº bp**	**Average sequence length**	**SD sequence length**
PITChip 1	5423	1208792	222.90	106.98
PITChip 2	4127	1009349	244.57	104.31
PITChip 3	6008	1361299	226.58	103.66

Characteristics after quality filtering							
Sample	**Nº sequences**	**Total nº bp**	**Average sequence length**	**% remaining quality filtered sequences**	**OUT**	**Chao1**	**ACE**
PITChip 1	2704	598416	221.30	49.86	890	1499.19	1480.86
PITChip 2	2124	499109	234.98	51.46	694	1241.81	1203.35
PITChip 3	2999	652638	217.61	49.91	938	1426.52	1451.73

The modified RDP classifier was used to assign sequences that passed quality filtering to the 144 different level 2 taxonomic groups and higher phylogenetic levels. The resulting classification summary was used to construct a plot to correlate PITChip probe intensities with pyrosequencing reads at two taxonomic ranks, namely genus level and class level. This revealed satisfying agreement of the two approaches, especially at class level, with a Pearson's correlation of 0.7 (Fig. 4). In contrast, correlation at higher phylogenetic resolution was less pronounced, mostly due to the higher number of phylogenetic groups detected by the PITChip, probably indicating a higher dynamic range of this tool as compared to pyrosequencing at the sequencing depth used in this study.

The RDP data was used as well to generate microbial profiles (data not shown). Relative contribution was calculated of level 1 groups in the microbiota profiles generated by the PITChip (samples PITChip 1, PITChip 2 and PITChip 3) and by 454 titanium pyrosequencing (samples Pyroseq 1, Pyroseq 2 and Pyroseq 3). In general, PITChip profiles were more diverse than those generated by pyrosequencing, indicating a higher depth of analysis (Table 4). Moreover, several predominant groups of the profiles seemed to have different weight depending on the technique. For example, both *Bacteroidetes* and *Bacilli* seemed more predominant in the pyrosequencing profiles than in the PITChip data. On the other hand *Clostridium* cluster IV appeared to be more abundant in the PITChip profiles while *Clostridium* cluster XIVa was comparable between profiles. Groups like *Clostridium* cluster XIVb and *Gammaproteobacteria* did not appear or with very low relative abundance in the pyrosequencing profiles.

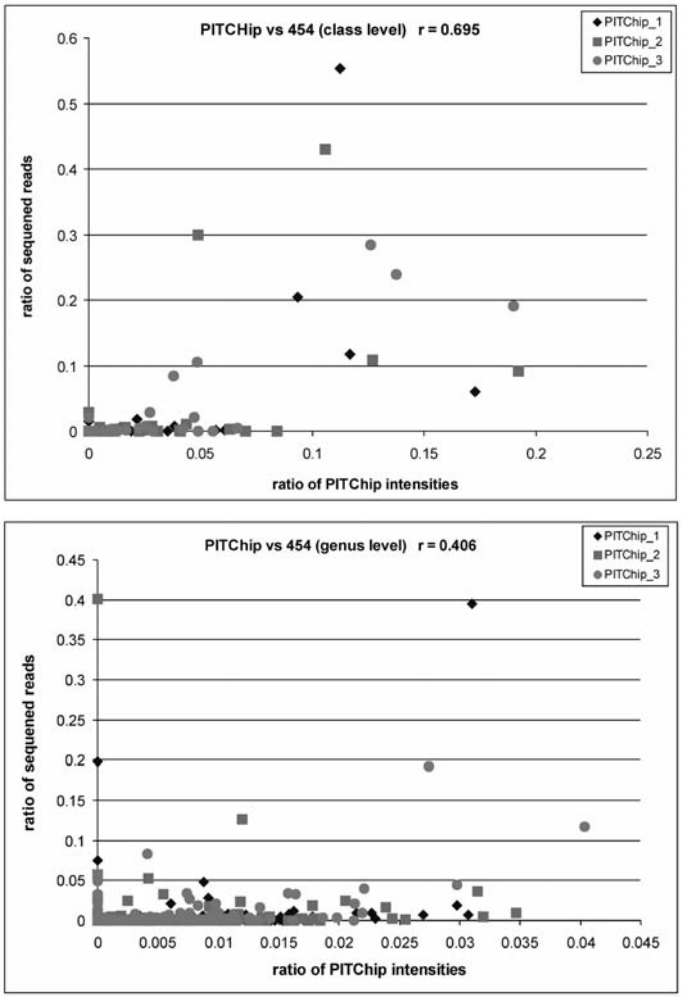

Figure 4. Comparisons of ratios of PITChip spot intensities and number of pyrosequencing reads for two taxonomic ranks, class- and genus level. The average Pearson's correlations are shown for each rank.

It was observed that in the PITChip profiles of the three samples used for the benchmarking, the *Proteobacteria* group was significantly higher ($p = 0.05$) than the *Bacteroidetes*, but this was not the case in the pyrosequencing profiles. The *Actinobacteria* were also one of the predominant groups in the PITChip profiles and both the *Proteobacteria* and the *Actinobacteria* were significantly ($p \leq 0.001$) higher with the PITChip analysis than with the sequencing analysis (data not shown).

Table 4. Comparison of Simpson's index of diversity for samples analyzed both by the PITChip and by pyrosequencing.

Sample	Simpson's index of diversity
Pyroseq_1	4.82
PITChip_1	79.18
Pyroseq_2	6.34
PITChip_2	73.43
Pyroseq_3	10.40
PITChip_3	80.37

Subsequently, the relative abundances of microbial groups observed based on PITChip and pyrosequencing analyses were in several cases compared to those obtained by group-specific qPCR, focusing on specific groups including *Firmicutes*, *Bacteroidetes* and *Bifidobacterium*. Average relative abundances of *Firmicutes* and *Bacteroidetes* were not significantly different between either of the three techniques (Fig. 5). Only for the level 2 group *Bifidobacterium* a statistically significantly higher ($p = 0.001$) relative abundance was found with the PITChip as compared to the other two approaches, albeit at a relatively low level of abundance, suggesting superior sensitivity of the microarray tool (Fig. 5).

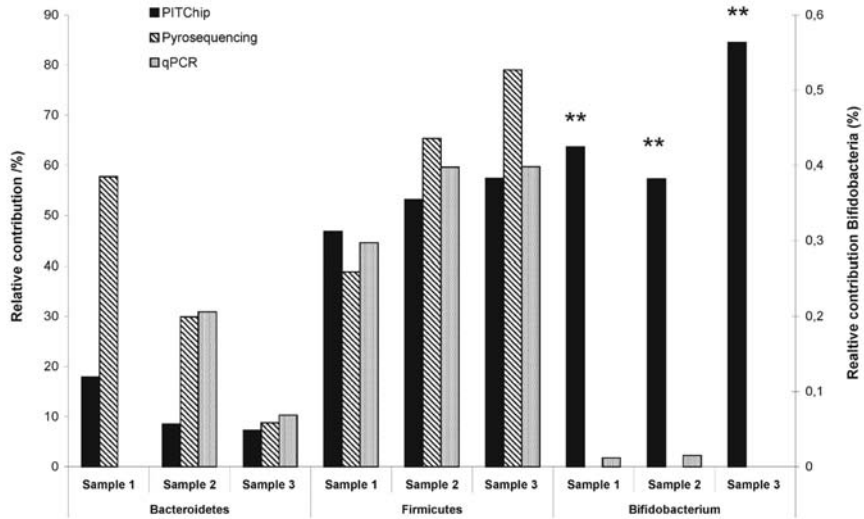

Figure 5. Comparison of the relative contribution of three different microbial groups obtained using PITChip, pyrosequencing and qPCR techniques. The average of PITChip relative contributions for the *Bifidobacterium* is significantly higher than observed by pyrosequencing or qPCR (** indicates that the difference is significant, with a p-value of 0.001).

Application of the PITChip: analysis of piglet digesta samples

In order to demonstrate the ability of the PITChip to provide high-resolution data on the effect of dietary treatments on pig intestinal microbiota, ileal and colonic digesta samples were analyzed from a total of 18 piglets that were included in an animal experiment testing the influence of different organic acidifiers. A first redundancy analysis of the microbial composition data obtained with the PITChip was performed projecting the summed hybridization signals of 144 phylogenetic groups on the first two RDA axes that had the highest explanatory potential, accounting for 29.5% and 6.1% of the total variance in the microbiota profiles, respectively. This revealed that both the sampling site and the addition of a mix of organic acids (OA1 or OA2) to the diet after weaning had a significant effect ($p < 0.05$) on the microbial composition as compared to the control diet (Fig. 6A). In order to focus only on the effect of the treatment, a subsequent analysis was done treating sampling site, i.e. distal ileum and proximal colon, as co-variables (Fig. 6B). In this second redundancy analysis, separation of samples taken from animals given one of either organic acid mixes and those from control animals was mostly along the first RDA axis, with a significant difference between organic acid treatments and control ($p = 0.014$). Furthermore, although samples from the two organic acids mixtures clustered separately, their difference was not found significant ($p = 0.26$). Co-variables ileum/colon explained 19% of the variation on the data.

In order to identify the bacterial groups responsible for the separate clustering of control piglets and treatment piglets, the summed hybridization signals of each of the 144 level 2 groups were subjected to a Student's T test, comparing each of the treatment groups (OA1 and OA2) with the control group (Table 5).

Interestingly, all the observed changes corresponded to an increase in the hybridization signals in one or both of the treatment groups. In the distal ileum the hybridization intensity of several microbial groups showed a significant change when piglets received organic acid mix OA2. These groups included mainly members of the *Bacteroidetes* phylum, in addition to *Mucispirillum schaedleri*- and *Sphingobacterium thalpophilum*-like organisms within the *Deferribacteres* and *Sphingobacteria*. Organic acids mix 1 (OA1) also caused a significant increase in several groups, which were, however, distributed over a broader range of phyla than with OA2, belonging to the *Bacteroidetes*, *Proteobacteria*, *Spirochaetes* and *Sphingobacteria*. In the proximal colon the microbiota composition seemed more stable, showing a significant increase in only a limited number of microbial groups when the organic acids were added in the diet (Table 5).

Simpson's reciprocal index of diversity indicated that the microbial composition of treated piglets appeared to be more diverse than the one of the control piglets, but this difference was not significant (data not shown). As it could be expected, colon microbial diversity was higher than ileum microbial diversity (187.5 ± 31.9 versus 143.3 ± 21.3).

In order to globally compare overall microbiota profiles of the treated groups and the control group, as well as at class-level (i.e. level 1), pair-wise similarities were calculated using Pearson's correlation. This analysis revealed that total microbiota profiles of the control subjects and those receiving OA1 showed a higher similarity ($90.3 \pm 3.07\%$), but not significant, than the profiles from pigs receiving OA2 ($89.3 \pm 3.37\%$) in the distal ileum (Fig. 7A). A similar trend was observed for the proximal colon samples ($87.1 \pm 4.78\%$ between control and OA1

and 84.2 ± 5.58% between control and OA2; Fig. 7B). Similarities of the microbial profiles of the two treatments were in the same range, and amounted to 90.4 ± 3.35% and 86.8 ± 3.89% for distal ileum and proximal colon, respectively. Similarities within the treatment groups were 91 ± 1.31%, 91.2 ± 0.92% and 90.6 ± 0.77% for the control, OA1 and OA2 groups respectively, for the distal ileum, and 87.3 ± 4.87%, 89.3 ± 3.22% and 85.5 ± 2.43% for the same treatment groups for the proximal colon.

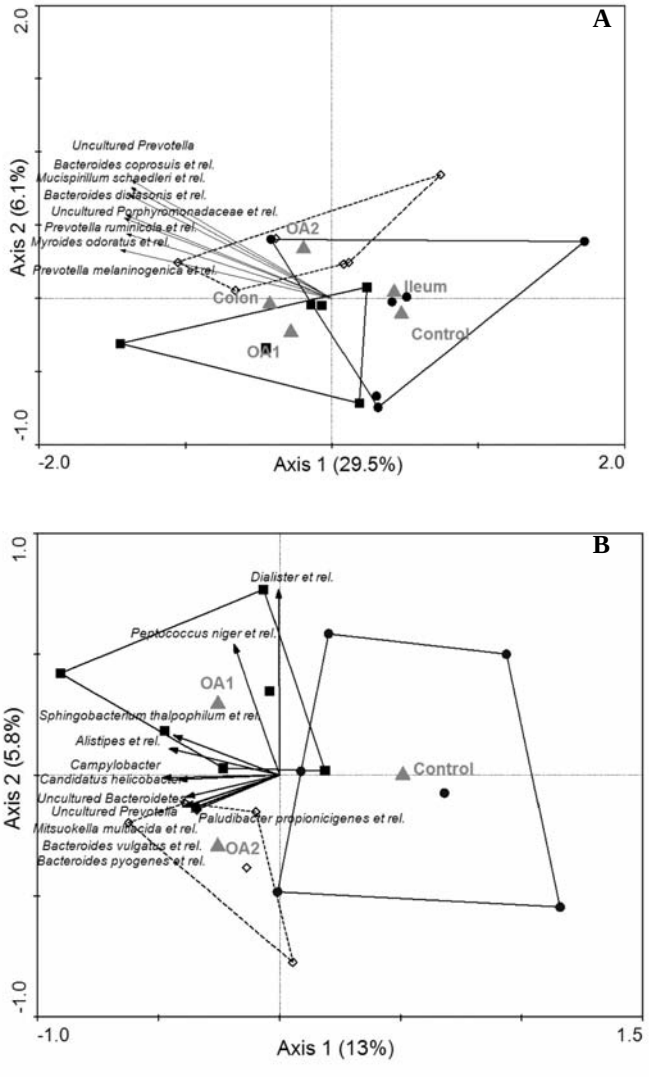

Figure 6. RDA triplots of the microbiota composition in ileal and colonic digesta samples of piglets receiving a control diet (●), piglets receiving either mix of organic acids 1 (OA1, ■) or mix of organic acids 2 (OA2, ◊), expressed as the summed hybridization signal of 144 phylogenetic groups (level 2). In A, both sampling sites (ileum, colon) and treatments (control, OA1 and OA2) are treated as nominal variables represented by centroids (▲). In B, sampling sites are treated as co-variables and treatments as nominal variables. In both plots microbial groups contributing at least 60% and 29%, respectively, to the explanatory axes are represented as vectors, and the nominal environmental variables (treatments: OA1, OA2 and control) are represented by centroids (▲). Percentage values at the axes indicate contribution of the principal components to the explanation of the total variance of the species in the dataset. Co-variables ileum/colon explained 19% of the variation. Monte Carlo permutation test indicated that the control diet had a significant effect on the variation of microbiota composition with a p-value of 0.014.

Table 5. SSU rRNA-based groups for which the hybridization signals were found to be significantly different between samples from piglets that were fed a control diet and samples from piglets receiving one of two mixes of organic acids (OA1 and OA2). The p-values were calculated in comparison to the control and using the sum of intensities (hybridization signals) of 144 level 2 phylogenetic groups. All significant changes are indicated in bold and corresponded to an increase in relative abundance due to the organic acid treatment.

Higher taxonomic Group	Group	Distal Ileum		Proximal Colon	
		p-value		p-value	
		(OA1)	(OA2)	(OA1)	(OA2)
Bacilli	*Lactobacillus gasseri*-like	0.628	0.292	0.414	**0.022**
Bacteroidetes	*Alistipes*-like	**0.005**	0.060	0.130	0.146
	Bacteroides coprosuis-like	0.278	**0.032**	0.471	0.285
	Bacteroides distasonis-like	0.239	**0.014**	0.821	0.518
	Bacteroides fragilis-like	0.188	**0.001**	0.760	0.551
	Bacteroides pyogenes-like	0.253	**0.025**	0.858	0.585
	Bacteroides vulgatus-like	0.252	**0.012**	0.748	0.508
	Paludibacter propionicigenes-like	0.252	**0.011**	0.707	0.485
	Prevotella ruminicola-like	0.265	**0.039**	0.637	0.388
	Uncultured *Bacteroidetes*	0.207	**0.013**	0.628	0.568
	Uncultured *Porphyromonadaceae*	0.226	**0.033**	0.575	0.382
	Uncultured *Prevotella*	0.247	**0.003**	0.505	0.375
Betaproteobacteria	Uncultured *Betaproteobacteria*	**0.050**	0.955	0.975	0.840
Clostridium cluster IX	*Mitsuokella multiacida*-like	0.122	0.332	0.271	**0.015**
Deferribacteres	*Mucispirillum schaedleri*-like	0.279	**0.032**	0.469	0.279
Epsilonproteobacteria	*Campylobacter*	0.072	0.106	**0.041**	0.100
	Helicobacter	**0.015**	0.085	0.172	0.631
Flavobacteria	*Chryseobacterium*-like	0.572	0.705	**0.024**	0.503
Gammaproteobacteria	*Actinobacillus*-like	0.079	0.842	0.383	**0.021**
Sphingobacteria	*Sphingobacterium thalpophilum*-like	**0.009**	**0.023**	**0.050**	0.383
Spirochaetes	*Treponema*-like	**0.040**	0.269	0.340	0.177

Furthermore, we calculated Pearson's correlation for those different level 1 groups, for which significant changes were observed at level 2 with the addition of the organic acids, for both sampling sites (Fig. 7A and 7B). This analysis indicated that in the ileum, profiles of *Betaproteobacteria*, *Deferribacteres*, *Epsilonproteobacteria* and *Spirochaetes* in the treated pigs were very similar to those in the control pigs (> 95%; Fig. 6A). In contrast, ileal profiles within the *Bacteroidetes* and *Sphingobacteria* had lower similarity between the treatment and the control pigs (≤ 90%; Fig. 7A). In the proximal colon, changes in profiles of *Epsilon-* and *Gammaproteobacteria*, *Flavobacteria*, and *Sphingobacteria* and *Bacilli* were only minor (> 91%), whereas the profiles of *Clostridium* cluster IX had a lower similarity with the control group (< 89%; Fig. 7B), albeit not significant.

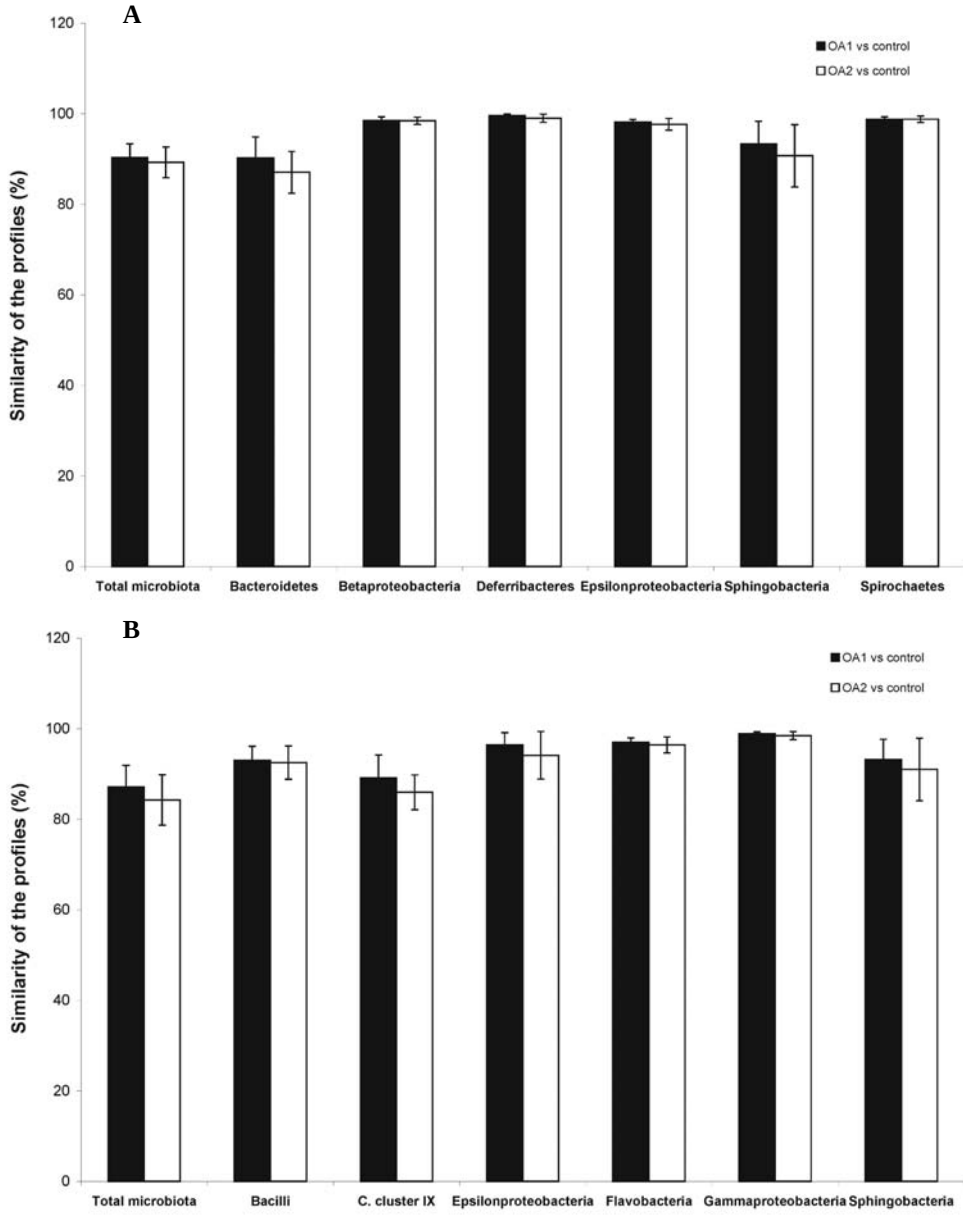

Figure 7. Similarity of the microbial profiles between the control piglets and those of one of the two treatment groups: OA1 (filled bars) and OA2 (empty bars). Similarity in the distal ileum (**A**) was calculated for the total microbiota, *Bacteroidetes*, *Betaproteobacteria*, *Deferribacteres*, *Epsilonproteobacteria*, *Sphingobacteria* and *Spirochaetes;* in the proximal colon (**B**) similarities were calculated for total microbiota, *Bacilli*, *Clostridium* cluster IX, *Epsilonproteobacteria*, *Flavobacteria*, *Gammaproteobacteria* and *Sphingobacteria*.

Discussion

The high complexity of microbial ecosystems and the low coverage of culture-dependent methods have increased the need of more comprehensive molecular techniques to study compositional dynamics of GI tract-associated microbiota and their interactions with the host. During the last years several high-throughput probe design strategies have been reported for the development of diagnostic microarray platforms based on 16S rRNA sequences [18, 52-55]. However, none of them had the capacity to target all of the more than one million SSU rRNA gene sequences present in today's databases (http://www.arb-silva.de), showing the necessity for a design of ecosystem-specific phylogenetic microarrays, like the PITChip. In the pig GI tract database created in ARB, unique phylotypes were defined as groups of sequences that have a sequence similarity of 98% or less with any other SSU rRNA sequence in the database. A cut-off of 98-99% identity is used commonly [56-58] to delimit species, although this value is somewhat arbitrary and has a major effect on the obtained diversity.

Benchmarking of PITChip profiling of porcine intestinal microbiota

Pigs are known for being very similar to humans with respect to the anatomy, physiology and metabolism of the digestive system, being a valuable biomedical model [59]. As in humans, the pig large intestine is an extremely densely populated microbial ecosystem, with total counts of more than 10^{11}- 10^{12} CFU/g digesta in the large intestine [12]. Moreover, the gut microbiota of pigs mainly consists of members of the *Firmicutes* and *Bacteroidetes* divisions [2], as has been found also for mice and humans (more than 90% of all phylogenetic types) [56, 60, 61].

In our microarray analysis, *Firmicutes* dominated in the 3 colon samples with approximately 53% of the total signal (Fig. 5). Contrary to what was found by pyrosequencing, the *Proteobacteria* were the second most abundant group in all samples, detected at higher relative abundance than *Bacteroidetes*. This is in disagreement with other studies, which found that *Firmicutes* and *Bacteroidetes* are the most predominant bacterial groups in the intestinal tract of mammals [2, 56, 61]. In a molecular study of human intestinal microbiota, Wang and co-workers reported as well higher numbers of *Proteobacteria* than of *Bacteroidetes*, albeit only for one subject, which probably was related to a pathology [62]. Furthermore, relative abundance of *Proteobacteria* observed in this study was in line with values previously published for the pig GI tract [2]. Although PITChip analysis showed lower contribution (not significant) of *Bacteroidetes* (11%) as compared to pyrosequencing and qPCR (19% and 20% respectively), the value was consistent to what was found in other studies in pigs [2, 40]. Interestingly, this proportion is lower than that of *Bacteroidetes* found in humans [60] and mice [63] (20-40%).

Bifidobacterium levels described previously for the porcine GI tract, though always low, are very variable and range between 0 and 10^9 CFU/g [64-66]. Therefore, even though significant differences in *Bifidobacterium* levels were detected by PITChip analysis (0.4%) as compared to those found by qPCR and pyrosequencing (0.1% and 0%, respectively), all values are well within the range of those commonly found in the porcine GI tract.

The PITChip used as a high-throughput analysis tool

Because of the ban on the use of antibiotics in animal production in the last years other possible

solutions are being studied. Acidifiers seem to be one of the most promising alternatives to replace antibiotics in post-weaning pig diets because of the large body of evidence regarding their positive effects [67-69].

In the present pilot experiment, PITChip analysis of distal ileum and proximal colon samples from piglets fed with diets supplemented with two different mixes of organic acids during three weeks after weaning, indicated that there was a significant effect of the supplementation on the microbial community as revealed by multivariate analysis. Observed hybridization signals were significantly higher in animals receiving the organic acid supplement for mainly members of the *Bacteroidetes* but also *Firmicutes*, *Proteobacteria*, *Deferribacteres*, *Spirochaetes* and *Sphingobacteria*. In previous studies it has been reported that the addition of organic acids had beneficial effects on piglet microbiota, reducing the numbers of pathogenic bacteria [67, 70] and increasing the number of some beneficial populations including *Lactobacillus* species [71, 72]. This is consistent with our results that showed an increase of *Lactobacillus gasseri* related species. However, we also observed a significant increase in potentially pathogenic bacteria, like *Campylobacter* related species [73]. The fact that most of the phylotypes that increased with the addition of the organic acids belonged to the *Bacteroidetes* could also indicate a beneficial effect of the organic acid treatment, as *Bacteroidetes* is one of the predominant microbial groups in healthy pigs [2, 40]. The increase in populations related to the acid-utilizing *Mitsiokella multiacida* has been reported before when acidifiers were added to the diet [74]. On the other hand, *Bacteroides fragilis* is a major microbial factor, together with other members of the microbiota in the production of mixed infections [75, 76]. The results of the PITChip analysis also confirmed the fact that the microbial diversity in the colon is significantly higher than in the ileum [7, 77].

The work described in this chapter presents the successful development of a high-throughput tool for the comprehensive analysis of pig GI tract microbiota. Diagnostic microarrays such as the PITChip are tools that only provide identification of those microorganisms in environmental samples for which specific probes can be designed based on present knowledge, reinforcing the need for a high-quality and comprehensive, probe target sequence database [78]. To this end, the non-redundant database of pig-associated microbiota generated in the present study provided the necessary basis for the development of the PITChip, allowing a fast and high-resolution fingerprinting of pig gastrointestinal samples. The PITChip allows studying the effect of different parameters on gut microbiota composition, including inter-individual variation, amplitude and direction of spatio-temporal dynamics of porcine microbiota in response to different dietary treatments. A first application of the PITChip in comparing the microbiota from piglets fed with diets with different organic acids additives showed significant differences and supported previous studies with respect to the effect of acidifiers on piglet intestinal microbiota.

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References

1. **Hill, J. E., Seipp, R. P., Betts, M., Hawkins, L., Van Kessel, A. G., Crosby, W. L., and Hemmingsen, S. M.** (2002) Extensive Profiling of a Complex Microbial Community by High-Throughput Sequencing. *Appl. Environ. Microbiol.* 68: 3055-3066.
2. **Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Moller, K.** (2002) Culture-Independent Analysis of Gut Bacteria: the Pig Gastrointestinal Tract Microbiota Revisited. *Appl. Environ. Microbiol.* 68: 673-690.
3. **Pryde, S. E., Richardson, A. J., Stewart, C. S., and Flint, H. J.** (1999) Molecular analysis of the microbial diversity present in the colonic wall, colonic lumen, and cecal lumen of a pig. *Appl. Environ. Microbiol.* 65: 5372-5377.
4. **Gaskins, H. R.** (2001) Intestinal bacteria and their influence on swine growth, in *Swine Nutrition*. Lewis, A. J. and Southern, L. L. (ed.). p. 585-608. CRC Press, Boca Raton, FL.
5. **Ewing, W. and Cole, D.** (1994) The Living Gut. An Introduction to Micro-organisms in Nutrition. Context Publication.
6. **Jensen, B. B. and Jorgensen, H.** (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl. Environ. Microbiol.* 60: 1897-904.
7. **Berg, R. D.** (1996) The indigenous gastrointestinal microflora. *Trends Microbiol.* 4: 430-435.
8. **Isolauri, E., Sutas, Y., Kankaanpaa, P., Arvilommi, H., and Salminen, S.** (2001) Probiotics: effects on immunity. *Am. J. Clin. Nutr.* 73: 444S-450S.
9. **Chowdhury, S. R., King, D. E., Willing, B. P., Band, M. R., Beever, J. E., Lane, A. B., Loor, J. J., Marini, J. C., Rund, L. A., Schook, L. B., Van Kessel, A. G., and Gaskins, H. R.** (2007) Transcriptome profiling of the small intestinal epithelium in germfree versus conventional piglets. *BMC Genomics*. 8: 215.
10. **Danielsen, M., Hornshoj, H., Siggers, R. H., Jensen, B. B., van Kessel, A. G., and Bendixen, E.** (2007) Effects of bacterial colonization on the porcine intestinal proteome. *J. Proteome. Res.* 6: 2596-604.
11. **Willing, B. P. and Van Kessel, A. G.** (2007) Enterocyte proliferation and apoptosis in the caudal small intestine is influenced by the composition of colonizing commensal bacteria in the neonatal gnotobiotic pig. *J. Anim. Sci.* 85: 3256-66.
12. **Ewing, W. and Cole, D.** (1994) The Living Gut: An Introduction to Micro-organisms in Nutrition. Context Publication, Ireland, UK.
13. **Stewart, C. S.** (1997) Microorganisms in hindgut fermentors, in *Gastrointestinal Microbiology*. Mackie, R. I., White, B. A., and Isaacson, R. E. (ed.). p. 142-186. Chapman and Hall, New York.
14. **Tannock, G. W.** (2001) Molecular assessment of intestinal microflora. *Am. J. Clin. Nutr.* 73: 410S-414.
15. **Vaughan, E. E., Schut, F., Heilig, H. G., Zoetendal, E. G., de Vos, W. M., and Akkermans, A. D.** (2000) A molecular view of the intestinal ecosystem. *Curr. Issues Intest. Microbiol.* 1: 1-12.
16. **Guschin, D. Y., Mobarry, B. K., Proudnikov, D., Stahl, D. A., Rittmann, B. E., and Mirzabekov, A. D.** (1997) Oligonucleotide microchips as genosensors for determinative and environmental studies in microbiology. *Appl. Environ. Microbiol.* 63: 2397-2402.
17. **Gentry, T. J., Wickham, G. S., Schadt, C. W., He, Z., and Zhou, J.** (2006) Microarray applications in microbial ecology research. *Microb. Ecol.* 52:159-175
18. **DeSantis, T. Z., Brodie, E. L., Moberg, J. P., Zubieta, I. X., Piceno, Y. M., and Andersen, G. L.** (2007) High-density universal 16S rRNA microarray analysis reveals broader diversity than typical clone library when sampling the environment. *Microb. Ecol.* 53: 371-383.
19. **Wagner, M., Smidt, H., Loy, A., and Zhou, J.** (2007) Unravelling microbial communities with DNA-microarrays: challenges and future directions. *Microb. Ecol.* 53: 498-506.
20. **Bodrossy, L. and Sessitsch, A.** (2004) Oligonucleotide microarrays in microbial diagnostics. *Curr. Opin. Microbiol.* 7: 245-54.
21. **Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H., and Wagner, M.**

(2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* 68: 5064-5081.

22. **Mitterer, G., Huber, M., Leidinger, E., Kirisits, C., Lubitz, W., Mueller, M. W., and Schmidt, W. M.** (2004) Microarray-based identification of bacteria in clinical samples by solid-phase PCR amplification of 23S ribosomal DNA sequences. *J. Clin. Microbiol.* 42: 1048-57.
23. **Palmer, C., Bik, E. M., Digiulio, D. B., Relman, D. A., and Brown, P. O.** (2007) Development of the human infant intestinal microbiota. *PLoS Biol.* 5: e177.
24. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
25. **Ludwig, W., Strunk, O., Westram, R., Richter, L., Meier, H., Yadhukumar, Buchner, A., Lai, T., Steppi, S., Jobb, G., Forster, W., Brettske, I., Gerber, S., Ginhart, A. W., Gross, O., Grumann, S., Hermann, S., Jost, R., Konig, A., Liss, T., Lussmann, R., May, M., Nonhoff, B., Reichel, B., Strehlow, R., Stamatakis, A., Stuckmann, N., Vilbig, A., Lenke, M., Ludwig, T., Bode, A., and Schleifer, K.-H.** (2004) ARB: a software environment for sequence data. *Nucleic Acids Res.* 32: 1363-1371.
26. **Collins, M. D., Lawson, P. A., Willems, A., Cordoba, J. J., Fernandez-Garayzabal, J., Garcia, P., Cai, J., Hippe, H., and Farrow, J. A.** (1994) The phylogeny of the genus Clostridium: proposal of five new genera and eleven new species combinations. *Int. J. Syst. Bacteriol.* 44: 812-26.
27. **SantaLucia, J., Jr.** (1998) A unified view of polymer, dumbbell, and oligonucleotide DNA nearest-neighbor thermodynamics. *Proc. Natl. Acad. Sci. U. S. A.* 95(4): 1460-5.
28. **Rajilić-Stojanović, M.** (2007) Diversity of the human gastrointestinal microbiota: novel perspectives from high throughput analyses, in *Laboratory of Microbiology*. Wageningen University, Wageningen, The Netherlands.
29. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
30. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) Calculating melting temperatures for perfectly matched hybrids between oligonucleotides and their target sequences. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
31. **Bolstad, B. M., Irizarry, R. A., Astrand, M., and Speed, T. P.** (2003) A comparison of normalization methods for high density oligonucleotide array data based on variance and bias. *Bioinformatics*. 19: 185-93.
32. **Carter, R. L.** (1989) On the partitioning of squared Euclidean distance and its application in cluster analysis. *Phsychometrika*. 54: 9-23.
33. **Felske, A. and Weller, R.** (2000) Cloning 16S rRNA genes and utilization to type bacterial communities. *Molecular Microbial Ecology Manual*. Akkermans, A. D. L., Van Elsas, J. D., and De Bruijn, F. J. (ed.). Vol. 5, 3.3.3: 1-16. Kluwer Academic Publishers, Dordrecht, The Netherlands.
34. **Andersson, A. F., Lindberg, M., Jakobsson, H., Backhed, F., Nyren, P., and Engstrand, L.** (2008) Comparative analysis of human gut microbiota by barcoded pyrosequencing. *PLoS ONE*. 3: e2836.
35. **Huse, S. M., Huber, J. A., Morrison, H. G., Sogin, M. L., and Welch, D. M.** (2007) Accuracy and quality of massively parallel DNA pyrosequencing. *Genome Biol.* 8: R143.
36. **Adlerberth, I., Ahrne, S., Johansson, M. L., Molin, G., Hanson, L. A., and Wold, A. E.** (1996) A mannose-specific adherence mechanism in *Lactobacillus plantarum* conferring binding to the human colonic cell line HT-29. *Appl. Environ. Microbiol.* 62: 2244-2251.
37. **Wang, Q., Garrity, G. M., Tiedje, J. M., and Cole, J. R.** (2007) Naive Bayesian classifier for rapid assignment of rRNA sequences into the new bacterial taxonomy. *Appl. Environ. Microbiol.* 73: 5261-5267.
38. **Garrity, G. M., Bell, J. A., and Lilburn, T. G.** (2004) Taxonomic Outline of the Procaryotes. Bergey's Manual of Systematic Bacteriology. Second Edition, Release 5.0. Springer-Verlag, New York.
39. **Suzuki, M. T., Taylor, L. T., and DeLong, E. F.** (2000) Quantitative analysis of small-subunit rRNA

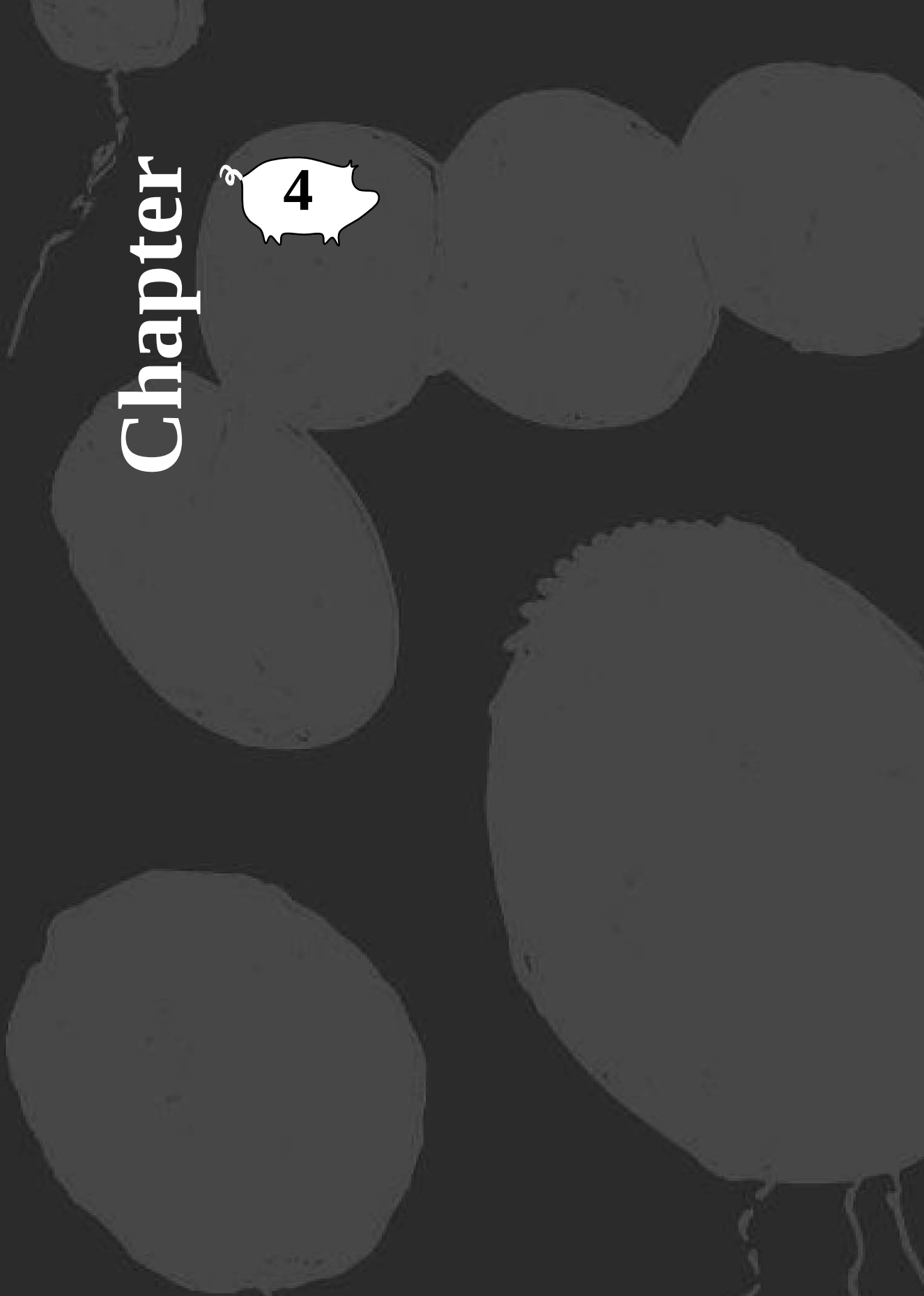
genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* 66: 4605-4614.

40. **Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., and Wang, K.** (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* 47: 367-373.
41. **Matsuki, T., Watanabe, K., Fujimoto, J., Miyamoto, Y., Takada, T., Matsumoto, K., Oyaizu, H., and Tanaka, R.** (2002) Development of 16S rRNA-Gene-Targeted Group-Specific Primers for the Detection and Identification of Predominant Bacteria in Human Feces. *Appl. Environ. Microbiol.* 68: 5445-5451.
42. **Chao, A. and Bunge, J.** (2002) Estimating the number of species in a stochastic abundance model. *Biometrics*. 58: 531-9.
43. **Chao, A. and Lee, S. M.** (1992) Estimating the Number of Classes Via Sample Coverage. *J. Am. Stat. Assoc.* 87: 210-217.
44. **Chao, A., Ma, M. C., and Yang, M. C. K.** (1993) Stopping Rules and Estimation for Recapture Debugging with Unequal Failure Rates. *Biometrika*. 80: 193-201.
45. **Sun, Y., Cai, Y., Liu, L., Yu, F., Farrell, M. L., McKendree, W., and Farmerie, W.** (2009) ESPRIT: estimating species richness using large collections of 16S rRNA pyrosequences. *Nucleic Acids Res*. 37: e76.
46. **Lepš, J. and Šmilauer, P.** (2003) Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
47. **Jongman, R. H. G., Ter Braak, C. J. F., and van Tongeren, O. F. R.** (1995) Data Analysis in Community and Landscape Ecology. Cambridge University Press, New York.
48. **Gittins, R.** (1985) Canonical Analysis. A Review with Applications in Ecology. Springer-Verlag, Berlin.
49. **Ter Braak, C. J. F.** (1990) Update notes: CANOCO version 3.1. p. GLW-Agricultural Mathematics Group, Wageningen.
50. **Simpson, E. H.** (1949) Measurements of diversity. *Nature*. 163: 688.
51. **Panjkovich, A. and Melo, F.** (2005) Comparison of different melting temperature calculation methods for short DNA sequences. *Bioinformatics*. 21: 711-22.
52. **Watanabe, K., Kodama, Y., and Harayama, S.** (2001) Design and evaluation of PCR primers to amplify bacterial 16S ribosomal DNA fragments used for community fingerprinting. *J. Microbiol. Methods*. 44: 253-262.
53. **Chee, M., Yang, R., Hubbell, E., Berno, A., Huang, X. C., Stern, D., Winkler, J., Lockhart, D. J., Morris, M. S., and Fodor, S. P. A.** (1996) Accessing genetic information with high-density DNA arrays. *Science*. 274: 610-614.
54. **Palmer, C., Bik, E. M., Eisen, M. B., Eckburg, P. B., Sana, T. R., Wolber, P. K., Relman, D. A., and Brown, P. O.** (2006) Rapid quantitative profiling of complex microbial populations. *Nucleic Acids Res.* 34: e5.
55. **Wilson, K. H., Wilson, W. J., Radosevich, J. L., DeSantis, T. Z., Viswanathan, V. S., Kuczmarski, T. A., and Andersen, G. L.** (2002) High-density microarray of small-subunit ribosomal DNA probes. *Appl. Environ. Microbiol.* 68: 2535-2541.
56. **Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A.** (2005) Diversity of the human intestinal microbial flora. *Science*. 308: 1635-1638.
57. **Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., and Nelson, K. E.** (2006) Metagenomic analysis of the human distal gut microbiome. *Science*. 312: 1355-1359.
58. **Hayashi, H., Sakamoto, M., and Benno, Y.** (2002) Phylogenetic analysis of the human gut microbiota using 16S rDNA clone libraries and strictly anaerobic culture-based methods. *Microbiol. Immunol.* 46: 535-548.
59. **Pang, X. Y., Hua, X. G., Yang, Q., Ding, D. H., Che, C. Y., Cui, L., Jia, W., Bucheli, P., and Zhao, L. P.** (2007) Inter-species transplantation of gut microbiota from human to pigs. *ISME J.* 1: 156-162.
60. **Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I.** (2005)

Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U. S. A.* 102: 11070-11075.

61. **Ley, R. E., Peterson, D. A., and Gordon, J. I.** (2006) Ecological and evolutionary forces shaping microbial diversity in the human intestine. *Cell*. 124: 837-848.
62. **Wang, X., Heazlewood, S. P., Krause, D. O., and Florin, T. H.** (2003) Molecular characterization of the microbial species that colonize human ileal and colonic mucosa by using 16S rDNA sequence analysis. *J. Appl. Microbiol.* 95: 508-20.
63. **Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I.** (2006) Microbial ecology: Human gut microbes associated with obesity. *Nature*. 444: 1022-1023.
64. **Brown, I., Warhurst, M., Arcot, J., Playne, M., Illman, R. J., and Topping, D. L.** (1997) Fecal numbers of bifidobacteria are higher in pigs fed *Bifidobacterium longum* with a high amylose cornstarch than with a low amylose cornstarch. *J. Nutr.* 127: 1822-1827.
65. **Molbak, L., Thomsen, L. E., Jensen, T. K., Knudsen, K. E. B., and Boye, M.** (2007) Increased amount of *Bifidobacterium thermacidophilum* and *Megasphaera elsdenii* in the colonic microbiota of pigs fed a swine dysentery preventive diet containing chicory roots and sweet lupine. *J. Appl. Microbiol.* 103: 1853-1867.
66. **Mikkelsen, L. L., Bendixen, C., Jakobsen, M., and Jensen, B. B.** (2003) Enumeration of bifidobacteria in gastrointestinal samples from piglets. *Appl. Environ. Microbiol.* 69: 654-658.
67. **Partanen, K. H. and Mroz, Z.** (1999) Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12: 117-145.
68. **Piva, A., Morlacchini, M., Casadei, G., Gatta, P. P., Biagi, G., and Prandini, A.** (2002) Sodium butyrate improves growth performance of weaned piglets during the first period after weaning. *Ital. J. Anim. Sci.* 1: 35-41.
69. **Mroz, Z.** (2005) Organic Acids as Potential Alternatives to Antibiotic Growth Promoters for Pigs. *Adv. Pork. Prod.* 16: 169-182.
70. **Overland, M., Granli, T., Kjos, N. P., Fjetland, O., Steien, S. H., and Stokstad, M.** (2000) Effect of dietary formates on growth performance, carcass traits, sensory quality, intestinal microflora, and stomach alterations in growing-finishing pigs. *J. Anim. Sci.* 78: 1875-1884.
71. **Ravindran, V. and Kornegay, E. T.** (1993) Acidification of Weaner Pig Diets - a Review. *J. Sci. Food. Agric.* 62: 313-322.
72. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
73. **Modolo, J. R., Margato, L. F. F., Gottschalk, A. F., and Lopes, C. A. D.** (1999) Incidence of Campylobacter in pigs with and without diarrhea. *Braz. J. Microbiol.* 30: 19-21.
74. **Tsukahara, T., Koyama, H., Okada, M., and Ushida, K.** (2002) Stimulation of butyrate production by gluconic acid in batch culture of pig cecal digesta and identification of butyrate-producing bacteria. *J. Nutr.* 132: 2229-2234.
75. **Sears, C. L., Myers, L. L., Lazenby, A., and Vantassell, R. L.** (1995) Enterotoxigenic *Bacteroides fragilis. Clin. Infect. Dis.* 20: S142-S148.
76. **Zhang, G. M., Svenungsson, B., Karnell, A., and Weintraub, A.** (1999) Prevalence of enterotoxigenic *Bacteroides fragilis* in adult patients with diarrhea and healthy controls. *Clin. Infect. Dis.* 29: 590-594.
77. **Simpson, J. M., McCracken, V. J., White, B. A., Gaskins, H. R., and Mackie, R. I.** (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods*. 36: 167-79.
78. **Loy, A. and Bodrossy, L.** (2006) Highly parallel microbial diagnostics using oligonucleotide microarrays. *Clin. Chim. Acta*. 363: 106-19.

Chapter 4



Activity and Composition of Piglet Fecal Microbiota after *in vitro* Fermentation in the Presence of Plant Extracts and Bioactive Compounds

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Abstract

A standard diet (control substrate) was incubated *in vitro* together with feces of weaning piglets, supplemented with 23 different PENS at different concentrations. Microbiota dynamics was analyzed using 16S ribosomal RNA gene-targeted DGGE and PITChip (Pig Intestinal Tract microarray) profiling, and fermentation parameters and metabolites were measured. An oregano oil water-soluble extract had a significant effect on fermentation characteristics and microbiota composition. Cumulative gas production, organic matter loss and maximum rate of gas production were lower. Halftime of gas production was delayed and ammonia ($p < 0.05$) and total volatile fatty acid concentration decreased. Oregano oil water-soluble extract lowered overall microbiota diversity and relative abundance of members of *Clostridium* clusters IV and XIVa species but increased the relative abundance of *Bacilli* and *Actinobacteria* groups. In most cases, no significant effect of PENS was observed when included in the diet at doses commonly used in animal production. This study reports the use of two unique techniques (cumulative gas production technique and PITChip) and provides information on fermentation characteristics and effect on fecal microbiota of plant extracts and natural substances. This data, in combination with *in vivo* experiments, is essential for the optimization of dietary strategies involving these plant extracts and natural substances.

Introduction

The gastrointestinal tract (GI) tract of pigs hosts complex microbial communities of commensal and potentially pathogenic species, predominantly comprising facultative and strictly anaerobic microorganisms. These bacterial communities ferment both the ingested feed as well as components secreted by the host into the GI tract. A variety of host-related and environmental factors (e.g. stress, age) and diet, as main source of substrates for metabolism, can modulate bacterial interactions [1] and cause changes in microbiota composition and activity. Furthermore, the autochthonous and non-pathogenic microbiota of the GI tract is considered to be one of the body's important natural defenses against potential pathogens, which is generally referred to as "colonization resistance" [2].

Bacterial colonization of the GI tract of neonatal piglets leads to the establishment of a stable microbiota during the suckling period. However, the introduction of solid food at the time of weaning causes important shifts in microbiota composition [3]. The weaning is a difficult period for piglets due to the experience of important changes in their lives: the separation from the mother, mixing with pigs from other litters in a new environment and the introduction of a less digestible solid diet. Moreover, weaning at an early age, which is common practice in commercial swine production, increases the general level of stress leading to diarrhea and growth reduction [4]. During the last decades, antimicrobial compounds have been used to enhance growth and suppress the activity of the gut microbiota in weaning pigs [5]. However, the appearance of antibiotic resistance in human commensal bacteria [6] motivated a full ban of these additives in the European Union from January 2006, and has triggered the search for alternative strategies. One of these strategies is the stimulation of the autochthonous microbiota to reinforce the colonization resistance, by prebiotics, probiotics and synbiotics [7-10].

Plant extracts and natural substances (PENS) have become an alternative to replace antibiotics in piglets' diets because of their potential benefits in animal and human health [11-13]. It can be expected that these PENS will have a differential effect on the host depending on their mode of action, including their fermentability by intestinal microbiota. To allow for the simultaneous evaluation of many compounds, an *in vitro* screening assessment should be carried out that at the same time is indicative of what could happen *in vivo* [14]. In theory, highly fermentable substances are fermented in the proximal GI tract, less rapidly fermentable substances are fermented in the distal GI tract, whereas non-fermentable PENS will be excreted in the fecal matter [15] and/or taken up by the epithelium. Therefore, before implementing these additives in dietary strategies, it is necessary to assess and combine both *in vitro* and *in vivo* screening assays in order to evaluate the composition and activity of the indigenous microbiota present along the GI tract towards PENS.

To complement analyses assessing the effect of PENS on epithelial cell membrane damage induced by enterotoxigenic *Escherichia coli* K88 in an *in vitro* pig intestinal cell model [16], the present work aimed to test a selection of PENS for their effect on *in vitro* fermentation kinetics (as an additive to a common control substrate), but also to study the effects of these dietary components on bacterial community composition. The effects of PENS addition to a substrate were tested in an *in vitro* batch culture system (cumulative gas production technique) using piglet feces as an inoculum [17]. A subset of samples was selected based on a significant

effect of PENS on fermentation characteristics and on their predicted effect on host and/or microbiota. These samples were analyzed for the effect of PENS on bacterial composition. For this purpose, molecular approaches were used based on PCR-amplified 16S ribosomal RNA (rRNA) gene fragments that were fingerprinted by DGGE (denaturing gradient gel electrophoresis) and phylogenetic microarray (Pig Intestinal Tract Chip) analysis.

Materials and methods

Animals, housing and dietary treatment

Eighteen piglets were selected from four different litters. Healthy individuals were selected for uniformity and on the basis of body weight (minimum BW was 8 kg at weaning). After weaning, at 26 days, animals were placed into one pen and were allowed to adapt to their diets for an 11-days period before fecal samples were collected. The animals received a barley- and wheat-based diet (defined as *EU-reference diet* for studies in the framework of the European *Feed for Pig Health* project) in the form of a meal and had unlimited access to water. The main dietary ingredients are summarized in Table 1. Handling of animals and experimental layout were approved by the ethical committee of Wageningen University and Research Centre, and were executed in accordance with the Dutch legislation on the use of experimental animals.

Table 1. Feed composition of the EU-reference diet

Feedstuff	Amount (%)
Potato protein (Prostar)	5.00
Barley	30.00
Wheat	29.55
Wheat bran	2.50
Sunflower bran CP 20-24	2.50
Peas < 22% CP	5.00
Maize gluten meal > 60	2.20
Maize starch	4.00
Whey proteins P.MSA-27% RE/2% RV	8.00
Soycomil	4.00
Melasse (sugarcane) > 47.5% SU	1.00
Soy oil	1.98
Palm oil	1.10
Limestone	1.20
Mono Calcium Phosphate	0.78
Salt	0.28
Lysine-HCl (L, 79%)	0.34
Methionine (DL, 99%)	0.11
Threonine (L, 98%)	0.03
Tryptophane (L, 98%)	0.03
Farmix (mineral-vitamin premix)	0.40
TOTAL	100

Sampling and preparation of inoculum

Handling of samples and preparation of inoculum was done as described before [18], however a description is given in this section. Fresh fecal samples were obtained from individual animals via rectal stimulation at day 11 post-weaning. Samples were directly placed into screw top flasks pre-flushed with CO_2, and kept on ice during transportation. Fecal samples from individual animals were pooled in a glass beaker (continuously flushed with CO_2) and a pre-warmed saline solution was added (dilution factor 1:10 w/w). This material was homogenized for 60 sec using a hand-mixer and filtered over two layers of cheese cloth. The resulting suspension was used to inoculate the fermentation batches. After inoculation of all bottles, the remainder of the inoculum was sampled for dry matter (DM), organic matter (OM), volatile fatty acids (VFA), ammonia (NH_3), lactate and microbiological analyses. The characteristics of the inoculum are summarized in Table 2.

Table 2. Key characteristics of the fecal inoculum used in the successive runs.*

						Molar VFA proportions[1]			
Dilution factor	**DM** (g/kg)	**OM** (g/kg)	**pH**	**TVFA**	**NH_3**	**AP**	**PP**	**BP**	**BCP**
1:10	20.1	464.9	6.27	16.32	6.37	55.2	22.9	11.7	6.2

* Fermentation end metabolites in mmol/l diluted pooled inocula, DM = dry matter, OM = organic matter, TVFA = sum of branched and unbranched volatile fatty acids; NH_3 = ammonia; [1] Molar proportions in % of TVFA; Acetate (AP), propionate (PP), butyrate (BP) and branched chain fatty acids (BCP = *i*butyrate + *i*valerate/ TVFA).

Preparation of substrates

In this experiment 24 PENS were tested as additives to the EU-reference diet. All additives were tested at different levels of inclusion, according to their presumed efficacy levels in the feed (Table 3).

The different PENS used in this trial were classified into two groups. A series of 11 PENS were included for their known fermentable properties, whereas 13 PENS were included for their reported microbiota-modulating properties. Mannuronic block, laminaran, oligo-laminaran, xylan and oligo-xylan are dietary fiber sources isolated from seaweed, which have potential prebiotic properties. Soycomil, a soy based protein extract used in weaning pigs diets, could, in addition to contain fermentable proteins, have a modulating effect on the intestinal microbiota through the isoflavones present in soy protein [45]. Carob pulp is obtained from the seed pods of the carob tree (*Ceratonia siliqua*) and contains in addition to insoluble and soluble fibers some polyphenolic compounds (e.g. gallic acid, gallotannins, flavonol glycosides) [46]. Raftilose P95 and Topinambur 40 are fructan polymers derived from roots of the chicory (*Chicorium intybus*) and the Jerusalem artichoke (*Helianthus tuberosus*), respectively. Citrus pulp, an industrial by-product, contains next to dietary fiber also some polyphenolic and flavonoid components

with potential antimicrobial effects [27]. With respect to the non-fermentable PENS, a range of essential oils and other natural compounds were included for their potential effects on microbial activity. For one of these products, oregano oil, three different preparations were included in this study, namely oregano oil of either Mediterranean or Dutch origin, as well as an aqueous extract, to evaluate the potential effect of source and extraction on biological activity.

Table 3. Summary of PENS tested, their inclusion levels, their mode of action as reported in literature with reference between parentheses, and the provider.

Name of PENS	Amount/ addition	Mode of action (Reference)	Provider[†]
Products used as control substrates			
EU-reference diet (C)	0.50 ± 0.02 g		SFR
Avilamycin (added to C)	+ 40 mg/kg	Antimicrobial [19]	SFR
Fermentable PENS			
Mannuronic block	+ 10, 20, 50 g/kg	Prebiotic [16, 20, 21]	CEVA (No. 1)
Laminaran	+ 10, 20, 50 g/kg	Prebiotic [22]	CEVA (No. 4)
Oligo-laminaran	+ 10, 20, 50 g/kg	Prebiotic [22]	CEVA (No. 5)
Xylan	+ 10, 20, 50 g/kg	Prebiotic [22]	CEVA (No. 6)
Oligo-xylan	+ 10, 20, 50 g/kg	Prebiotic [23]	CEVA (No. 7)
Carobpulp	+ 20, 50 g/kg	Antibacterial [24, 25]	IRTA
Raftilose P95	+ 20, 50 g/kg	Prebiotic [26]	FBN
Topinambur 40	+ 20, 50 g/kg	Prebiotic [11, 15]	FBN
Citrus Pulp	+ 20, 50 g/kg	Prebiotic [27]	SFR
Guargum (5000)	+ 20, 50 g/kg	Prebiotic [25, 28, 29]	SFR
Soycomil	+ 20, 50 g/kg	Microbiota modulatory [30, 31]	SFR
Non-fermentable PENS (etheric oils and other extracts)			
Oregano oil (D)[*]	+ 50, 100, 200 mg/kg	Antibacterial [32-34]	ANU&MIB
Oregano oil (M)[*]	+ 50, 100, 200 mg/kg	Antibacterial [32-34]	ANU&MIB
Oregano oil (W)[*]	+ 20%	Antibacterial [32-34]	ANU&MIB
Allicin	+ 50, 100, 200 mg/kg	Antibacterial [35, 36]	Langley
Etheric oil	+ 4.4, 8.8 mg/kg	Antibacterial [37, 38]	FBN
Saponin	+ 50, 100, 200 mg/kg	Antibacterial [39]	FBN
Onion oil extract	+ 0.12, 0.24 mg/kg	Antioxidant, anti inflammatory, anticancer [32, 33]	FBN
Garlic extract	+ 0.07, 0.14 mg/kg	Antibacterial	FBN
Herbal mixture	+ 50, 100, 200 mg/kg	Antibacterial	FBN
Sanguinarine	+ 50, 100, 200 mg/kg	Antibacterial, antioxidant, anti-inflammatory [40]	ASG
Benzoic acid	+ 50, 100, 200 mg/kg	Bacterial growth inhibitor [41, 42]	IRTA
Cinnamaldehyde	+ 50, 100, 200 mg/kg	Fungicide [43]	IRTA
Naringin	+ 50, 100, 200 mg/kg	Antioxidant, anticancer [44]	IRTA

[*]D= Dutch type; M= Mediterranean type; W= water-soluble extract. [†]SFR = Schothorst Feed Research, Lelystad, NL; CEVA = French Regional Technical Centre for Seaweed Valorisation, FR; IRTA = Institute for Food and Agricultural Research and Technology, Reus, ES; FBN = Research Institute for Biology of Farm Animals, Dummerstorf, DE; ANU/MIB = Animal Nutrition Group & Laboratory of Microbiology, Wageningen University, Wageningen, NL; Langley = University of Bristol, Bristol, UK; ASG=Animal Sciences Group, Wageningen University and research Centre, Lelystad, NL;

Per PENS-concentration three replicate bottles were included in the *in vitro* run. The EU-reference diet without any additive was used as the negative control. The antibiotic avilamycin was added to the EU-reference diet at an inclusion level of 40 mg/kg as a positive control. Substrate and additives were weighed into the bottles, and a vitamin/mineral, sodium-bicarbonate based buffer solution [18] was added. Before inoculation, bottles were pre-warmed at 38°C.

Cumulative gas production

Once inoculated, the bottles were incubated for a 72-h period after which cumulative gas production was measured manually [18]. A modified bi-phasic Michaelis-Menten equation was fitted to the gas production profiles [47]. In addition, the maximum rate of gas production (R_{max}), the time at which this rate is reached (T_{max}) and the time at which 50% of the asymptotic gas production is reached (halftime, $T_{1/2}$) were calculated [29].

Fermentation end-products

At the end of the fermentation process, the pH of the fermentation fluids was recorded and samples were taken for VFA (in vials containing 0.75 ml of a stock solution composed of 25 ml of 85% ortho-phosphoric acid dissolved in 200 ml Millipore and 300 ml of a 4 g/l 2-methyl valeric acid solution), NH_3 (in vials containing 0.75 ml of a 10% trichloroacetic acid solution) and molecular microbiological analyses. Vials were stored at -20°C for further analysis (in case of microbiological samples vials were store at -80°C). Organic matter loss (OM Loss) was determined by filtration of fermentation fluids through sintered glass filter crucibles (Schott Duran, porosity #2, Mainz, Germany) and subsequent drying (103°C) and ashing (530°C) of the residual unfermented substrate.

Chemical analyses

VFA produced was analyzed by gas chromatography (GC; Fisons HRGC Mega 2, CE Instruments, Milan, Italy) with a split / split-less injector operated in split mode (split ratio 1:10) and fitted to a flame ionization detector (FID), using a capillary column (EC-1000, Alltech; 30 m, i.d. 0.53 mm, film thickness 1.00 μm) with helium as carrier gas (50 kPa pressure). The starting temperature of the column was set at 110°C for two minutes followed by an 18°C/min increase to 200°C that was maintained for 1 min. 2-methylvaleric acid was included as internal standard. Ammonia was determined according to the method described by Houdijk et al [48]. In short, supernatants were deproteinized using 10% trichloroacetic acid. Ammonia forms a blue complex with phenol and hypochlorite in an alkaline environment, which was measured colorimetrically at 623 nm using a UV spectrophotometer (Beckman-Coulter DU 64, Fullerton, USA).

Statistical analysis of in vitro gas production data

Fermentation kinetic parameters and fermentation end-products were measured for the effects of PENS when added to a control substrate at different levels (Equation 1) using the GLM procedure of SAS 9.1 (SAS Institute Inc.).

$$Y = \mu + L_i (P_k) + \varepsilon_{ijkl} \quad (1)$$

Y is the dependent variable, μ the overall mean, $L_i (P_k)$ is the level of PENS inclusion (L) nested within PENS (P) and ε_{ijkl} is the error term. Differences between the main effects were analyzed using Tukey-Kramer's multiple comparison procedure in the LSMEANS statement in SAS.

DNA isolation

DNA isolation from *in vitro* incubated samples was done using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad, CA, USA). Prior to DNA extraction, the samples were centrifuged and 0.2 g of pellet were taken and processed using the kit mentioned above. Quality and amount of extracted DNA were assessed by agarose gel electrophoresis in the presence of ethidium bromide.

PCR and DGGE analysis

Universal primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 (Table 4) were used to amplify the V6 to V8 regions of the bacterial 16S rRNA gene. PCRs were performed using a GoTaq® DNA Polymerase kit from Promega (Madison, WI, USA.). Each PCR mixture (50 μl) contained 1.25 U of GoTaq® DNA polymerase, Green GoTaq® reaction buffer containing 1.5 mmol/l $MgCl_2$, 0.2 mmol/l of each deoxynucleoside triphosphate, 0.2 μmol/l of the primers, 1 μl of DNA solution (~1 ng/μl) and UV-sterilized water. The samples were amplified in a thermocycler T1 (Whatman Biometra, Göttingen, Germany) using the following program: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 20 sec, extension at 68°C for 40 sec and final extension at 68°C for 7 min. The size of the PCR products was checked by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

To study the *Lactobacillus*-specific GI tract community, Lac1/Lac2 primers were used (Table 4). The cycling conditions were as described above.

The PCR amplicons were separated by DGGE according to the specifications of Heilig et al. [52] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in an 8% (v/v) polyacrylamide gel (37.5:1 acrylamide-bisacrylamide and dimensions: 200 by 200 by 1 mm) and 0.5× Tris-acetate-EDTA (TAE) (pH 8) buffer. The gels were prepared using a gradient maker and a pump (Econopump; Bio-Rad Laboratories). The denaturing gradient used was 30-60% for both total bacterial and *Lactobacillus*-group specific PCR products. Electrophoresis was done for 16 h at 85 V in 0.5× TAE buffer at a constant temperature of 60°C, and gels were stained with $AgNO_3$ as described before [53], scanned at 400 dpi and analyzed using Bionumerics software package version 4.5 (Applied MathS, Kortrijk, Belgium).

Table 4. DNA oligonucleotides primers used in this study.

Primers	Sequence (5'-3')	References
S-D-Bact-0968-a-S-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGG GGAACGCGAAGAACCTTAC	[49]
S-D-Bact-1401-a-A-17	CGGTGTGTACAAGACCC	[49]
Lac1 (Lab-352-S)	AGCAGTAGGGAATCTTCA	[50]
Lac2 (Lab-679-A-GC)	CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGG GATTYCACCGCTACACATG	[50]
T7prom-Bact-27-for	TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGG CTCAG	[51]
Uni-1492-rev	CGGCTACCTTGTTACGAC	[51]

PITChip analysis

Phylogenetic analysis of incubated samples was done using the Pig Intestinal Tract Chip (PITChip, Chapter 3). This is a phylogenetic microarray with more than 2900 oligonucleotides based on 16S rRNA gene sequences targeting over 627 porcine intestinal microbial phylotypes [54]. The protocol for hybridization and analysis of the generated data was essentially performed as described before for the HITChip (Human Intestinal Tract chip) [55].

PCR: The 16S rRNA gene was amplified using the primers T7prom-Bact-27-for and Uni-1492-rev (Table 4). For each reaction 10 ng of DNA samples was used as a template in a final volume of 50 µl. Samples were denatured at 94°C for 2 min followed by 35 cycles of 94°C (30 sec), 52°C (40 sec), 72°C (90 sec) and a final extension at 72°C for 7 min. After purification of the PCR products (High Pure PCR Cleanup Micro Kit, Roche Diagnostics GmbH, Mannheim, Germany) the DNA concentration was measured using a NanoDrop spectrophotometer.

RNA production and labeling: *In vitro* transcription of the 16S rRNA gene amplicons carrying the T7-promoter was performed using the Riboprobe System (Promega, La Jolla, USA). For the reaction in the presence of rATP, rGTP, rCTP and a 1:1 mix of rUTP and aminoallyl-rUTP (Ambion, Austin, TX, USA), 500 ng of the PCR product were used. The transcription reaction was incubated at room temperature for two hours, followed by digestion with Qiagen RNAse-free DNAse (Qiagen, Hilden, Germany) to remove remaining DNA. RNA was purified (RNeasy Mini-Elute Kit, Qiagen, Hilden, Germany) and the concentration was determined using a NanoDrop spectrophotometer. RNA samples containing modified amino-allyl nucleotides were coupled with CyDye using the Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK) dissolved in DMSO to a final concentration of 0.5 nmol/µl. For the labeling reaction 20 µl of CyDye were added to 2 µg of purified RNA and 25 mmol/l of sodium

bicarbonate buffer (pH 8.7) in a final volume of 40 µl. Samples were incubated during 90 min, in the dark and at room temperature. After this time the reaction was stopped by adding 15 µl of 4 mol/l hydroxyl-amine and incubating in the dark for 15 min. RNAse-free water was added to 100 µl and the labeled RNA was quantified.

Hybridization: Microarrays synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) in 8×15K format were used for hybridization with two samples, labeled with Cy3 and Cy5, respectively. The Cy3/Cy5-labeled target mixtures were fragmented with 10× fragmentation reagent (Ambion, Austin, TX, USA). Hybridization on the arrays was performed essentially as described previously [55] at 62.5°C for 16 h in a rotation oven (Agilent). Slides were washed at room temperature in 2× SSC with 0.3% SDS for 10 min, followed by 0.1× SSC with 0.3% SDS at 38°C for 10 min and 0.06× SSPE for 5 min [56].

Data extraction: Data was extracted from microarray images using the Agilent Feature Extraction software, version 9.1. Data normalization and the further microarray analysis were performed using a set of R based scripts (http://r-project.org) in combination with a custom designed relational database, which runs under the MySQL database management system (http://www.mysql.com) [54, 55].

Statistical analysis of microbial fingerprints

Similarities between DGGE profiles were determined by calculating the similarity indices of the corresponding densitometric curves using the Pearson's product moment correlation [57]. The UPGMA algorithm was used as implemented in the Bionumerics analysis software for the construction of dendograms. Similarity of the total microbiota composition based on the PITChip profiles was assessed as well by calculating Pearson's product moment correlation that reflects the degree of linear relationship between analyzed data sets. Ward's minimum variance method was used for the generation of hierarchical clustering of probe profiles by calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance – E2) [58].

Diversity of microbial profiles obtained by PITChip analysis was expressed as Simpson's reciprocal index of diversity (1/D) [59]. This diversity was calculated with the equation $\lambda = 1/\Sigma Pi^2$, where Pi is the proportion of i^{th} taxon. This is the proportion of each probe signal compared to the total signal for each sample. A higher Simpson's index value indicates a higher degree of diversity.

To determine whether observed differences between datasets were statistically significant, Student's t-test was applied. Calculated p-values were two tailed. If the p-value was lower than 0.05, the difference between data was considered significant and p-values lower than 0.01 were considered indicative of highly significant differences.

Results

Effect of PENS on in vitro fermentation

The different PENS used in this trial were classified in two groups according to their presumed fermentability by intestinal microbiota (Table 3). Fermentation characteristics of the substrate as such (Control, EU-reference diet), the substrate in the presence of a commonly used in-feed antimicrobial (avilamycin) or in the presence of added PENS, are summarized in Tables 5 and 6. The addition of avilamycin did not have any significant effect on fermentation characteristics, including kinetic parameters of gas production and concentrations of fermentation end products (Tables 5 and 6).

Fermentable PENS: Addition of Guar gum (5000) resulted in numerically the lowest cumulative gas production (OMCV) and lowest maximum rate of gas production (Rmax) compared to the other fermentable PENS (Table 5). This was found in a range of Guar gum (5000) concentrations (data not shown). In contrast, addition of carob pulp and Topinambur 40 yielded the highest OMCV and Rmax. Seaweed products (poly- and oligosaccharides), considered as highly fermentable dietary fiber, did not cause significant changes in the fermentation kinetics at any of the concentrations used in this study. None of the seaweed fractions affected the OMCV distinctively compared to the control. With increasing levels of laminaran the OMCV tended to increase but the effect was non-significant ($p > 0.668$) (data not shown). The other seaweed products (mannuronic block, oligo-laminaran, oligo-xylan) showed no dose-response like trends. Curve fit parameters like Rmax, Tmax and $T_{1/2}$ were deviating from the control substrate, but differences showed no consistent pattern and the effects were non-significant.

As to fermentation end products, VFA concentrations showed some inconsistent and non-significant deviations from the control substrate (Table 6). In case of NH_3, higher inclusion levels of Guar gum (5000) and Raftilose P95 caused a numerical decrease in NH_3-concentration (data not shown). No effects of PENS addition on the molar proportions of VFA and on the branched chain fatty acids (BCP) were observed compared to control. However, the molar proportion of branched chain fatty acids (Table 6) tended to be negatively related to the proportion of acetate ($BCP = 20.1 – 0.32xAP$; $R^2 = 0.61$).

Non-fermentable PENS: Addition of the pure oregano oil types (Mediterranean and Dutch) as well as the other etheric oils, did not alter the fermentation characteristics significantly. However, addition of the water-soluble oregano oil extract affected the fermentation characteristics drastically (Table 5). The cumulative gas production (OMCV) and organic matter loss (OMloss) were significantly lower ($p < 0.0001$) compared to the control and control + avilamycin, to the Mediterranean oregano oil and to the Dutch type. Rmax was also lower and the halftime (T½) was considerably delayed by about 14 h ($p < 0.0001$ for both parameters).

Neither the addition of the pure oregano oil types nor the addition of the other extracts affected the fermentation end-products (Table 6). In general, the proportion of BCP was higher for the control with avilamycin, the Mediterranean oregano oil and the Dutch oregano oil, compared to the control without addition of PENS, but differences were not significant. However, in case of the water-soluble form of oregano oil the total VFA (tVFA) and NH_3 concentrations

Table 5. *In vitro* fermentation characteristics of substrate as affected by different plant extracts and other natural substances using feces as inoculum.*

PENS	pH	OMCV (ml)	A (ml)	B	C (h)	Rmax (ml/h)	Tmax (h)	OMLoss (%)
Products used as control substrates								
EU-reference diet (C)	6.50^{a}	248.6^{ab}	254.0^{a}	2.48^{ab}	14.92^{cb}	12.5^{ab}	10.57^{b}	82.9^{bcd}
C+Avilamycin	6.52^{a}	247.0^{ab}	253.8^{a}	2.44^{ab}	14.85^{cb}	12.4^{ab}	10.38^{cb}	83.8^{abcd}
Fermentable PENS								
Carob pulp	6.50^{a}	259.2^{a}	265.1^{a}	2.43^{ab}	13.62^{cb}	14.1^{a}	9.47^{cb}	83.0^{bcd}
Citrus pulp	6.50^{a}	244.9^{ab}	250.7^{a}	2.52^{ab}	14.37^{cb}	13.0^{ab}	10.28^{cb}	83.9^{abc}
Guargum (5000)	6.50^{a}	231.3^{b}	237.6^{a}	2.44^{ab}	14.92^{cb}	11.7^{b}	10.34^{cb}	83.6^{abcd}
Laminaran	6.50^{a}	254.3^{ab}	261.6^{a}	2.38^{ab}	14.77^{cb}	12.7^{ab}	10.04^{cb}	83.9^{abc}
Mannuronic block	6.51^{a}	250.0^{ab}	255.9^{a}	2.36^{ab}	14.22^{cb}	12.8^{ab}	9.68^{cb}	83.8^{abcd}
Oligo-laminaran	6.49^{a}	251.6^{ab}	257.9^{a}	2.49^{ab}	14.86^{cb}	12.8^{ab}	10.54^{cb}	83.9^{abc}
Oligo-xylan	6.50^{a}	249.0^{ab}	256.4^{a}	2.39^{ab}	14.51^{cb}	12.7^{ab}	9.97^{cb}	84.1^{ab}
Raftilose P95	6.48^{a}	254.0^{ab}	262.0^{a}	2.36^{ab}	14.51^{cb}	12.9^{ab}	9.88^{cb}	83.7^{abcd}
Soycomil	6.52^{a}	248.1^{ab}	254.3^{a}	2.53^{ab}	14.78^{cb}	12.8^{ab}	10.58^{b}	82.6^{d}
Topinambur 40	6.50^{a}	259.2^{a}	265.4^{a}	2.25^{b}	12.90^{c}	14.3^{a}	8.41^{c}	84.3^{a}
Xylan	6.51^{a}	246.2^{ab}	252.2^{a}	2.50^{ab}	14.94^{cb}	12.5^{ab}	10.61^{b}	82.9^{bcd}
Non-fermentable PENS (etheric oils and other extracts)								
Allicin	6.52^{a}	241.6^{ab}	246.8^{a}	2.47^{ab}	14.56^{cb}	12.4^{ab}	10.25^{cb}	83.1^{abcd}
Etheric oil	6.52^{a}	249.3^{ab}	253.9^{a}	2.52^{ab}	14.44^{cb}	13.1^{ab}	10.36^{cb}	83.1^{abcd}
Garlic extract	6.51^{a}	247.0^{ab}	253.0^{a}	2.47^{ab}	14.83^{cb}	12.5^{ab}	10.48^{cb}	83.3^{abcd}
Onion oil extract	6.50^{a}	258.0^{a}	264.1^{a}	2.50^{ab}	14.61^{cb}	13.3^{ab}	10.38^{cb}	82.8^{cd}
Oregano oil (D)[1]	6.52^{a}	252.7^{ab}	257.4^{a}	2.55^{ab}	14.15^{cb}	13.6^{ab}	10.21^{cb}	82.9^{bcd}
Oregano oil (M)[1]	6.51^{a}	249.8^{ab}	255.1^{a}	2.53^{ab}	14.70^{cb}	12.9^{ab}	10.53^{cb}	83.4^{abcd}
Oregano oil (W)[1]	6.41^{b}	166.2^{c}	196.7^{b}	2.45^{ab}	34.53^{a}	4.2^{c}	24.03^{a}	69.4^{e}
Benzoic acid	6.51^{a}	246.5^{ab}	251.8^{a}	2.45^{ab}	14.30^{cb}	12.8^{ab}	10.02^{cb}	83.2^{abcd}
Cinnamaldehyde	6.51^{a}	251.4^{ab}	255.9^{a}	2.55^{ab}	14.60^{cb}	13.1^{ab}	10.55^{cb}	83.4^{abcd}
Herbal mixture	6.51^{a}	251.1^{ab}	256.4^{a}	2.44^{ab}	14.33^{cb}	13.1^{ab}	10.03^{cb}	83.2^{abcd}
Naringine	6.51^{a}	250.5^{ab}	255.4^{a}	2.58^{ab}	14.51^{cb}	13.3^{ab}	10.56^{cb}	83.5^{abcd}
Sanguinarine	6.50^{a}	251.4^{ab}	256.3^{a}	2.65^{a}	15.39^{b}	12.8^{ab}	11.40^{b}	82.9^{bcd}
Saponin	6.51^{a}	250.9^{ab}	256.7^{a}	2.49^{ab}	14.75^{cb}	12.9^{ab}	10.48^{cb}	83.7^{abcd}
SEpooled[2]	0.01	5.0	5.3	0.07	0.42	0.4	0.41	0.2
MSD[3]	0.04	26.4	28.2	0.35	2.22	2.1	2.16	1.3
p-values[4]								
PENS	< 0.001	< 0.001	< 0.001	0.014	< 0.001	< 0.001	< 0.001	< 0.001
Level (PENS)	0.399	0.373	0.365	0.425	0.928	0.635	0.468	< 0.001
Rep	0.746	0.506	0.426	0.478	0.286	0.308	0.423	0.001

*OMCV = cumulative gas production (ml/g OM initial weight); A = asymptotic gas production (ml); B = switching characteristic; C = half time of asymptotic gas production (h; T½); Rmax = maximal rate of gas production (ml/h); Tmax = time of occurrence of Rmax (h); OMLoss = organic matter loss (%). [1]D= Dutch type; M= Mediterranean type; W= water-soluble extract. [2]SEpooled = pooled standard error; [3]MSD = Minimum Significant Difference. [4]Main effects PENS (n = 26), Level (n = 3) nested within PENS and Rep (n = 3). Different superscripts indicate a significant difference between any pair of treatments ($p < 0.05$).

were lowered considerably compared to the control ($p < 0.0001$).

Table 6. End products from the *in vitro* fermentation of substrate as affected by different plant extracts and other natural substances using feces as inoculum.*

PENS	NH_3-N (mmol/g OM)	TVFA (mmol/g OM)	Acetate (%)	Propionate (%)	Butyrate (%)	BCP (%)
Products used as control substrates						
EU-reference diet (C)	5.38^{abcdef}	7.99^{a}	47.1^{b}	25.6^{a}	17.7^{a}	4.94^{a}
C+Avilamycin	5.40^{abcde}	8.25^{a}	46.3^{b}	25.9^{a}	17.3^{a}	5.52^{a}
Fermentable PENS						
Carob pulp	5.20^{efghi}	8.17^{a}	45.9^{b}	26.3^{a}	16.9^{a}	5.82^{a}
Citrus pulp	5.18^{ghi}	7.97^{a}	48.1^{b}	26.0^{a}	16.6^{a}	4.77^{a}
Guargum (5000)	5.15^{hi}	8.11^{a}	45.3^{b}	27.0^{a}	17.1^{a}	5.61^{a}
Laminaran	5.20^{fghi}	7.90^{a}	46.2^{b}	26.6^{a}	17.2^{a}	5.19^{a}
Mannuronic block	$5.26^{bcdefghi}$	8.00^{a}	47.6^{b}	25.8^{a}	16.9^{a}	5.13^{a}
Oligo-laminaran	$5.27^{bcdefghi}$	8.13^{a}	46.1^{b}	26.2^{a}	17.4^{a}	5.41^{a}
Oligo-xylan	$5.22^{cdefghi}$	8.35^{a}	46.0^{b}	26.8^{a}	16.7^{a}	5.51^{a}
Raftilose P95	5.07^{i}	7.98^{a}	45.9^{b}	27.2^{a}	17.2^{a}	4.99^{a}
Soycomil	$5.31^{abcdefgh}$	7.74^{a}	47.1^{b}	25.4^{a}	18.1^{a}	4.97^{a}
Topinambur 40	5.21^{defghi}	7.89^{a}	47.4^{b}	25.8^{a}	17.3^{a}	4.79^{a}
Xylan	$5.37^{abcdefg}$	8.40^{a}	45.8^{b}	27.2^{a}	16.2^{a}	5.70^{a}
Non-fermentable PENS (etheric oils and other extracts)						
Allicin	5.38^{abcdef}	8.08^{a}	46.4^{b}	26.5^{a}	17.0^{a}	5.28^{a}
Etheric oil	5.42^{abc}	7.74^{a}	48.1^{b}	26.0^{a}	17.0^{a}	4.49^{a}
Garlic extract	5.38^{abcdef}	8.31^{a}	45.8^{b}	26.5^{a}	16.8^{a}	5.70^{a}
Onion oil extract	5.46^{a}	8.11^{a}	45.9^{b}	26.4^{a}	17.3^{a}	5.38^{a}
Oregano oil (D)[1]	5.40^{abcde}	8.41^{a}	46.3^{b}	26.3^{a}	16.5^{a}	5.87^{a}
Oregano oil (M)[1]	5.40^{abcd}	8.12^{a}	46.1^{b}	26.1^{a}	17.3^{a}	5.48^{a}
Oregano oil (W)[1]	3.27^{j}	3.79^{b}	66.1^{a}	21.1^{b}	8.9^{b}	1.16^{b}
Benzoic acid	5.41^{abc}	8.08^{a}	47.2^{b}	25.8^{a}	17.0^{a}	5.18^{a}
Cinnamaldehyde	5.40^{abcd}	8.15^{a}	46.8^{b}	25.8^{a}	17.5^{a}	5.19^{a}
Herbal mixture	5.40^{abcde}	8.17^{a}	46.2^{b}	26.6^{a}	16.8^{a}	5.47^{a}
Naringine	$5.35^{abcdefg}$	8.19^{a}	46.6^{b}	26.5^{a}	16.6^{a}	5.46^{a}
Sanguinarine	5.40^{abcde}	8.09^{a}	44.9^{b}	26.4^{a}	17.5^{a}	5.95^{a}
Saponin	5.43^{ab}	8.24^{a}	45.2^{b}	26.3^{a}	17.5^{a}	5.90^{a}
SEpooled[2]	0.04	0.20	0.73	0.39	0.41	0.34
MSD[3]	0.20	1.07	3.9	2.1	2.2	1.81
p-values[4]						
PENS	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001	< 0.001
Level (PENS)	< 0.001	0.465	0.104	0.232	0.109	0.038
Rep	0.468	0.752	0.185	0.608	0.581	0.124

*TVFA = sum of branched and unbranched VFA (mmol/g OM initial weight); NH3 = ammonia (mmol/g OM initial weight); Acetic, propionic and butyric proportions (% of TVFA); BCP = Branched Chain Proportion ((iBu +iVal)/TVFA). [1]D= Dutch type; M= Mediterranean type; W= water-soluble extract. [2]SEpooled = pooled standard error; [3]MSD = Minimum Significant Difference. [4]Main effects PENS (n = 26), Level (n = 3) nested within PENS and Rep (n = 3). Different superscripts indicate to a difference between any pair of treatments ($p < 0.05$).

Moreover, there was a distinct shift in the molar proportions of VFA, with a relative increase in acetic acid ($p < 0.0001$), mainly at the expense of butyric acid ($p < 0.0001$) as well as propionic acid ($p < 0.0001$). The proportion of BCP was significantly lower compared to the control and other treatments ($p \leq 0.0001$).

Additive-associated dynamics of fecal slurry bacteria

The effect of the presence of PENS at different inclusion levels on fecal microbiota was assessed by community fingerprinting of selected samples using two 16S rRNA gene-based techniques, namely DGGE as well as PITChip phylogenetic microarray analysis of PCR-amplified 16S rRNA gene fragments. For DGGE analysis, the selection of samples was based on *in vitro* fermentation results (substances with a significant effect on one or more fermentation parameters) as well as on the nature of the predicted effect (antimicrobial, antioxidant, anticancer, etc) of the PENS on the microbiota.

The analysis of piglet fecal bacteria associated with the presence of additives showed almost identical DGGE profiles for most of the PENS, with an average Pearson's similarity of 95.1% (Fig. 1). Only the addition of water-soluble oregano oil resulted in a remarkable change on the microbiota profile. Cluster analysis based on Pearson's similarity coefficients showed two clearly different clusters with an average similarity of only 23% (Fig. 1).

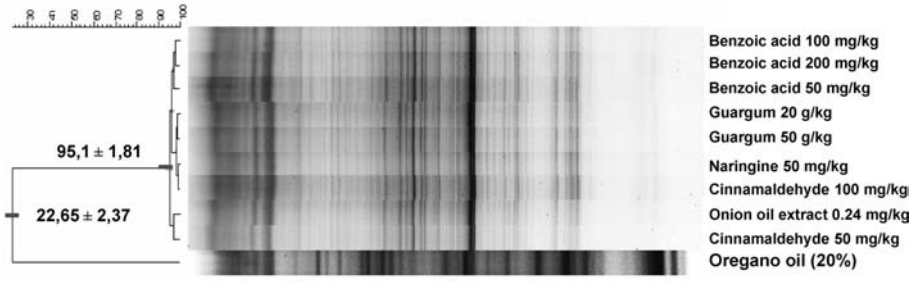

Figure 1. Similarity dendogram of DGGE profiles of bacterial community associated with different PENS, generated by UPGMA clustering based on Pearson's similarity coefficients. The upper cluster contains examples of samples fermented in the presence of different PENS whereas the lower cluster contains only the water-soluble oregano oil sample.

For in-depth analysis of the effect of the different PENS used during the *in vitro* fermentation, microbiota composition in selected representative samples was profiled as well by PITChip analysis. These profiles were obtained based on the signal intensity of 2985 oligonucleotide probes targeting 627 porcine microbial phylotypes so far reported in literature and/or public nucleotide sequence databases. In agreement with the results obtained by DGGE analysis, the PENS-based clustering was also observed by hierarchical clustering of the microarray-based profiles of fermentation batches incubated in the presence of water-soluble oregano oil or oregano oil Dutch type, the control fermentation and the inoculum (Fig. 2).

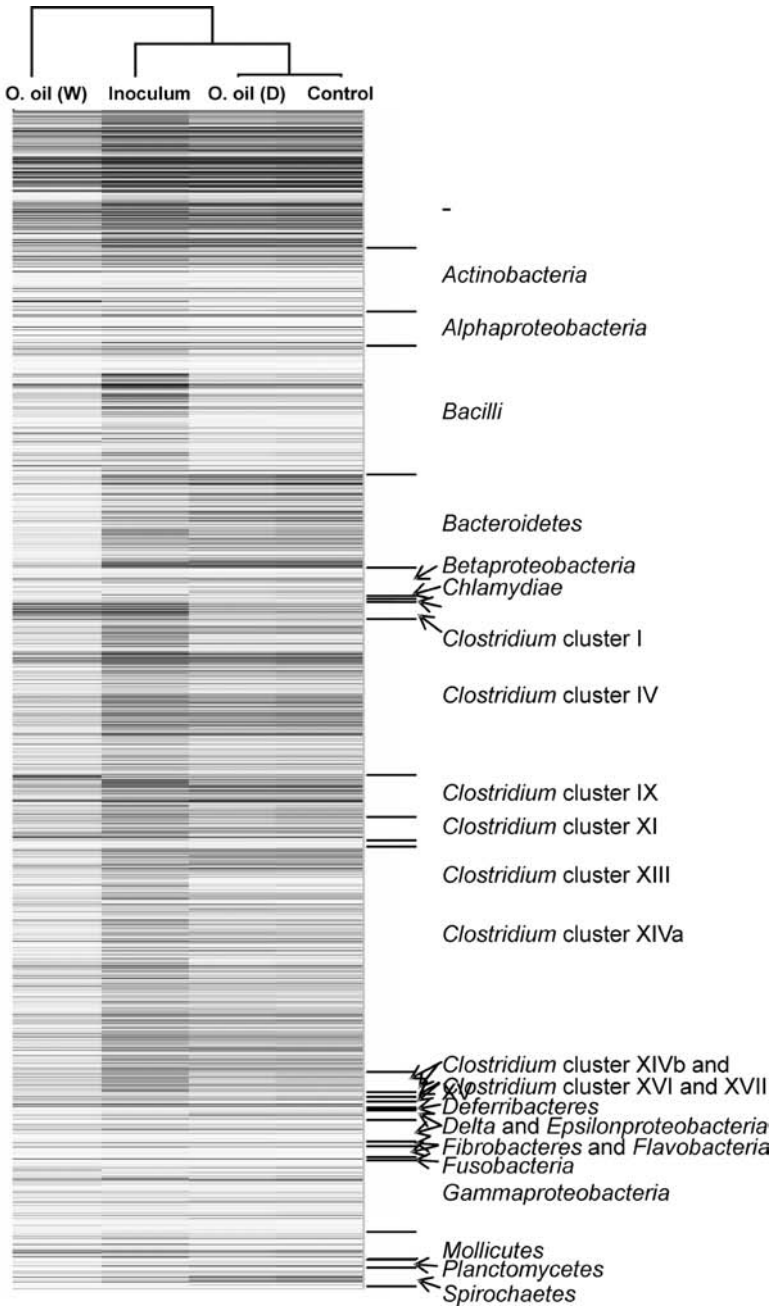

Figure 2. PITChip phylogenetic fingerprints of the microbiota of piglets' feces fermented *in vitro* using different additives: oregano oil W and oregano oil D. The highest phylogenetic level of specificity of probes is shown on the right panel of the figure.

Pearson's correlation analysis showed that the microbiota composition after incubation in presence of the oregano oil Dutch type was very similar to the one present in the control sample (99.2%). However, as already seen with DGGE analysis, the profile after incubation in presence of the water-soluble oregano oil was more different (76.6%) from the control. Moreover, PITChip analysis revealed a small but measurable change in the composition of the feces due to the *in vitro* fermentation, as the inoculum showed a somewhat different profile than the control (inoculum after fermenting the EU reference diet) (90.1%). These differences are also reflected in the diversity of PITChip profiles. The profile of feces fermenting water-soluble oregano oil extract seemed to be less diverse (106.1) compared to the other three samples: feces fermenting oregano oil Dutch type (277.4), feces fermenting the EU-reference diet (298) and inoculum (319.7).

To gain further insight in the fluctuations in relative abundance of phylogenetic groups present in the fermented samples, changes in the summed signal intensities for 144 genus-level bacterial groups represented on the PITChip were studied. Overall, genus-level bacterial groups with higher relative abundance (> 2%) belonged to the classes of *Clostridia*, *Bacilli* and *Actinobacteria*. Remarkably, feces fermented in the presence of the water-soluble oregano oil extract had an overall lower relative abundance of members of *Clostridium* cluster XIVa (*Bryantella*- and *Lachnospira pectinoschiza*-like organisms) and *Clostridium* cluster IV (*Ruminococcus callidus*- and *Sporobacter termiditis*-like species) but higher relative abundance of *Bacilli* and *Actinobacteria* groups, including members of the genus *Lactobacillus* as well as uncultured *Bacilli*.

To further identify those groups that significantly changed in relative abundance after incubation in the presence of the control substrate with or without additives, Student's T-tests were performed (Table 7).

On one hand, this showed that not many bacterial groups (7 out of 144) were significantly affected by the *in vitro* fermentation of the control substrate when compared to the inoculum. On the other hand, this analysis confirmed that there was an important change in the composition when the feces fermented the substrate in the presence of water-soluble oregano oil extract, and 32 bacterial groups had a significant increase or decrease in relative abundance (compared to the control). These groups were mainly members of the *Bacteroidetes* and *Firmicutes*. In contrast, when the fecal slurry was incubated in the presence of oregano oil Dutch type, no bacterial group was significantly affected as compared to the control incubation.

Discussion

Effect of PENS on in vitro fermentation

Addition of fermentable carbohydrates, probiotics and other natural feed additives to the diet of weaning piglets can significantly affect microbiota composition and functionality. This is known to be an efficient way to improve the microbial balance in both the small and large intestines of piglets, preventing intestinal disorders [60, 61]. In this study we used a manual *in vitro* technique to measure kinetics of fermentation and to assess activity of microbial populations [18, 62].

Table 7. PITChip-based analysis of bacterial groups significantly affected by either the *in vitro* fermentation without additive or by the in vitro fermentation in the presence of oregano oil (W) or oregano oil (D). The p-values were calculated comparing to the control and using the sum of intensities of 144 phylogenetic groups. The effect caused on the bacterial group intensity is indicated by Inc (increase) or Dec (decrease). Significant values are marked in bold.

Higher taxonomic group	Group	Oregano oil (W)		Oregano oil (D)		Inoculum	
		p-value	Effect	p-value	Effect	p-value	Effect
Bacilli	*Lactobacillus gasseri*-like	0.472	Inc	0.579	Dec	**< 0.001**	Inc
	Uncultured *Bacilli*	**0.039**	Dec	0.674	Dec	0.894	Inc
Bacteroidetes	*Alistipes*-like	**0.001**	Dec	0.276	Dec	**0.013**	Dec
	Bacteroides distasonis-like	**0.003**	Dec	0.611	Dec	**0.043**	Dec
	Bacteroides pyogenes-like	**0.007**	Dec	0.784	Dec	0.052	Dec
	Bacteroides vulgatus-like	**0.024**	Inc	0.813	Inc	0.110	Inc
	Paludibacter propionicigenes-like	**0.030**	Dec	0.802	Dec	0.120	Dec
	Prevotella melaninogenica-like	**< 0.001**	Dec	0.814	Inc	**0.004**	Dec
	Uncultured *Bacteroidetes*	**< 0.001**	Inc	0.369	Inc	**0.001**	Inc
	Uncultured *Porphyromonadaceae*	**0.011**	Dec	0.676	Dec	0.090	Dec
	Uncultured *Prevotella*	**< 0.001**	Inc	0.771	Dec	**< 0.001**	Inc
Clostridium cluster I	*Clostridium perfringens*-like	0.324	Inc	0.768	Dec	**0.039**	Inc
Clostridium cluster IV	*Anaerotruncus*-like	**0.018**	Inc	0.763	Dec	0.721	Inc
	Clostridium cellulosi-like	**0.046**	Dec	0.815	Dec	0.876	Inc
	Clostridium leptum-like	**0.005**	Dec	0.698	Dec	0.602	Inc
	Eubacterium siraeum-like	**0.013**	Inc	0.857	Dec	0.625	Inc
	Faecalibacterium-like	**0.003**	Dec	0.664	Inc	0.549	Inc
	Faecalibacterium prausnitzii-like	**0.016**	Dec	0.820	Dec	0.190	Inc
	Papillibacter cinnamivorans-like	**0.042**	Dec	0.929	Inc	0.755	Inc
	Ruminococcus callidus-like	**< 0.001**	Inc	0.812	Dec	0.386	Inc
	Sporobacter termitidis-like	**< 0.001**	Inc	0.560	Dec	0.266	Inc
	Subdoligranulum-like	**0.009**	Dec	0.757	Dec	0.536	Dec
Clostridium cluster IX	*Dialister*-like	**0.011**	Inc	0.926	Dec	0.523	Dec
Clostridium cluster XIVa	*Bryantella*-like	**0.003**	Dec	0.876	Dec	0.530	Inc
	Butyrivibrio crossotus-like	**0.015**	Inc	0.862	Dec	0.555	Inc
	Clostridium lactifermentans-like	**0.013**	Dec	0.789	Inc	0.610	Inc
	Clostridium symbosium-like	**0.009**	Dec	0.790	Dec	0.579	Dec
	Coprococcus eutactus-like	**0.029**	Dec	0.800	Dec	0.694	Dec
	Lachnobacillus bovis-like	**0.014**	Dec	0.889	Inc	0.592	Inc
	Lachnospira pectinoschiza-like	**0.013**	Inc	0.842	Dec	0.831	Inc
	Uncultured *Clostridia XIVa*	**0.026**	Inc	0.900	Dec	0.847	Inc
Clostridium cluster XIVb	Uncultured *Clostridia XIVb*	**0.015**	Dec	0.667	Dec	0.584	Inc
Gammaproteobacteria	*Actinobacillus*-like	**0.032**	Inc	0.898	Dec	0.217	Dec
	Pasteurella	**0.045**	Inc	0.939	Inc	0.197	Inc

Different plant-derived diet additives (PENS) were tested for their potential effect on activity of microbiota associated with the fermentation of the feedstuff. To complement information on kinetics and product formation of the *in vitro* fermentation, we analyzed several samples using complementary molecular fingerprinting techniques that allowed us to determine microbiota

dynamics. In general, most of the additives had little or no effect on fermentation parameters or microbiota composition.

Fermentable PENS: Of all fermentable additives tested, Topinambur 40 (source of inulin) gave the largest reduction in Tmax with an increase in Rmax, indicating that it was fermented faster and more by the fecal microbiota than the other additives, but differences were not significant. VFA levels showed as well non-significant deviations from the control substrate. Ammonia concentration decreased ($p < 0.05$) upon addition of higher amounts of citrus pulp, Guar gum (5000) and Raftilose P95 (indigestible carbohydrates) demonstrating reduced protein breakdown. This is of interest, as reduced proteolytic fermentation can lead to the reduced formation of potentially toxic metabolites such as ammonia, amines, volatile phenols and indoles [63, 64]. This result confirmed earlier observations that such prebiotic carbohydrates affect proteolytic fermentation [65-68].

It is known that seaweeds contain a large quantity of insoluble and soluble dietary fiber [69]. Some water-soluble fibers, such as resistant starch from potatoes and undigested oligosaccharides, are metabolized to short-chain fatty acids (SCFA; acetic acid, propionic acid and butyric acid) and lactic acid by colonic bacteria [70]. Therefore, a significant increase in SCFA concentrations was expected in this study after the *in vitro* fermentation in the presence of seaweed components. Nevertheless, the inclusion of different seaweed fractions at varying doses to the control substrate did not affect the fermentation characteristics. This lack of effect is probably due to the inclusion levels. For the study described here, it was decided to set the levels of PENS inclusion on the basis of concentrations generally accepted in diet formulations, and PENS were added at 1-5% of the reference diet. The most pronounced effect was the reduction of ammonia production, albeit not significant, when the glucan laminaran was added. This was in line with observations by Kuda [71], who found that laminaran reduced ammonia produced by rat intestinal microbiota *in vitro*.

The control diet used in this study was composed mainly of barley, wheat and soybean meal. Such ingredients contain considerable levels of non-digestible oligosaccharides (NDOs), therefore, the use of such control diets may have diluted or masked the effects of NDOs added [72]. Hence, it is tempting to speculate that the absence of any effect of treatment for most PENS studied here is likely related to the low levels of inclusion. Therefore, the choice of the height of inclusion level to be used in this type of batch culture system should be considered in further research.

Non-fermentable PENS: Essential oils were included in the trial because many of them are known to have strong antibacterial effects [73]. Nevertheless, the only essential oil included in the experiment that had a significant effect on the fermentation characteristics and fermentation end products was water-soluble oregano oil. The lower cumulative gas production (OMCV) and organic matter loss (OMloss) pointed to a significant reduction of microbial activity when oregano oil water-soluble (20%) was used as additive, in comparison with the controls (with and without avilamycin) and with the other two types of oregano oil (Dutch and Mediterranean). Rmax was also lower and the halftime (T½) was considerably delayed. These results were in line with observations described in literature [74]. The fact that only one of the oregano

oil preparations had a significant effect on the fermentation might be attributed to the high concentration used (2000 mg/kg) in comparison with the 200 mg/kg used for the other two types. Furthermore, a number of microbial metabolites were decreased in the presence of the water-soluble oregano oil compared to the control, namely ammonia ($p < 0.05$) as well as total VFA concentration. This was consistent with the known antimicrobial activity of carvacrol (active component of oregano oil) [73, 75] and in line with the findings of Busquet et al. [76] and Oh et al. [77] who reported that high doses of different plant essential oils, tested in *in vitro* fermentation of mixed ruminal microorganisms, resulted in a general inhibition of rumen microbial fermentation. However, the observed decrease in pH at 2000 mg/kg is not consistent with these previous results. This pH decrease could be caused by the significant increase in acetate concentration by approximately 50% when compared to the control and other PENS. The BCP was significantly lower for the water-soluble oregano oil extract compared to the control and other treatments. This together with the lower ammonia content indicated a significantly lower proteolytic activity.

Additive-associated dynamics of fecal microbiota

The most striking observation with respect to microbiota composition after incubation in the presence or absence of a number of different PENS was a big difference between the composition between batches where feces were incubated in the presence of the water-soluble oregano oil, and feces incubated in the presence of any other PENS. This latter group of samples clustered together and the similarity between the two clusters was only 23% based on DGGE analysis, confirming previously reported changes on microbiota composition caused by oregano oil supplementation [33, 78, 79].

The analysis of microbiota composition of the inoculum, as well as after *in vitro* fermentation in the absence or presence of two different oregano oil preparations (water-soluble form and Dutch type), using the PITChip, allowed us to confirm the *in vitro* fermentation kinetics, metabolite production and DGGE results and to add more detailed information to these findings. The lower microbial diversity in the presence of the high concentration oregano oil water-soluble form (Fig. 1 and 2) confirmed its antimicrobial effect. In addition, it was possible to determine that feces incubated in the presence of the water-soluble oregano oil extract had lower relative abundance of *Bryantella*- and *Lachnospira pectinoschiza*-like organisms and *Ruminococcus callidus*- and *Sporobacter termiditis*-like species, but higher relative abundance of *Lactobacillus gasseri*-like phylotypes and yet-uncultured *Bacilli*. This confirms previous studies that showed that feeding diets supplemented with plant extracts can influence the microbiota in the digestive tract of early-weaned piglets by increasing the number of lactobacilli and the ratio of lactobacilli and enterobacteria in the caecum [80, 81].

In conclusion, this study evaluated the effect of plant-derived additives on fecal microbiota composition and activity during *in vitro* fermentation of a standard feed-stuff. When included in the diet at doses commonly used in animal production, in most cases no significant effect of PENS could be observed. This is in contrast to previous findings of studies where products were tested at higher doses, or in the absence of feed stuff. To this end, the information provided here, in combination with *in vivo* experiments, is essential for optimization of effective dietary strategies involving plant extracts and other natural substances such as studied here.

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References

1. **Raibaud, P.** (1992) Bacterial interactions in the gut, in *Probiotics: Scientific Basis*. Fuller, R. (ed.). p. 9-28. Chapman & Hall, London.
2. **van der Waaij, D., Berghuis-de Vries, J. M., and Lekkerkerk, L.-v.** (1971) Colonization resistance of the digestive tract in conventional and antibiotic-treated mice. *J. Hyg. (Lond).* 69: 405-11.
3. **Bauer, E., Williams, B. A., Smidt, H., Mosenthin, R., and Verstegen, M. W. A.** (2006) Influence of dietary components on development of the microbiota in single-stomached species. *Nutr. Res. Rev.* 19: 63-78.
4. **Pluske, J. R., Hampson, D. J., and Williams, I. H.** (1997) Factors influencing the structure and function of the small intestine in the weaned pig: a review. *Livest. Prod. Sci.* 51: 215-236.
5. **Cromwell, G. L.** (2002) Why and how antibiotics are used in swine production. *Anim. Biotechnol.* 13: 7-27.
6. **Smith, D. L., Harris, A. D., Johnson, J. A., Silbergeld, E. K., and Morris, J. G., Jr.** (2002) Animal antibiotic use has an early but important impact on the emergence of antibiotic resistance in human commensal bacteria. *PNAS.* 99: 6434-6439.
7. **Bezkorovainy, A.** (2001) Probiotics: determinants of survival and growth in the gut. *Am. J. Clin. Nutr.* 73: 399S-405S.
8. **Nemcova, R., Bomba, A., Gancarcikova, S., Herich, R., and Guba, P.** (1999) Study of the effect of *Lactobacillus paracasei* and fructooligosaccharides on the faecal microflora in weanling piglets. *Berl. Munch. Tierarztl. Wochenschr.* 112: 225-8.
9. **Collins, M. D. and Gibson, G. R.** (1999) Probiotics, prebiotics and synbiotics: approaches for modulating the microbial ecology of the gut. *Am. J. Clin. Nutr.* 69: 1052S-1057S.
10. **Shim, S. B.** (2005) Effects of prebiotics, probiotics and synbiotics in the diet of young pigs. Wageningen University, Wageningen.
11. **Kaur, N. and Gupta, A. K.** (2002) Applications of inulin and oligofructose in health and nutrition. *J. Biosci.* 27: 703-14.
12. **Buddington, R. K., Kelly-Quagliana, K., Buddington, K. K., and Saavedra, J. M.** (2002) Non-digestible oligosaccharides and defense functions: lessons learned from animal models. *Br. J. Nutr.* 87: S223-230.
13. **Lallès, J.-P., Bosi, P., Smidt, H., and Stokes, C. R.** (2007) Nutritional management of gut health in pigs around weaning. *Proc. Nutr. Soc.* 66: 260-268.
14. **Williams, B. A., Bosch, M. W., Awati, A., Konstantinov, S. R., Smidt, H., Akkermans, A. D. L., Verstegen, M. W. A., and Tamminga, S.** (2005) In vitro assessment of gastrointestinal tract (GIT) fermentation: Fermentable substrates and microbial activity. *Anim. Res.* 54: 191-201.
15. **Gibson, G. R.** (1999) Dietary modulation of the human gut microflora using the prebiotics oligofructose and inulin. *J. Nutr.* 129: 1438S-41S.
16. **Roselli, M., Britti, M. S., Le Huerou-Luron, I., Marfaing, H., Zhu, W. Y., and Mengheri, E.** (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by enterotoxigenic Escherichia coli K88 in pig intestinal cells. *Toxicol. In Vitro*. 21: 224-9.
17. **Theodorou, M. K., Williams, B. A., Dhanoa, M. S., McAllan, A. B., and France, J.** (1994) A simple gas production method using a pressure transducer to determine the fermentation kinetics of ruminant feeds. *Anim. Feed Sci. Tech.* 48: 185-197.
18. **Williams, B. A., Bosch, M. W., Boer, H., Verstegen, M. W. A., and Tamminga, S.** (2005) An in vitro batch culture method to assess potential fermentability of feed ingredients for monogastric diets. *Anim. Feed Sci. Tech.* 123: 445-462.
19. **Pierce, K. M., Callan, J. J., McCarthy, P., and O'Doherty, J. V.** (2005) Performance of weanling pigs offered low or high lactose diets supplemented with avilamycin or inulin. *Anim. Sci.* 80: 313-318.
20. **Fujihara, M. and Nagumo, T.** (1992) The effect of the content of D-mannuronic acid and L-guluronic acid blocks in alginates on antitumor activity. *Carbohydr. Res.* 224: 343-7.

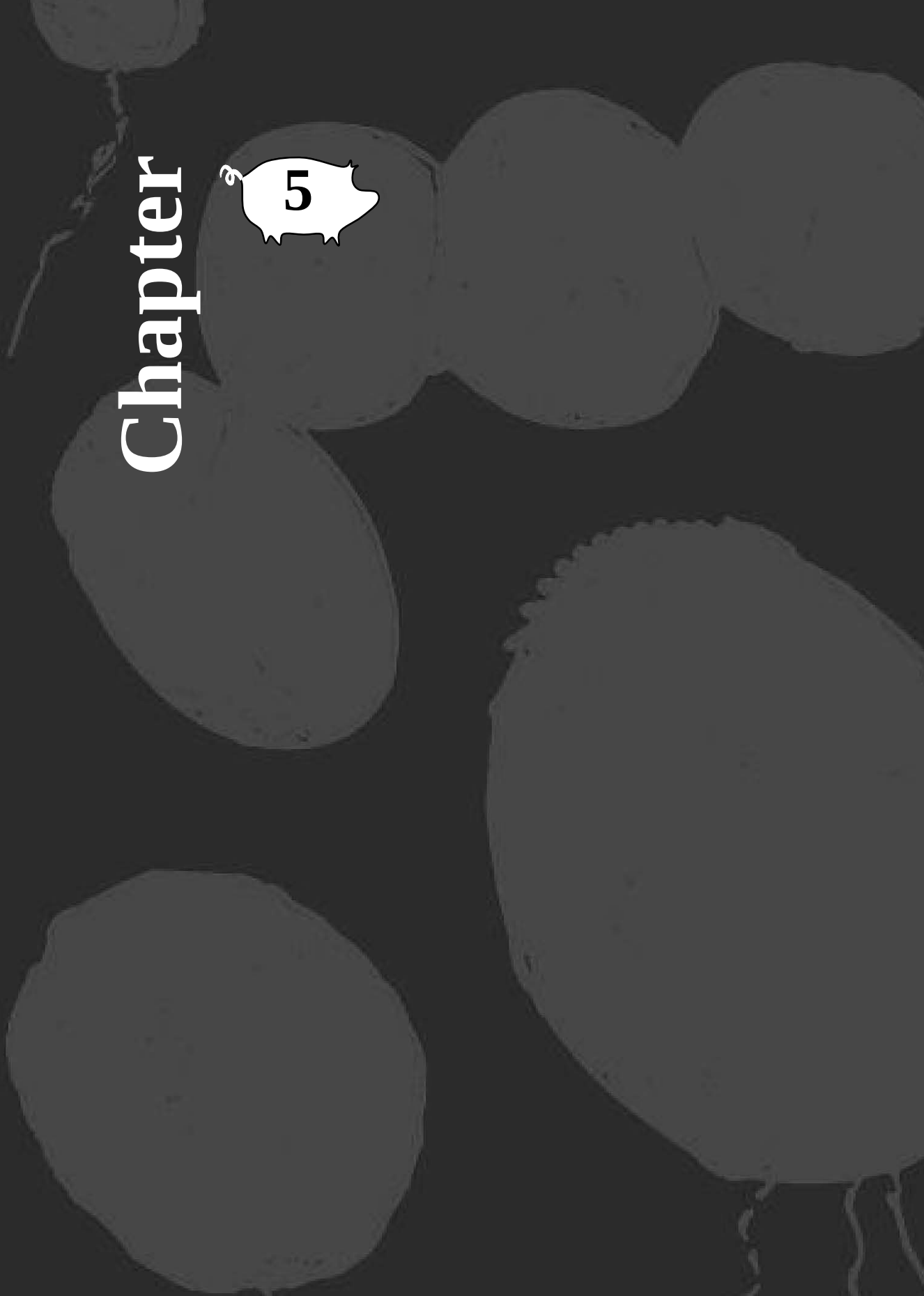
21. **Roussel, M., Benvegnu, T., Lognoné, V., Le Deit, H., Soutrel, I., Laurent, I., and Plusquellec, D.** (2005) Synthesis and Physico-Chemical Properties of Novel Biocompatible Alkyl D-Mannopyranosiduronate Surfactants Derived from Alginate. *Eur. J. Org. Chem.* 14: 3085-3094.
22. **Michel, C. and Macfarlane, G. T.** (1996) Digestive fates of soluble polysaccharides from marine macroalgae: involvement of the colonic microflora and physiological consequences for the host. *J. Appl. Bacteriol.* 80: 349-69.
23. **Campbell, J. M., Fahey, G. C., Jr., and Wolf, B. W.** (1997) Selected indigestible oligosaccharides affect large bowel mass, cecal and fecal short-chain fatty acids, pH and microflora in rats. *J. Nutr.* 127: 130-6.
24. **Andrés-Elias, N., Pujols, J., Badiola, I., and Torrallardona, D.** (2007) Effect of nucleotides and carob pulp on gut health and performance of weanling piglets. *Livest. Sci.* 108: 280-283.
25. **Van Nevel, C. J., Decuypere, J. A., Dierick, N. A., and Molly, K.** (2005) Incorporation of galactomannans in the diet of newly weaned piglets: effect on bacteriological and some morphological characteristics of the small intestine. *Arch. Anim. Nutr.* 59: 123-38.
26. **Koning, C., Jonkers, D. M., Stobberingh, E., Mulder, L., Rombouts, F. M., and Stockbrugger, R.** (2008) The Effect of a Multispecies Probiotic on the Intestinal Microbiota and Bowel Movements in Healthy Volunteers Taking the Antibiotic Amoxycillin. *Am. J. Gastroenterol.* 103: 178-189.
27. **Cvetnic, Z. and Vladimir-Knezevic, S.** (2004) Antimicrobial activity of grapefruit seed and pulp ethanolic extract. *Acta Pharmaceut.* 54: 243-50.
28. **Salyers, A. A., West, S. E., Vercellotti, J. R., and Wilkins, T. D.** (1977) Fermentation of mucins and plant polysaccharides by anaerobic bacteria from the human colon. *Appl. Environ. Microbiol.* 34: 529-33.
29. **Bauer, E., Williams, B. A., Voigt, C., Mosenthin, R., and Verstegen, M. W. A.** (2001) Microbial activities of faeces from unweaned and adult pigs, in relation to selected fermentable carbohydrates. *Anim. Sci.* 73: 313-322.
30. **Wellock, I., Fortomaris, P., Houdijk, J., and Kyriazakis, I.** (2008) Effects of dietary protein supply, weaning age and experimental enterotoxigenic *Escherichia coli* infection on newly weaned pigs: performance. *Animal*. 2: 825-833.
31. **Lawlor, P. G., Lynch, P. B., Caffrey, P. J., O'Reilly, J., and O'Connell, M. K.** (2005) Measurements of the acid-binding capacity of ingredients used in pig diets. *Ir. Vet J.* 58: 448-452.
32. **Suhr, K. I. and Nielsen, P. V.** (2003) Antifungal activity of essential oils evaluated by two different application techniques against rye bread spoilage fungi. *J. Appl. Microbiol.* 94: 665-74.
33. **Hammer, K. A., Carson, C. F., and Riley, T. V.** (1999) Antimicrobial activity of essential oils and other plant extracts. *J. Appl. Microbiol.* 86: 985-90.
34. **Burt, S. A. and Reinders, R. D.** (2003) Antibacterial activity of selected plant essential oils against *Escherichia coli* O157:H7. *Lett. Appl. Microbiol.* 36: 162-7.
35. **Yamada, Y. and Azuma, K.** (1977) Evaluation of the in vitro antifungal activity of allicin. *Antimicrob. Agents Chemother.* 11: 743-9.
36. **Ankri, S. and Mirelman, D.** (1999) Antimicrobial properties of allicin from garlic. *Microbes Infect.* 1: 125-9.
37. **Kaya, C. A. and Tuncer, S. D.** (2009) The Effects of an Organic Acids and Etheric Oils Mixture on Fattening Performance, Carcass Quality and Some Blood Parameters of Broilers. *J. Anim. Vet. Adv.* 8: 94-98.
38. **Dorman, H. J. and Deans, S. G.** (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88: 308-16.
39. **Killeen, G. F., Madigan, C. A., Connolly, C. R., Walsh, G. A., Clark, C., Hynes, M. J., Timmins, B. F., James, P., Headon, D. R., and Power, R. F.** (1998) Antimicrobial Saponins of *Yucca schidigera* and the Implications of Their *in Vitro* Properties for Their *in Vivo* Impact. *J. Agric. Food Chem.* 46: 3178-3186.
40. **Kosina, P., Walterova, D., Ulrichova, J., Lichnovsky, V., Stiborova, M., Rydlova, H., Vicar, J., Krecman, V., Brabec, M. J., and Simanek, V.** (2004) Sanguinarine and chelerythrine: assessment of safety on pigs in ninety days feeding experiment. *Food Chem. Toxicol.* 42: 85-91.

41. **Mroz, Z.** (2005) Organic Acids as Potential Alternatives to Antibiotic Growth Promoters for Pigs. *Adv. Pork. Prod.* 16: 169-182.
42. **Torrallardona, D., Badiola, I., and Broz, J.** (2007) Effects of benzoic acid on performance and ecology of gastrointestinal microbiota in weanling piglets. *Livest. Sci.* 108: 210-213.
43. **Michiels, J., Missotten, J., Fremaut, D., De Smet, S., and Dierick, N.** (2007) *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. *Livest. Sci.* 109: 157-160.
44. **Ueng, Y.-F., Chang, Y.-L., Oda, Y., Park, S. S., Liao, J.-F., Lin, M.-F., and Chen, C.-F.** (1999) *In vitro* and *in vivo* effects of Naringin on cytochrome P450-dependent monooxygenase in mouse liver. *Life Sci*. 65: 2591-2602.
45. **Dastidar, S. G., Manna, A., Kumar, K. A., Mazumdar, K., Dutta, N. K., Chakrabarty, A. N., Motohashi, N., and Shirataki, Y.** (2004) Studies on the antibacterial potentiality of isoflavones. *Int. J. Antimicrob. Agents*. 23: 99-102.
46. **Kumazawa, S., Taniguchi, M., Suzuki, Y., Shimura, M., Kwon, M. S., and Nakayama, T.** (2002) Antioxidant activity of polyphenols in carob pods. *J. Agric. Food Chem.* 50: 373-7.
47. **Groot, J. C. J., Cone, J. W., Williams, B. A., Debersaques, F. M. A., and Lantinga, E. A.** (1996) Multiphasic analysis of gas production kinetics for in vitro fermentation of ruminant feeds. *Anim. Feed Sci. Tech.* 64: 77-89.
48. **Houdijk, J.** (1998) Effect of non-digestible oligosaccharides in young pig diets, in *Animal nutrition*. Wageningen University, Wageningen, The Netherlands.
49. **Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H.** (1996) Sequence heterogeneities of genes encoding 16S rRNA in *Paenibacillus polymixa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178: 5636-5643.
50. **Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., and Hammes, W. P.** (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67: 2578-2585.
51. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
52. **Heilig, H. G. H. J., Zoetendal, E. G., Vaughan, E. E., Marteau, P., Akkermans, A. D. L., and de Vos, W. M.** (2002) Molecular diversity of *Lactobacillus* spp. and other lactic acid bacteria in the human intestine as determined by specific amplification of 16S ribosomal DNA. *Appl. Environ. Microbiol.* 68: 114-123.
53. **Sanguinetti, C. J., Dias Neto, E., and Simpson, A. J. G.** (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques*. 17: 915-919.
54. **Pérez Guiérrez, O., van den Bogert, B., Derrien, M., Molenaar, D., de Vos, W. M., and Smidt, H.** (2010) Design of a high throughput diagnostic microarray for the characterization of pig gastrointestinal tract microbiota. *In preparation*.
55. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
56. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) Calculating melting temperatures for perfectly matched hybrids between oligonucleotides and their target sequences. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
57. **Häne, B. G., Jäger, K., and Drexler, H. G.** (1993) The Pearson product-moment correlation coefficient is better suited for identification of DNA fingerprint profiles than band matching algorithms. *Electrophoresis*. 14: 967-972.
58. **Carter, R. L.** (1989) On the partitioning of squared Euclidean distance and its application in cluster analysis. *Phsychometrika*. 54: 9-23.
59. **Simpson, E. H.** (1949) Measurements of diversity. *Nature*. 163: 688.

60. **Bikker, P., Dirkzwager, A., Fledderus, J., Trevisi, P., le Huerou-Luron, I., Lalles, J. P., and Awati, A.** (2006) The effect of dietary protein and fermentable carbohydrates levels on growth performance and intestinal characteristics in newly weaned piglets. *J. Anim. Sci.* 84: 3337-45.
61. **Williams, B. A., Verstegen, M. W. A., and Tamminga, S.** (2001) Fermentation in the large intestine of single-stomached animals and its relationship to animal health. *Nutr. Res. Rev.* 14: 207-227.
62. **Williams, B. A.** (2000) Cumulative gas - production techniques for forage evaluation, in *Forage Evaluation in Ruminant Nutrition*. Givens, D. I., Owen, E., Axford, R. F. E., and Omed, H. M. (ed.). p. 189-213. CAB International, Wallingford.
63. **Yokoyama, M. T., Tabori, C., Miller, E. R., and Hogberg, M. G.** (1982) The effects of antibiotics in the weanling pig diet on growth and the excretion of volatile phenolic and aromatic bacterial metabolites. *Am. J. Clin. Nutr.* 35: 1417-24.
64. **Macfarlane, G. T., Gibson, G. R., Beatty, E., and Cummings, J. H.** (1992) Estimation of short-chain fatty acid production from protein by human intestinal bacteria based on branched-chain fatty acid measurements. *FEMS Microbiol. Ecol.* 101: 81-88.
65. **Knudsen, K. E. B. and Hansen, I.** (1991) Gastrointestinal implications in pigs of wheat and oat fractions: 1. Digestibility and bulking properties of polysaccharides and other major constituents. *Br. J. Nutr.* 65: 217-232.
66. **Mathew, A. G., Jones, T., and Franklin, M. A.** (1994) Effect of creep feeding on selected microflora and short-chain fatty acids in the ileum of weanling pigs. *J. Anim. Sci.* 72: 3163-8.
67. **Canibe, N., Steien, S. H., Overland, M., and Jensen, B. B.** (2001) Effect of K-diformate in starter diets on acidity, microbiota, and the amount of organic acids in the digestive tract of piglets, and on gastric alterations. *J. Anim. Sci.* 79: 2123-2133.
68. **Kluess, J.** (2004) Evaluation of ileal digesta in weaning piglets fed different diets. Free University of Berlin, Berlin.
69. **Suzuki, T., Ohsugi, Y., Yoshie, Y., Shirai, T., and Hirano, T.** (1996) Dietary fiber content, water-holding capacity and binding capacity of seaweeds. *Fish Sci*. 62: 7.
70. **Cherbut, C., Michel, C., and Lecannu, G.** (2003) The prebiotic characteristics of fructooligosaccharides are necessary for reduction of TNBS-induced colitis in rats. *J. Nutr.* 133: 21-7.
71. **Kuda, T.** (2005) Inhibitory effects of laminaran and low molecular alginate against the putrefactive compounds produced by intestinal microflora in vitro and in rats. *Food Chem*. 91: 745-749.
72. **Henry, R. J. and Saini, H. S.** (1989) Characterization of cereal sugars and oligosaccharides. *Cereal Chem*. 66: 362-365.
73. **Burt, S.** (2004) Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* 94: 223-53.
74. **Davidson, P. M. and Naidu, A. S.** (2000) Phyto-phenols, in *Natural Food Antimicrobial Systems*. Naidu, A. S. (ed.). CRC Press, Boca Raton, FL, USA.
75. **Oviedo-Rondón, E. O., Clemente-Hernández, S., Salvador, F., Williams, P., and Losa, R.** (2006) Essential oils on mixed coccidia vaccination and infection in broilers. *Int. J. Poult. Sci.* 5: 723-730.
76. **Busquet, M., Calsamiglia, S., Ferret, A., and Kamel, C.** (2006) Plant extracts affect in vitro rumen microbial fermentation. *J. Dairy Sci.* 89: 761-71.
77. **Oh, H. K., Sakai, T., Jones, M. B., and Longhurst, W. M.** (1967) Effect of various essential oils isolated from Douglas fir needles upon sheep and deer rumen microbial activity. *Appl. Microbiol*. 15: 777-84.
78. **Elgayyar, M., Draughon, F. A., Golden, D. A., and Mount, J. R.** (2001) Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *J. Food Prot.* 64: 1019-24.
79. **Chaudhry, N. M. A., Saeed, S., and Tariq, P.** (2007) Antibacterial Effects of Oregano (*origanum vulgare*) against gram negative Bacilli. *Pakistan J. Bot.* 39: 609-613.
80. **Manzanilla, E. G., Perez, J. F., Martin, M., Kamel, C., Baucells, F., and Gasa, J.** (2004) Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* 82: 3210-8.

81. **Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., and Gasa, J.** (2006) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *J. Anim. Sci.* 84: 2725-34.

Chapter 5



Dynamics of Piglet Ileal Microbiota in Response to Carvacrol

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Abstract

Essential oils are being considered as possible alternatives to in-feed antibiotic growth promoters in pig nutrition. The effect of carvacrol, the active component of oregano oil, on ileal microbiota dynamics was studied in piglets. Animals were fitted with an ileal T-shaped cannula at 17-18 days of age, and 26 days after birth (day 0) they were weaned and offered either a control diet or a control diet plus carvacrol (150 g/ton of diet). Samples from ileal digesta were collected at days -10, -3, 1 to 7, 10, 13, 14 and 15. DNA was extracted and total amounts of bacteria and lactobacilli were measured by real time PCR. Detailed phylogenetic analysis by the Pig Intestinal Tract Chip (PITChip) was performed on a representative selection of samples. Specific differences in microbial communities were identified at each time point for the two treatment groups. An antimicrobial effect of carvacrol was observed at different days as the phylogenetic analysis showed significantly lower relative intensity of several microbial taxa, among which there were several *Streptococcus*- and *Bacteroides*-like species. Furthermore, carvacrol was found to stabilize total bacteria numbers during the weaning transition.

Introduction

In modern pig production weaning of piglets occurs at a very young age and is associated with very severe stress. The main reasons are an abrupt removal of the young piglets from the dam, mixing of different litters, dietary changes (from a milk to a cereal-based diet) and contact with new bacteria. These all result in changes in the intestinal microbial community, a suppressed immune system and an increased prevalence of intestinal pathogens [1, 2]. As a result, piglets stop eating and become easy targets for the development of post-weaning diarrhea with resultant performance losses [3]. In an attempt to control these problems, antibiotics are included in the feed as growth promoters [4]. This has been common practice for many years, until the use of antibiotics was banned in the European Union due to an increased concern on emergence of antimicrobial resistance in humans [5, 6]. For this reason there is a strong interest in finding alternatives to modify pig gastrointestinal (GI) tract microbiota and help piglets in their weaning transition.

Due to their antimicrobial properties, plant extracts have been used during many years for different purposes, including their application in traditional medicine and as food preservatives [7, 8]. It has been reported that these plant extracts can also influence the gut microbiota when used in piglets' diets [9-11]. In many cases, the exact origin of the antimicrobial activity of plant extracts has not been unequivocally elucidated, but it is often attributed to a number of secondary plant metabolites, which include saponins, tannins and essential oils [12, 13]. Essential oils are aromatic oily liquids obtained from non-woody plant material, mainly produced by steam distillation. Many of these extracts have strong antimicrobial activities against a wide range of Gram-positive and Gram-negative bacteria, yeasts and moulds [14-16]. Hence, they can be used as a substitute to antibiotics in the animal feed industry. Active components of essential oils are also known to have anti-oxidative and anti-inflammatory effects [17-19].

In general, the antimicrobial activity of the active components of essential oils is highest in oxygenated cyclic hydrocarbons, and particularly in phenolic structures such as thymol and carvacrol [20, 21]. This could be due to the hydroxyl group and the dislocated electrons that permit the interaction with water through hydrogen bridges, making them particularly active against a wide range of microorganisms [7, 22]. Hence, carvacrol, the active component of oregano oil, can act both on Gram-positive and Gram-negative bacteria [13, 22-25]. Recently, another mechanism for the broad action of carvacrol was proposed in which the hydroxyl group of the phenol acts as a carrier of monovalent cations and protons through the cytoplasmic membrane [26].

The work presented here studied the effect of carvacrol on the global microbiota composition of piglets weaned at an early age. In addition, the development of the microbial community during the weeks after weaning was determined. To this end, ileal digesta was continuously sampled through T-shaped cannulas, and a phylogenetic microarray custom-designed towards comprehensive profiling of the pig intestinal microbiota was used to provide a detailed insight in the piglets' GI tract microbial dynamics.

Materials and Methods

Animals & Housing

Twelve Landrace piglets selected from four different sows were fitted with an ileal T-shaped cannula at an age of 17 to 18 days (day -10; body weight, BW, 6.13 ± 0.46 kg). Before surgery piglets were fasted for 2 h (with water *ad libitum*) and were medicated (approx. ½ hour before surgery) with 4 mg/kg BW azaperone and 0.04 mg/kg BW atropine. Anesthesia was induced with isoflurane. A T-cannula was inserted in the intestinal lumen, exteriorized through a hole, fastened to the exterior part and closed with a stopper. After surgery the animals were placed back with the sows. Ten days later (day 0) piglets were weaned and per litter randomly assigned to one of the two dietary treatments, control *vs*. carvacrol. After weaning (26 days after birth) the animals were housed individually in metabolic cages (L x W x H: 90 x 60 x 45 cm) at ambient temperature (24°C).

Dietary treatment and sample collection

This study involved two periods: the pre-weaning period from day -10 (17-18 days after birth) when piglets were cannulated, and the post-weaning period from day 0 (26 days after birth) to day 15. Before weaning piglets were suckled by the sow, no creep feed was provided. At weaning, animals were randomly assigned to either an EU-reference diet (Table 1; control) or an EU-reference diet with added carvacrol (150 ppm; provided by Pancosma S.A.S., Bellegarde s/Valserine, France). Diets were offered in pelleted form and in increasing portions twice daily. Water was offered *ad libitum*. Samples of approximately 1g of ileal chyme were taken twice per day (11.30 h and 16.30 h) at the moment of surgery (day -10), at day -3, day 1 to day 7, day 10, day 13, day 14 and day 15. Samples were stored in eppendorf tubes at -20°C. At day 16 post-weaning, animals were slaughtered, 5 h after the last meal.

DNA isolation

A total of 117 samples, from 10 piglets that had either a control or carvacrol supplemented diet, were selected for DNA extraction. Samples (stored at -20ºC) were defrosted and DNA was extracted from 100 mg of digesta using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad,CA, USA). A NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) was used to determine DNA concentrations and electrophoresis in agarose gel 1.2% (w/v) containing ethidium bromide was used to check the quality of DNA.

Quantitative PCR (qPCR)

Quantitative 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). The reaction mixture (25 µL) consisted of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 5 µL of template DNA diluted in water (10 or 100 times) to avoid PCR inhibition. A standard curve was generated using the serially diluted 16S ribosomal RNA (rRNA) gene amplicons obtained from a pure culture of *Lactobacillus sobrius*. All reactions were done in triplicate. Universal primers, Bact 1369 (5´-CGGTGAATACGTTCYCGG-3´)

and Prok 1492 (5´-GGWTACCTTGTTACGACTT-3´) [27] 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 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 sec, 56ºC for 30 sec and 72ºC for 30 sec.

Primers Lacto F (5´-TGGAAACAGRTGCTAATACCG- 3´) and Lacto R (5´-GTCCA TTGTGGAAGATTCCC 3´) [28] were used to quantify the *Lactobacillus* group present in the sample using the following PCR program: initial denaturation step of 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 62ºC for 60 sec.

Table 1. Feed composition of the EU- reference diet.

Feedstuff	Amount (%)
Potato protein (Prostar)	5.00
Barley	30.00
Wheat	29.55
Wheat bran	2.50
Sunflower bran CP 20-24	2.50
Peas < 22% CP	5.00
Maize gluten meal > 60	2.20
Maize starch	4.00
Whey proteins P.MSA-27% RE/2% RV	8.00
Soycomil	4.00
Melasse (sugarcane) > 47.5% SU	1.00
Soy oil	1.98
Palm oil	1.10
Limestone	1.20
Mono Calcium Phosphate	0.78
Salt	0.28
Lysine-HCl (L, 79%)	0.34
Methionine (DL, 99%)	0.11
Threonine (L, 98%)	0.03
Tryptophane (L, 98%)	0.03
Farmix (mineral-vitamin premix)	0.40
TOTAL	100

PITChip analysis

The phylogenetic analysis of the digesta samples was done using the PITChip, a diagnostic microarray (Chapter 3), composed of approximately 3000 DNA oligonucleotide probes targeting bacterial 16S rRNA variable regions V1 and V6 of in total 627 bacterial phylotypes previously described for the porcine gastrointestinal tract (Chapter 3) [29]. Hybridization, washing and data analysis was essentially done as described previously for the human intestinal tract chip [30]. Primers T7prom-Bact-27-for (5´-TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGGCTCAG-3´) and

Uni-1492-rev (5´-CGGCTACCTTGTTACGAC-3´ [31] were used for the amplification of bacterial 16S rRNA gene fragments from 10 ng of DNA extracted from intestinal samples in a final volume of 50 m). The PCR program included initial denaturation at 94°C for 2 min followed by 35 cycles of 30 sec at 94°C, 40 sec at 52°C, and 90 sec at 72°C, and a final extension of 7 min at 72°C. PCR products were purified using the NucleoSpin Extract II kit (Macherey-Nagel, Düren, Germany), and a NanoDrop ND-1000 spectrophotometer was used to determine DNA concentrations.

In vitro transcription of the 16S rRNA genes carrying the T7-promoter was performed with the Riboprobe System (Promega, La Jolla, USA), using 500 ng of the gene amplicon plus rATP, rGTP, rCTP and a 1:1 mix of rUTP and aminoallyl-rUTP (Ambion, Austin, TX, USA). Following incubation for 2 h at room temperature, DNA was digested with the Qiagen RNAse-free DNAse kit (Qiagen, Hilden, Germany). RNA was purified (RNeasy Mini-Elute Kit, Qiagen, Hilden, Germany) and the concentration was quantified spectrometrically (NanoDrop).

For fluorescent labeling of RNA samples containing modified amino-allyl nucleotides, the CyDye Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK) dissolved in DMSO (84 µl) was used. Twenty microliters of either Cy3 or Cy5 dye were added to 2 mg of purified RNA in the presence of sodium bicarbonate buffer (25 mM, pH 8.7). Samples were incubated in a final volume of 40 µl for 90 min in the dark and at room temperature. The reaction was stopped by addition of 15 µl of 4 M hydroxyl-amine and incubation in the dark for 15 min. RNAse-free water was added to 100 µl and the labeled RNA was quantified.

Custom microarrays of the 8×15K format were synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA). To each array, two samples that were labeled as described above with Cy3 and Cy5 dye, respectively, were hybridized. The Cy3/Cy5-labeled target mixtures were fragmented with 10× fragmentation reagent (Ambion), and hybridization was performed for 16 h in a rotation oven (Agilent) at 62°C. Subsequently, slides were washed at room temperature for 10 min in 2× SSC with 0.3% SDS, followed by washing at 38°C for 10 min with 0.1× SSC with 0.3% SDS, and for 5 min in 0.06× SSPE [32]. The Agilent Feature Extraction software, version 9.1 (http://www.agilent.com) was used for data extraction from scan images. A set of R based scripts (http://r-project.org) in combination with a custom designed relational database, running under the MySQL database management system (http://www.mysql.com), was used for normalization of raw data, and further statistical analysis. To this end, Ward's minimum variance method was used for hierarchical clustering of probe profiles by calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance – E2) [33].

Statistical analysis

For the statistical analysis of 16S rRNA gene copy numbers of bacteria and lactobacilli as measured by qPCR, the number of gene copies was $\log_{10}$ transformed to achieve normal distribution and the mean and standard deviation were calculated. Comparisons between control and carvacrol treatments were calculated with ANOVA and were considered statistically significant if $p < 0.05$.

Simpson's reciprocal index of diversity (1/D) was used to measure diversity of microbial profiles obtained by PITChip analysis [34], and was calculated with the equation $l = 1/\Sigma Pi^2$,

where Pi is the proportion of i^{th} taxon. This is the proportion of each probe signal compared to the total signal for each sample. A higher Simpson's index value indicates a higher degree of diversity.

Multivariate analysis was applied for PITChip data interpretation. In order to relate changes in bacterial microbiota composition to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) [35]. This direct gradient analysis method allows ordering of samples and taxonomic groups (also referred to as species data in the statistical analysis) considering that the latter have linear relationships to environmental variables. As species data, the sums of signal intensities for 144 phylogenetic groups defined at approximately the genus level (90% 16S rRNA sequence identity threshold) were used. Treatment classes (i.e. control *vs*. carvacrol) were introduced as nominal environmental variables, and time was treated as a quantitative variable (vector). Redundancy analysis was performed focusing on inter-samples correlation and the Monte Carlo permutation test was applied to test for significance of the effect of treatment and/or time on microbiota composition [36]. Environmental variables were considered to interfere significantly with microbiota composition at p-values < 0.05. Diagrams were plotted as triplots using CanoDraw for Windows. Another multivariate analysis applied was Principle Response Curve (PRC). This is done by performing RDA but this time introducing the relation between sampling date and treatment as species variables and the sampling time points as co-variables [37].

Results

The present study aimed at determining the ileal microbiota dynamics during the first weeks of life, focusing on the weaning transition, as well as the effect of dietary carvacrol on the microbiota composition. To this end, newborn piglets were fitted with an ileal cannula to allow repeated sampling. Subsequently, the intestinal microbiota dynamics was analyzed in the recovered samples, using 16S rRNA gene-targeted quantitative PCR as well as diagnostic microarray analyses, without the need to sacrifice the animals. This notably reduced the impact of inter-individual differences that often interfere with the significance of the observations.

Total number of bacteria and Lactobacillus spp.

The total and *Lactobacillus* microbial populations were quantified in the distal ileum during the weaning period (from 3 days before weaning till day 15 post-weaning) using qPCR (Fig. 1). Total numbers of bacteria before weaning, expressed as log 16S rRNA gene copies/g of ileal chyme, were 11.0 ± 1.04 and these decreased more than one log unit (to 9.9 ± 0.54 log copies/g) one day after weaning. After the first day post-weaning the numbers of bacteria increased slowly until they stabilized at the end of the sampling period 15 days after weaning. After weaning (from day -3 to day 1) there was a reduction in total bacterial numbers that was almost significant ($p = 0.078$) and from day 1 bacterial numbers started increasing until reaching significant levels (compared to day 1) at day 4, 5 and 6 ($p < 0.05$). From day 6, total bacterial numbers started decreasing significantly again until day 13 when they stabilized. Interestingly,

piglets receiving carvacrol had a more stable transition from before weaning till day 15 after weaning. At weaning bacterial numbers only decreased from 10.7 ± 0.41 log copies/g of ileal chyme to 10.4 ± 0.55 log copies/g one day after weaning. There were no further changes until day 6 from when there was a significant decrease until day 13 and from then on, they remained stable till day 15 (Fig. 1A).

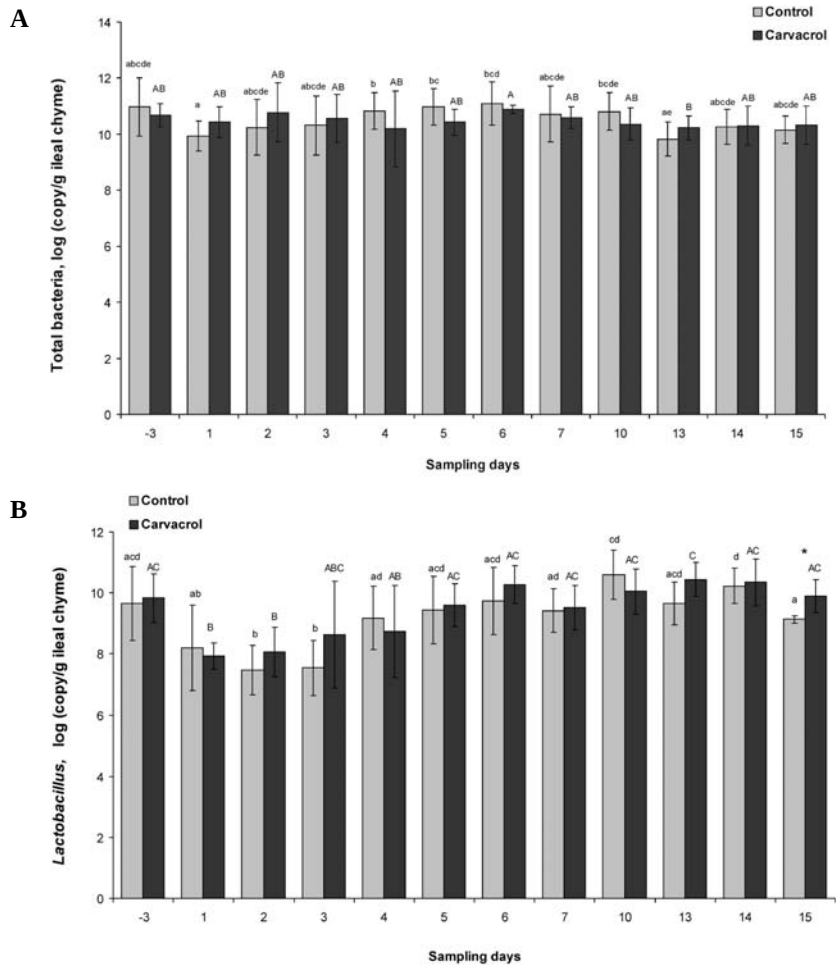

Figure 1. Quantitative PCR results for total bacteria (**A**) and *Lactobacillus* (**B**) in the ileum of piglets fed with a control diet (light bars) and piglets fed the control diet supplemented with 150ppm carvacrol (dark bars). Bacterial counts were measured at different time points, from 3 days before weaning (-3) until 15 days post-weaning, and were expressed as log 16S rRNA gene copies/g ileal chyme. * indicates a significant difference ($p < 0.05$) between the value corresponding to the control group and the value corresponding to the carvacrol group. Different letters indicate significant differences ($p < 0.05$), lowercase for the control group and uppercase for the carvacrol group. Bars represent means and SEM.

No significant differences in total bacterial loads related to experimental diets were found at any day of the studied period.

The weaning transition had a drastic effect on the *Lactobacillus* numbers. There was a decrease of more than 2 log units (from 9.6 ± 1.21 to 7.5 ± 0.81 log copies/g ileal chyme; $p = 0.010$) during weaning (from day -3 to day 2) and from that point onward, the numbers of lactobacilli increased until reaching significant differences at day 4 and the maximum value at day 10 (10.6 ± 0.81 log copies/g). During the last 5 days (from day 10 till day 15) a significant decrease ($p < 0.01$) was observed and numbers equivalent to the initial pre-weaning level were reached (9.1 ± 0.11 log copies/g) (Fig. 1B). Lactobacilli dynamics did not change much in piglets fed with carvacrol. However, there was a significant decrease in lactobacilli numbers from before weaning to day 1 ($p < 0.01$; from 9.8 ± 0.80 to 7.9 ± 0.42 log copies/g) and from there on, numbers increased reaching 10.3 ± 0.61 log copies/g at day 6, followed by slight oscillations without significant changes till the end of the experiment (Fig. 1B). Compared to the control group, the decrease in lactobacilli numbers was faster after weaning (already at day 1) and numbers were significantly higher in the carvacrol group at day 15 ($p = 0.03$).

Microarray profiling

In order to provide a more detailed assessment of microbial composition dynamics with time and treatment, a selection of samples taken at day -3, 1, 5, 10 and 15 was analyzed with the PITChip, a phylogenetic microarray. A multivariate analysis (RDA) of the phylogenetic data generated by the PITChip was performed projecting the summed hybridization signals of 144 phylogenetic groups on the two canonical axes that had the highest explanatory potential (they accounted for 20.4% of the total variance in the species data; Fig. 2). The redundancy analysis showed that time (day of sampling) had a significant influence on the microbial profiles ($p = 0.006$), whereas the addition of carvacrol to the diet of weaned piglets did not cause any statistically significant change as compared to the control group, but close to significance ($p = 0.07$). Eigenvalues of the first and second RDA axes constrained to the treatment classes were 0.184 and 0.02, respectively. Microbial groups that explained at least 26% of the differences among samples appear represented on the triplot and seemed to be more related to the control than to the carvacrol group (Fig. 2). Mainly groups within the *Bacteroidetes* were positively correlated with the control samples and negatively correlated with the samples from carvacrol-treated animals.

Principle Response Curve analysis, which allows to identify differences between experimental groups in time, showed that the treatment groups were more different towards the end of the experiment, whereas the microbiota composition of animals of the two different groups was more similar directly after weaning (Fig. 3).

The relative changes in diversity in time for each pig, both from the control and the carvacrol group, were determined based on the PITChip results (Fig. 4A). The 3 control piglets showed a decrease in diversity immediately after weaning, and at day 15, in 2 of the 3 piglets, it was higher again. Piglets fed with carvacrol did not show this decrease in diversity after weaning.

On the contrary, their diversity increased indicating a possible positive effect of carvacrol. The

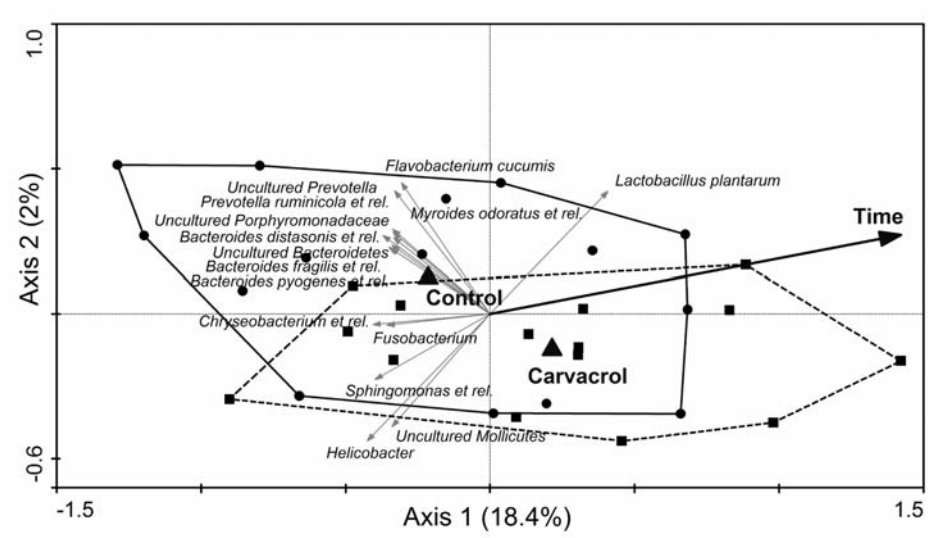

Figure 2. Redundancy analysis triplot of the ileum microbiota composition, as measured by PITChip analysis and expressed by the summed hybridization signals of 144 phylogenetic groups, for 15 pigs from the control group (●) and 15 pigs from the carvacrol treated group (■). Microbial groups that contributed at least 20% to the explanatory axes are represented as vectors. The environmental nominal variables Control and Carvacrol are centroids of the plot and the non-nominal variable time is represented as a vector, the length of which corresponds to variance that can be explained by the environmental variable. Monte Carlo permutation test indicated that changes in microbial profiles are significantly correlated to Time ($p < 0.01$).

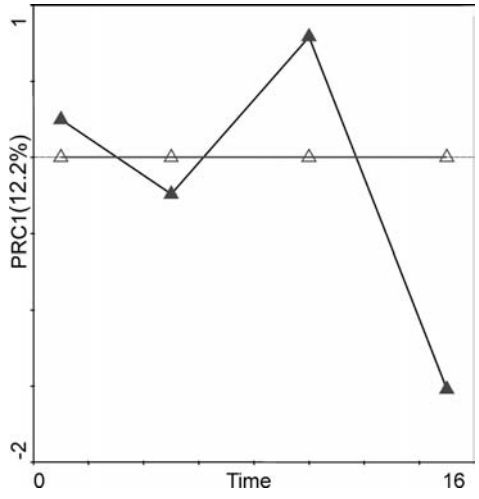

Figure 3. Diagram showing the first component of the principle response curve using the control dataset as a reference line. Filled triangles represent samples from pigs receiving carvacrol and empty triangles represent the control samples.

average for the 3 piglets also showed a decrease from day -3 to day 1 in the control group and an increase in the carvacrol group, although these changes were not significant (Fig. 4B). In the control piglets, the microbial diversity decreased in time from day -3 until reaching the lowest value at day 10 (0.72 ± 0.08; $p < 0.01$). Diversity was higher in piglets that received carvacrol than in piglets that received the control diet; this difference was significant at days 1 and 10.

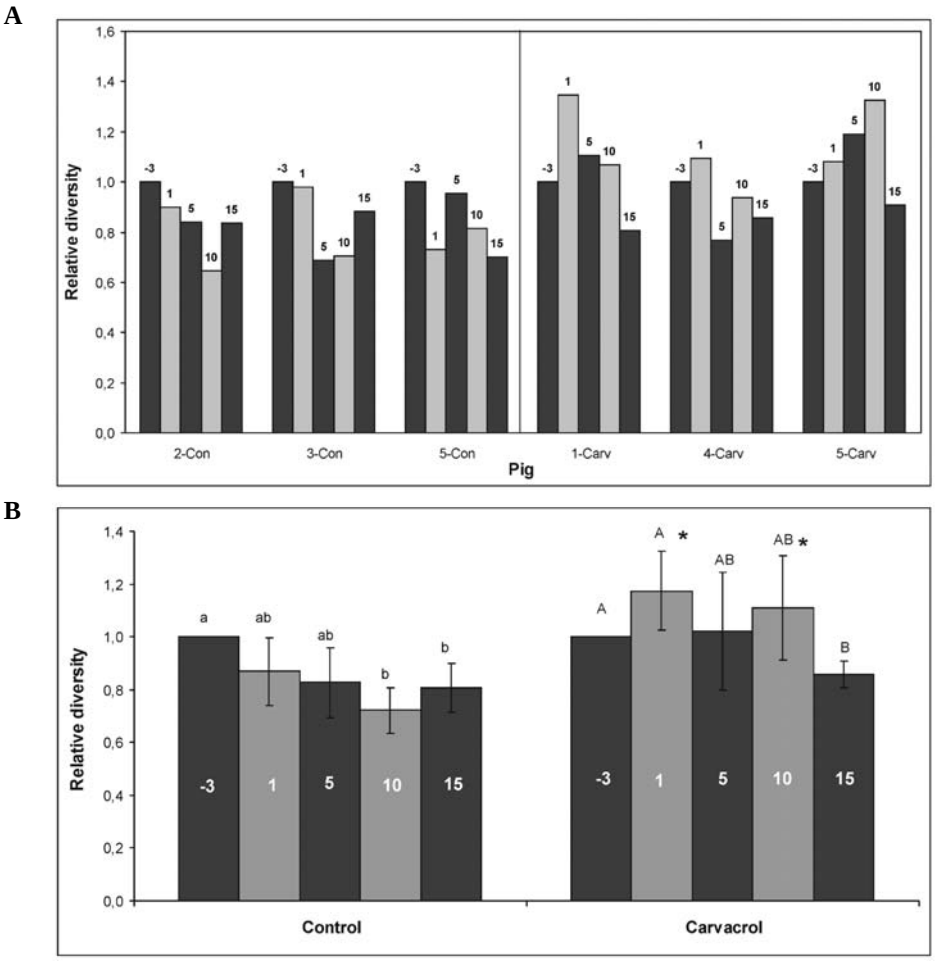

Figure 4. Relative diversity of PITChip microbial profiles along the sampling period. (**A**) Diversity for the individual piglets, with control piglets on the left half and treatet piglets on the right half. Time points are indicated above the bars. (**B**) Average diversity for both the control group and the carvacrol group. Time points are labeled in the interior of the bars. ∗ indicates that the difference between the treatment and the carvacrol is significant ($p < 0.05$) and letters indicate significant differences between time points (small for the control and capital for carvacrol), $p < 0.05$. Bars represent means and SEM.

To assess the effect of carvacrol supplementation on specific microbial groups detected by the PITChip, the sums of hybridization signals of the most abundant (> 1%) phylogenetic groups (level 2, 90% 16S rRNA sequence identity threshold) were compared for the two treatment groups using t-tests (Table 2). Comparison of the two treatment groups before weaning indicated that the lower diversity in piglets from the treatment group was most likely due to a significantly lower abundance of *Bacteroides* populations. One day after weaning, the bacterial profiles were very similar between the two treatment groups with no clear difference in *Bacteroides* species anymore. Five days after weaning, the carvacrol group had lower levels of *Bacteroidetes*, *Proteobacteria* and *Clostridium*. At day 10 post-weaning, piglets supplemented with the antimicrobial showed a higher intensity of several microbial groups, possibly indicating an improved response to the weaning period and the beginning of the recovery. Samples collected 15 days after weaning presented a significanty lower intensity of several bacterial groups compared to the control and this is in agreement with diversity results, as well as with the pronounced differences between treatment groups as visualized by the principle response curves (Fig. 3).

Comparisons of the microbial composition between pigs before weaning and each day after weaning, for both the control and the carvacrol groups, revealed a lower intensity of several genus-like groups (mainly from the *Bacteroidetes*) for the control piglets. In contrast, the carvacrol-treated piglets showed an increase in relative abundance of many bacterial groups, with a more stable weaning transition, while only a few microbial groups changed significantly between day -3 and the day 1 (data not shown). At day 15, many significant changes with respect to the pre-weaning profiles were observed in the treated group. The main trends included an increase in *Actinobacteria*, a decrease in *Bacteroidetes* and *Clostridium* cluster XIVa, and an increase in *Proteobacteria* (data not shown). These changes were not observed in piglets not receiving carvacrol.

Lactobacillus-like species decreased after weaning with both treatments and from day 5 onwards they started to increase again. These results were not statistically significant but the trend agreed with what was observed by group-specific qPCR.

Discussion

This study describes the effect of carvacrol supplementation to the diet of newborn piglets on the ileal microbial composition before and after weaning. According to previous studies carvacrol, the main component of oregano oil, has a strong antimicrobial effect [38, 39]. Consequently it has been hypothesized that carvacrol supplementation to piglets' diet could result in an improvement in pig performance and health around the stressful period of weaning [16].

In the present study, the ileal microbiota composition was followed from 3 days before until 15 days after weaning (Fig. 2). We observed a significant effect of sampling time on the microbial composition, in line with previous studies [40]. It has been shown previously that weaning is a difficult period for the pig that is reflected by major changes in the GI tract microbiota [41-43]. Fermentation-end products analysis reflecting an altered microbiota confirmed these observations (Pellikaan et al., in preparation).

Total volatile fatty acids (TVFA), ammonia (NH_3) and branched chain proportions

Table 2. SSU rRNA-based groups, for which relative contribution was above 1% and hybridization signals were significantly different for at least one of the sampling days between ileal chyme samples from piglets receiving the control diet and piglets receiving the control diet supplemented with 150ppm of carvacrol. p-values were determined with the sum of the hybridization signals of 144 phylogenetic groups. Significant values are marked in bold and the effect of the treatment is indicated as "up" (increase) or "down" (decrease).

Higher taxonomic group	Group	Day -3		Day 1		Day 5		Day 10		Day 15	
		Effect	p-value	Effect	p-value	Effect	p-value	Effect	p-value	Effect	p-value
Alphaproteobacteria	*Caulobacter*-like	up	0.294	up	0.392	down	0.925	down	0.782	**up**	**0.036**
	Rhodobacter-like	up	0.197	up	0.196	**down**	**0.002**	up	0.350	**down**	**0.039**
Bacilli	*Bacillus*-like	up	0.217	up	0.469	**down**	**0.001**	up	0.353	down	0.124
	Staphylococcus aureus-like	up	0.275	up	0.925	**down**	**0.013**	up	0.397	down	0.311
	Streptococcus intermedius-like	up	0.183	up	0.417	up	0.862	up	0.147	**down**	**0.026**
	Streptococcus salivarius-like	up	0.361	up	0.803	up	0.848	up	0.152	**down**	**0.032**
	Streptococcus suis-like	up	0.204	up	0.429	up	0.746	up	0.102	**down**	**0.011**
Bacteroidetes	*Bacteroides distasonis*-like	**down**	**< 0.001**	up	0.440	down	0.363	up	0.087	down	0.053
	Bacteroides fragilis-like	**down**	**0.006**	up	0.076	down	0.386	**up**	**0.029**	**down**	**0.024**
	Bacteroides pyogenes-like	**down**	**0.002**	down	0.960	down	0.317	up	0.151	down	0.072
	Prevotella ruminicola-like	**down**	**0.001**	up	0.334	down	0.332	up	0.084	down	0.061
	Unc. *Bacteroidetes*	**down**	**0.001**	down	0.150	down	0.341	up	0.128	down	0.055
	Unc. *Porphyromonadaceae*	**down**	**< 0.001**	up	0.497	down	0.368	up	0.083	down	0.057
	Unc. *Prevotella*	**down**	**0.021**	up	0.087	down	0.400	up	0.215	**down**	**0.038**
Clostridium cluster IV	*Eubacterium siraeum*-like	up	0.332	up	0.334	down	0.690	down	0.854	**up**	**0.033**
	Unc. *Clostridia* IV	up	0.573	down	0.297	down	0.398	up	0.100	**down**	**0.005**
Clostridium cluster IX	*Phascolarctobacterium faecium*-like	down	0.178	down	0.538	down	0.736	up	0.087	**down**	**0.036**
Clostridium cluster XIII	*Peptoniphilus indolicus*-like	up	0.287	down	0.611	**down**	**0.004**	up	0.310	down	0.133
Clostridium cluster XIVa	*Clostridium hylemonae*-like	up	0.373	up	0.425	**down**	**0.004**	up	0.161	down	0.067
	Clostridium oroticum-like	up	0.371	up	0.911	**down**	**0.004**	up	0.161	down	0.067
	Clostridium sphenoides-like	up	0.296	down	0.796	**down**	**0.001**	up	0.198	down	0.094
Clostridium cluster XIVb	Unc. *Clostridia* XIVb	down	0.642	up	0.546	down	0.329	**up**	**0.025**	down	0.119
Gammaproteobacteria	*Acinetobacter*-like	up	0.230	up	0.432	**down**	**0.004**	up	0.333	**down**	**0.029**
	Xanthomonas-like	up	0.126	down	0.956	up	0.958	down	0.669	**up**	**0.007**

(BCP) were found to be significantly affected by time, with a decline between pre-weaning (day -3) and the first day post-weaning (day 1) followed by a general increase in concentrations through to day 15. Such changes in metabolites in literature correlated with the abundance and composition of *Lactobacillus* spp., which was in line with the data on lactobacilli counts observed in this study (Fig. 1B) [40-42, 44]. The effect of carvacrol on the fermentation parameters, however, was not significant, contrasting with previous work [45].

The total numbers of bacteria did not reveal specific changes related to the different diets. This result could be explained by a decrease in beneficial bacteria and an increase in pathogens. The decrease in good microbes would leave behind a less competitive environment for pathogens (more available nutrients and attachment sites to the epithelium [46].

Although there was no significant effect of carvacrol on pig performance (Paul Bikker, personal communication) and fermentation characteristics, there was a clear antimicrobial effect on gut microbiota, where mainly the Gram-negative bacteria were found to be affected (Table 2). It has been reported that *Staphylococcus aureus* and *Escherichia coli* were inhibited by the addition of oregano oil to the diet of piglets [38, 47]. This observation was confirmed in the present work only for *S. aureus*-like populations that were significantly inhibited in the presence of carvacrol. However, *E. coli*-like species levels were not different between the control piglets and treated animals. This difference in results could be caused by the use of different doses of oregano oil, since it has been shown that the bactericidal activity of carvacrol depends on the concentration and the time of exposure [48]. Results from other studies provide support for the hypothesis that the low concentration of carvacrol could be the reason why only a mild effect of this compound was seen on microbial dynamics, contrasting with stronger effects previously reported [11, 49]. Similarly, we observed a dose-dependent effect of different oregano oil preparations in *in vitro* batch fermentation assays (Chapter 4 of this thesis). The microbial changes in time (from day -3 to day 15) suggested a beneficial effect of carvacrol when supplemented to piglets just after weaning. Moreover, the increase in diversity indicated a possible healthier status of piglets receiving carvacrol as diversity may be associated with resilience [50].

The objective of this study was to determine the effect of carvacrol addition to piglets' diets after weaning on animal performance and ileal microbial profiles. The results found indicated that there was an important effect of carvacrol on ileal microbiota, albeit not concomitant with modulation of piglets' performance. This may be linked to the low inclusion levels of carvacrol.

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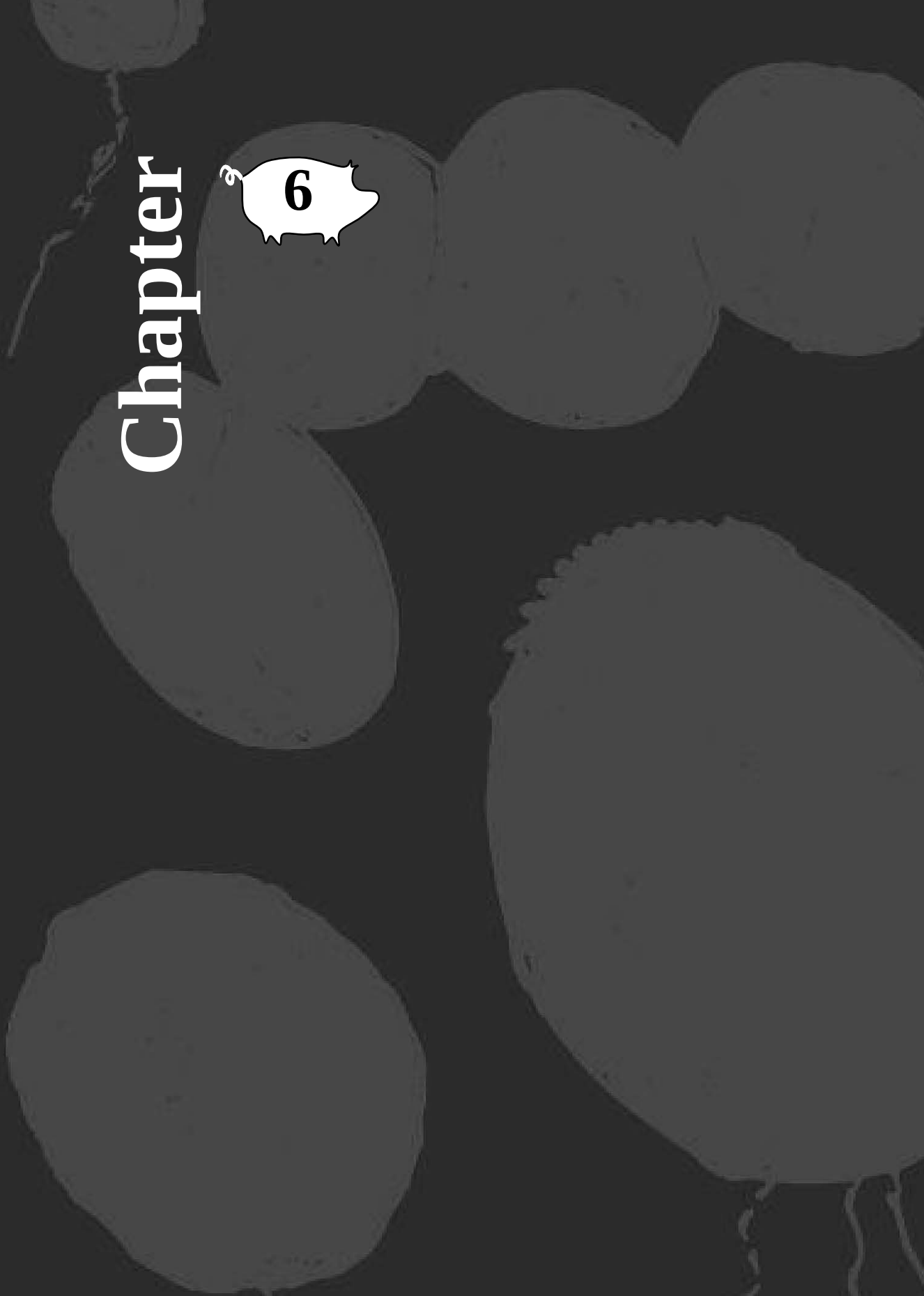
References

1. **Lallès, J. P., Boudry, G., Favier, C., Le Floc'h, N., Lurona, I., Montagne, L., Oswald, I. P., Pie, S., Piel, C., and Seve, B.** (2004) Gut function and dysfunction in young pigs: physiology. *Anim. Res.* 53: 301-316.
2. **Pluske, J. R., Le Dividich, J., and Verstegen, M. W. A.** (2003) Weaning the Pig: Concepts and Consequences. Wageningen Academic Publishers, Wageningen, The Netherlands.
3. **Dividich, J. L. and Herpin, P.** (1994) Effects of climatic conditions on the performance, metabolism and health status of weaned piglets: a review. *Livest. Prod. Sci.* 38: 79-90.
4. **van Nevel, C. J. and Demeyer, D. I.** (1988) Manipulation of rumen fermentation, in *The Rumen Microbial Ecosystem*. Hobson, P. N. (ed.). p. 387-447. Elsevier Applied Science, New York, USA.
5. **Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., and Waddell, J.** (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* 53(1): 28-52.
6. **Bager, F., Madsen, M., Christensen, J., and Aarestrup, F. M.** (1997) Avoparcin used as a growth promoter is associated with the occurrence of vancomycin-resistant *Enterococcus faecium* on Danish poultry and pig farms. *Prev. Vet. Med.* 31: 95-112.
7. **Davidson, P. M. and Naidu, A. S.** (2000) Phyto-phenols, in *Natural Food Antimicrobial Systems*. Naidu, A. S. (ed.). CRC Press, Boca Raton, FL, USA.
8. **Burt, S.** (2004) Essential oils: their antibacterial properties and potential applications in foods-a review. *Int. J. Food Microbiol.* 94: 223-53.
9. **Manzanilla, E. G., Pérez, J. F., Martin, M., Kamel, C., Baucells, F., and Gasa, J.** (2004) Effect of plant extracts and formic acid on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* 82: 3210-8.
10. **Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., and Gasa, J.** (2006) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *J. Anim. Sci.* 84: 2725-34.
11. **Michiels, J., Missotten, J., Fremaut, D., De Smet, S., and Dierick, N.** (2007) *In vitro* dose-response of carvacrol, thymol, eugenol and *trans*-cinnamaldehyde and interaction of combinations for the antimicrobial activity against the pig gut flora. *Livest. Sci.* 109: 157-160.
12. **Wallace, R. J.** (2004) Antimicrobial properties of plant secondary metabolites. *Proc. Nutr. Soc.* 63: 621-629.
13. **Calsamiglia, S., Busquet, M., Cardozo, P. W., Castillejos, L., and Ferret, A.** (2007) Essential oils as modifiers of rumen microbial fermentation. *J. Dairy Sci.* 90: 2580-2595.
14. **Baratta, M. T., Dorman, H. J. D., Deans, S. G., Figueiredo, A. C., Barroso, J. G., and Ruberto, G.** (1998) Antimicrobial and antioxidant properties of some commercial essential oils. *Flavour Frag. J.* 13: 235-244.
15. **Giordani, R., Regli, P., Kaloustian, J., Mikail, C., Abou, L., and Portugal, H.** (2004) Antifungal effect of various essential oils against Candida albicans. Potentiation of antifungal action of amphotericin B by essential oil from Thymus vulgaris. *Phytother. Res.* 18: 990-995.
16. **Lalles, J. P., Bosi, P., Janczyk, P., Koopmans, S. J., and Torrallardona, D.** (2009) Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. *Animal*. 3: 1625-1643.
17. **Grassmann, J., Schneider, D., Weiser, D., and Elstner, E. E.** (2001) Antioxidative effects of lemon oil and its components on copper induced oxidation of low density lipoprotein. *Arzneimittel Forsch.* 51: 799-805.
18. **Gallois, M., Rothkotter, H. J., Bailey, M., Stokes, C. R., and Oswald, I. P.** (2009) Natural alternatives to in-feed antibiotics in pig production: can immunomodulators play a role? *Animal*. 3: 1644-1661.
19. **Trevisi, P., Merialdi, G., Mazzoni, M., Casini, L., Tittarelli, C., De Filippi, S., Minieri, L., Lalatta-Costerbosa, G., and Bosi, P.** (2007) Effect of dietary addition of thymol on growth, salivary and gastric function, immune response, and excretion of Salmonella enterica serovar Typhimurium, in weaning pigs

challenged with this microbe strain. *Ital. J. Anim. Sci.* 6: 374-376.

20. **Ultee, A., Kets, E. P. W., and Smid, E. J.** (1999) Mechanisms of action of carvacrol on the food-borne pathogen *Bacillus cereus. Appl. Environ. Microbiol.* 65: 4606-4610.
21. **Ultee, A., Gorris, L. G. M., and Smid, E. J.** (1998) Bactericidal activity of carvacrol towards the food-borne pathogen *Bacillus cereus. J. Appl. Microbiol.* 85: 211-218.
22. **Dorman, H. J. and Deans, S. G.** (2000) Antimicrobial agents from plants: antibacterial activity of plant volatile oils. *J. Appl. Microbiol.* 88: 308-16.
23. **Chao, S. C., Young, D. G., and Oberg, C. J.** (2000) Screening for inhibitory activity of essential oils on selected bacteria, fungi and viruses. *J. Essent. Oil Res.* 12: 639-649.
24. **Cimanga, K., Kambu, K., Tona, L., Apers, S., De Bruyne, T., Hermans, N., Totte, J., Pieters, L., and Vlietinck, A. J.** (2002) Correlation between chemical composition and antibacterial activity of essential oils of some aromatic medicinal plants growing in the Democratic Republic of Congo. *J. Ethnopharmacol.* 79: 213-220.
25. **Griffin, S. G., Wyllie, S. G., Markham, J. L., and Leach, D. N.** (1999) The role of structure and molecular properties of terpenoids in determining their antimicrobial activity. *Flavour Frag. J.* 14: 322-332.
26. **Ultee, A., Bennik, M. H. J., and Moezelaar, R.** (2002) The phenolic hydroxyl group of carvacrol is essential for action against the food-borne pathogen *Bacillus cereus. Appl. Environ. Microbiol.* 68: 1561-1568.
27. **Suzuki, M. T., Taylor, L. T., and DeLong, E. F.** (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* 66: 4605-4614.
28. **Byun, R., Nadkarni, M. A., Chhour, K. L., Martin, F. E., Jacques, N. A., and Hunter, N.** (2004) Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *J. Clin. Microbiol.* 42: 3128-3136.
29. **Pérez Guiérrez, O., van den Bogert, B., Derrien, M., Molenaar, D., de Vos, W. M., and Smidt, H.** (2010) Design of a high throughput diagnostic microarray for the characterization of pig gastrointestinal tract microbiota. *Submitted to Environmental Microbiology*.
30. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
31. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
32. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) Calculating melting temperatures for perfectly matched hybrids between oligonucleotides and their target sequences. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
33. **Carter, R. L.** (1989) On the partitioning of squared Euclidean distance and its application in cluster analysis. *Phsychometrika*. 54: 9-23.
34. **Simpson, E. H.** (1949) Measurements of diversity. *Nature*. 163: 688.
35. **Lepš, J. and Šmilauer, P.** (2003) Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
36. **Gittins, R.** (1985) Canonical Analysis. A Review with Applications in Ecology. Springer-Verlag, Berlin.
37. **van den Brink, P. J., den Besten, P. J., bij de Vaate, A., and ter Braak, C. J. F.** (2009) Principal response curves technique for the analysis of multivariate biomonitoring time series. *Environ. Monit. Assess.* 152: 271-281.
38. **Elgayyar, M., Draughon, F. A., Golden, D. A., and Mount, J. R.** (2001) Antimicrobial activity of essential oils from plants against selected pathogenic and saprophytic microorganisms. *J. Food Prot.* 64: 1019-24.
39. **Chaudhry, N. M. A., Saeed, S., and Tariq, P.** (2007) Antibacterial Effects of Oregano (*origanum vulgare*) against gram negative Bacilli. *Pakistan J. Bot.* 39: 609-613.

40. **Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8: 1191-1199.
41. **Pieper, R., Janczyk, P., Zeyner, A., Smidt, H., Guiard, V., and Souffrant, W. B.** (2008) Ecophysiology of the developing total bacterial and Lactobacillus communities in the terminal small intestine of weaning piglets. *Microb. Ecol.* 56: 474-483.
42. **Pieper, R., Janczyk, P., Schumann, R., and Souffrant, W. B.** (2006) The intestinal microflora of piglets around weaning - with emphasis on lactobacilli. *Archiva Zootechnica*. 9: 28-40.
43. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
44. **Sghir, A., Antonopoulos, D., and Mackie, R. I.** (1998) Design and evaluation of a *Lactobacillus* group-specific ribosomal RNA- targeted hybridization probe and its application to the study of intestinal microecology in pigs. *Syst. Appl. Microbiol.* 21: 291-296.
45. **Busquet, M., Calsamiglia, S., Ferret, A., and Kamel, C.** (2006) Plant extracts affect in vitro rumen microbial fermentation. *J. Dairy Sci.* 89: 761-71.
46. **Guarner, F. and Malagelada, J. R.** (2003) Gut flora in health and disease. *Lancet*. 361: 512-519.
47. **Biondi, D., Cianci, P., Geraci, C., and Ruberto, G.** (1993) Antimicrobial activity and chemical composition of essential oils from sicilian aromatic plants. *Flavour Frag. J.* 8: 331-337.
48. **Sikkema, J., Debont, J. A. M., and Poolman, B.** (1994) Interactions of Cyclic Hydrocarbons with Biological-Membranes. *J. Biol. Chem.* 269: 8022-8028.
49. **Lee, K.-W., Everts, H., and Beynen, A. C.** (2004) Essential Oils in Broiler Nutrition. *Int. J. Poult. Sci.* 3: 738-752.
50. **Zoetendal, E. G., Akkermans, A. D. L., and De Vos, W. M.** (1998) Temperature gradient gel electrophoresis analysis of 16S rRNA from human fecal samples reveals stable and host-specific communities of active bacteria. *Appl. Environ. Microbiol.* 64: 3854-9.


Chapter
6

Dynamics of Gastrointestinal Microbiota Composition of Weaning Piglets Fed with Sodium Butyrate during the Suckling Period

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Abstract

Sodium butyrate (SB) is used as an acidifier in animal nutrition. Provided orally at different stages during growth, SB has been shown to exert a positive effect on body growth and maturation of the gastrointestinal (GI) tract in milk-fed pigs, helping them to overcome the post-weaning syndrome. As SB is known to influence the GI tract microbiota, an experiment was designed to evaluate the effect of pre-weaning administration of SB on the gastrointestinal microbiota of piglets before and after weaning. Forty eight piglets were blocked in pairs within litters and were assigned to one of the two treatments: a control diet and one containing 3 g/kg SB, from day 5 of life onward. There were three replicates and four litters per replicate. Animals were sacrificed at the end of the suckling period, at day 29, and 11 days after weaning, at day 40, and samples from the stomach, jejunum, ileum, caecum and colon were taken. Subsequently, the changes in the GI tract microbiota in response to SB were characterized using 16S ribosomal RNA (rRNA) gene-based analyses including PCR-Denaturing Gradient Gel Electrophoresis (DGGE), global profiling using the Pig Intestinal Tract Chip (PITChip), a phylogenetic microarray, and quantitative PCR (qPCR) using group-specific primers. It was observed that SB supplementation had a significant effect on total microbiota composition in the ileum, caecum and colon before weaning ($p = 0.03$) and only in the ileum after weaning ($p < 0.05$). *Lactobacillus* 16S rRNA gene copy numbers were higher ($p = 0.03$) in the stomach of non-weaned piglets. The PITChip analysis revealed that the SB administration most strongly affected populations within the *Clostridium* cluster XIVa group and the *Bacteroidetes*. This study reinforces previous reports on the antimicrobial effect of SB on piglet GI tract microbiota.

Introduction

Weaning of piglets in a natural environment is a period that lasts approximately 10 weeks, during which the piglet is progressively introduced to several new feed sources so that its fermentative capacity can develop gradually. However, due to economic demands, this process occurs abruptly at an early age in the production setting. This negatively impacts pig performance, and leads to reduced feed intake and depressed growth rates [1]. Moreover, it results in an increased susceptibility to disease and intestinal disorders like post-weaning diarrhea [2-4]. These are mainly related to dysbiosis in the microbiota of the gastrointestinal (GI) tract and it has been reported that the establishment of a diverse microbiota has an important role in preserving GI health by preventing colonization by pathogens [5].

In the past, the solution to weaning-associated problems was the use of antimicrobial growth promoters (AGP), to prevent GI disorders and to improve the post-weaning growth rate. Nevertheless, AGP have been forbidden in Europe due to public concerns over the possibility that they may select antibiotic-resistant genes that could be transferred to human pathogens [6]. An expanding alternative to dietary antibiotics are feed additives, such as organic acids, probiotics, prebiotics, and plant extracts [7]. The fact that organic acids are cheap and reduce the growth of some undesired bacteria such as coliforms and *Salmonella typhimurium* [8-10], explains their widespread use as alternatives to AGP. This protective action of organic acids has proven helpful especially when the transition from the maternal (milk) diet to the weaning diet is abrupt, as it occurs in piglets. Most studies have been done using formic, propionic, lactic, or citric acid, or the corresponding salts, and to a lesser extent, butyrate [11-14].

Butyrate is a short chain fatty acid (SCFA) produced by bacterial fermentation of carbohydrates in the colon of single-stomached species, including swine and man [15]. This organic acid is also released from milk fat (triglycerides) in the stomachs of suckled veal calves due to a continuous action of pre-gastric lipases. Butyrate is known to modulate the gut microbiota [16, 17] and to inhibit the growth of both Gram-positive and Gram-negative bacteria [18]. Moreover, butyrate is easily metabolized by β-oxidation in the mitochondria providing 60% to 70% of the total energy demand of colonocytes [19]. In animal practice, butyrate is added to feed in the form of sodium butyrate (SB) and it has been found to influence the inflammatory response and increase feed conversion efficiency in suckling pigs [12].

The objective of this study was to assess if SB administrated during the suckling period would have a positive effect on the pig, in order to facilitate the weaning transition. For that purpose, we studied changes in the small and large intestinal microbiota, both before and after weaning, including analyses of the diversity of the microbiota and identification of affected bacteria, using Denaturing Gradient Gel Electrophoresis (DGGE), quantitative PCR (qPCR), and the Pig Intestinal Tract Chip (PITCHip), a phylogenetic microarray developed for the comprehensive high-throughput profiling of porcine intestinal microbiota.

Materials and methods

Animals, experimental design and dietary treatment

The experimental procedures were carried out according to the guidelines of the French Ministry for Animal Research. This study involved two periods: the suckling period, from day 5 after birth until weaning at day 28 and the post-weaning period, from day 29 to day 40. The effect of SB supplementation before weaning on piglets GI tract microbiota was investigated.

The study was done with 48 crossbred (Pietrain × Large White × Landrace) piglets (body wight, BW = 2 kg ± 0.12 kg) from the experimental herd of the National Institute for Agricultural Research (INRA; Saint-Gilles, France). Piglets were grouped in 3 replicates, 4 litters per replicate. Piglets of the same birth weights and growth rates over the first 4 days of life were selected within litters and were assigned randomly to the two dietary treatments. During the suckling period a SB (treatment group) or saline solution (control group) were carefully administered by esophageal tube twice daily (at 09.00 h and 15.00 h) using a slightly curved brass tube (length, 12 cm; internal diameter, 3 mm; external diameter, 6 mm) [20]. The extremity ending with a bulbous part (length, 16 mm; wider external diameter, 12 mm) was made to avoid injury to the pigs. The other end was a luer-lock system fitting into 5–10 ml plastic syringes. Sodium butyrate was supplied at a concentration of 3 g/kg (0.3%) of the daily milk dry matter (DM) intake. Milk intake for each piglet was estimated during the suckling period from individual growth rates using the equation of Noblet and Etienne [21]. The piglets were kept with their littermates and the sow in the farrowing unit during this period. At day 29, the experimental pigs were transported to the weaning building where they were housed in individual cages (0.6 × 0.8 m) with a mesh floor. Air temperature (27–28°C) and light (08.00–21.00 h) were automatically controlled. From day 29 (weaning) till day 32, piglets were fed with a starter diet with a progressive increasing amount, from 10% to 80% of the INRA recommendation [22], as previously reported [20]. The reason of this feeding strategy was to minimize the risk of over-feeding and subsequent GI tract disorders and scours often seen with *ad libitum* feeding immediately after weaning. Feed intake amounted to 70 g/kg BW at day 32. The amounts of feed were 100% of the requirements from day 33 until the end of the trial. The weaning diets were offered twice daily (at 09.00 h and 15.00 h) as a mash with a feed:water ratio of 1:1 (w/w) during the first 3 days after weaning and 2:1 thereafter. Water was provided *ad libitum*.

Treatment solutions and diet

Two solutions were prepared for oral administration during the suckling period: a saline solution (9 g NaCl/l), used for the control group, and a SB solution (60 g/l; Sigma, St Quentin Fallavier, France) dissolved in saline solution, used for the treatment group. The pH of these solutions was adjusted to 7.0 with NaOH. The two weaning diets (Table 1) had the same composition except that maize starch in the control group diet was substituted by SB (3 g/kg DM) in the treatment group diet. No antimicrobial agent was added to the diets.

Table 1. Composition of the control weaning diet*

Ingredient	Content (g/kg)
Barley meal	300
Wheat meal	297
Field peas (44% starch)	50
Whey powder	80
Wheat bran	25
Soya protein concentrate[1]	40
Maize starch[2]	36
Potato protein, purified[3]	50
Maize gluten meal	22
Sunflower	25
Vegetable oil[4]	31
Molasses	10.1
Limestone	10.2
$CaHPO_4 \cdot H_2O$	7.8
NaCl	2.8
Trace mineral and vitamin premix[5]	4.0
L-Methionine (99%)	1.1
L-Lysine-HCl (79%)	3.4
L-Tryptophan	0.3
L-Threonine	0.3
Chromic oxide or titanium dioxide	4.0

*Calculated nutrient composition (as-fed basis, g/kg): DM, 888; crude protein, 191; crude fibre, 34; fat, 50; Ca, 7.2; Na, 2.5; P, digestible, 3.65; lysine, 12.5; methionine, 4.4; tryptophan, 2.5; threonine, 8.0; net energy, 10.0 MJ/kg.

[1]Soycomilw (ADM, LJ Koog aan de Zaan, The Netherlands). [2]Maize starch (4 %) partially substituted by sodium butyrate (3 g/kg) in the experimental treatment. [3]Protastarw (AVEBE, Veendam, The Netherlands). [4]Mixture of palm oil and soyabean oil (1:1, v/v). [5]Supplying (per kg diet): retinol, 525mg; cholecalciferol, 5mg; a-tocopherol, 7.4 mg; phylloquinone, 0.5 mg; thiamin, 1 mg; riboflavin, 4 mg; D-pantothenic acid, 9 mg; niacin, 12.5mg (available); biotin, 50mg; cyanocobalamin, 15mg; folic acid, 0.3 mg; pyridoxine, 1.5 mg; choline, 400 mg; Fe, 80 mg; Zn, 54 mg; Mn, 30 mg; Co, 0.15 mg; I, 0.14 mg; Se, 0.25 mg; antioxidants (E310, E320, R321), 50 mg; maize starch as a carrier.

Sampling

Piglets were weighed 3 times per week during the suckling period and day of weaning, in order to adjust the SB doses to their actual bodyweight. Fresh feces were collected at different time points: day 5 (start of the experiment), day 7, day 14, day 21 and day 28. Fecal samples were stored at -20°C till further analysis.

Piglets were slaughtered 4 h after the last meal, at the end of both periods (day 29 and day 40). The animals were stunned by electric shock and then exsanguinated. For each pig, a midline abdominal incision was made and the whole GI tract was gently removed and placed on ice for sampling digesta contents. The stomach and large intestine (LI) were separated from the

small intestine (SI) and the stomach was weighed full and empty. Samples from the stomach, jejunum, ileum, caecum and colon were collected and stored at -20 °C until analysis.

DNA isolation

In total 120 samples, from 12 piglets per treatment (control or SB-supplemented diet) distributed among the three replicate experiments, were selected for DNA extraction. Samples were defrosted and DNA was extracted from 200 mg of digesta using the Fast DNA Spin Kit (Qbiogene, Inc, Carlsbad,CA, USA). A NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA) was used to determine DNA concentrations and electrophoresis in agarose gels 1.2% (w/v) containing ethidium bromide was used to check the integrity of DNA.

PCR-DGGE analysis

Universal primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 (Table 2) were used to amplify the V6 to V8 variable regions of the bacterial 16S rRNA gene. To study the *Lactobacillus*-specific GI tract community, primers Lac1 and Lac2 (Table 2) were used to amplify the V3 region of the bacterial 16S rRNA gene. PCRs were performed using a GoTaq® DNA Polymerase kit from Promega (Madison, WI, USA.). Each PCR mixture (50 µl) contained 1.25 U of GoTaq® DNA polymerase, Green GoTaq® reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 µM of the primers, 1 µl of DNA solution (~1 ng/µl) and UV-sterilized water. The samples were amplified in a thermocycler T1 (Whatman Biometra, Göttingen, Germany) using the following program: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 20 sec, extension at 68°C for 40 sec and final extension at 68°C for 7 min. The integrity of the DNA was checked by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies).

Table 2. Oligonucleotide primers used in this study

Primers	Sequence 5'-3'	References
S-D-Bact-0968-a-S-GC	CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAACCTTAC	[23]
S-D-Bact-1401-a-A-17	CGGTGTGTACAAGACCC	[23]
Lac1 (Lab-352-S)	AGCAGTAGGGAATCTTCA	[24]
Lac2 (Lab-679-A-GC)	CGCCCGGGGCGCGCCCCGGGCGGCCCGGGGGCACCGGGGATTYCACCGCTACACATG	[24]
Lacto F	TGGAAACAGRTGCTAATACCG	[25]
Lacto R	GTCCATTGTGGAAGATTCCC	[25]
Bact1369F	CGGTGAATACGTTCYCGG	[26]
Prok 1492	GGWTACCTTGTTACGACTT	[26]
T7prom-Bact-27-for	TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGGC-TCAG	[27]
Uni-1492-rev	CGGCTACCTTGTTACGAC	[27]

The V6-V8 PCR amplicons were separated by DGGE according to the specifications of Muyzer et al. [28] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in an 8% (v/v) polyacrylamide gel (37.5:1 acrylamide-bisacrylamide and dimensions: 200 by 200 by 1 mm) and 0.5× Tris-acetate-EDTA (TAE)

(pH 8) buffer. The gels were poured from the bottom using a gradient maker and a pump (Econopump; Bio-Rad Laboratories). The denaturing gradient used was 30-60% and 30-55% for total bacterial and *Lactobacillus*-group specific amplicons, respectively. The gels were electrophoresed for 16 h at 85 V in 0.5x TAE buffer at a constant temperature of 60°C and stained with $AgNO_3$ [29]. Gel images were digitally normalized by comparison with an external standard pattern using Bionumerics software package version 4.5 (Applied MathS, Kortrijk, Belgium). This normalization enabled comparison between DGGE profiles from different gels that were run under identical denaturing and electrophoresis conditions [30]. For comparisons of the profiles, the Shannon Index of diversity [31], the number of bands (richness) and the Pearson's coefficient were calculated. Dendograms were constructed using an unweighed pair-group method with averaging algorithm (UPGMA).

Quantitative PCR

Quantitative PCR (qPCR) 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). The reaction mixture (25 µL) consisted of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 5 µL of template DNA diluted in water (10 or 50 times) to avoid PCR inhibition. A standard curve was generated using the serially diluted 16S rRNA gene amplicons obtained from *Lactobacillus rhamnosus.* Universal primers, Bact 1369 and Prok 1492 (Table 2) were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample by triplicate. PCR was performed with an initial denaturation step of 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 sec, 56ºC for 30 sec and 72ºC for 30 sec.

Primers Lacto F and Lacto R (Table 2) were used to quantify the *Lactobacillus* group present in the sample using the following PCR program: initial denaturation step of 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 62ºC for 60 sec.

PITChip analysis

The phylogenetic analysis of the digesta samples was done using the PITChip (Chapter 3 of this thesis). This is a phylogenetic microarray with more than 2900 oligonucleotides based on 16S rRNA gene sequences targeting 627 porcine intestinal microbial phylotypes. The protocol for hybridization and analysis of the generated data was performed essentially as described before [32]. Briefly, the 16S rRNA gene from pig intestinal samples was amplified using the primers T7prom-Bact-27-for and Uni-1492-rev (Table 2). For the reaction 10 ng of DNA samples were used as a template in a final volume of 50 µl. Samples were denatured at 94°C for 2 min followed by 35 cycles of 94°C (30 sec), 52°C (40 sec), 72°C (90 sec) and a final extension at 72°C for 7 min. After purification of the PCR products (NucleoSpin Extract II kit, Macherey-Nagel, Düren, Germany) the DNA concentration was measured using a NanoDrop spectrophotometer.

In vitro transcription of the 16S rRNA genes carrying the T7-promoter was performed using the Riboprobe System (Promega). For the reaction 500 ng of the gene amplicon plus rATP, rGTP, rCTP and a 1:1 mix of rUTP and aminoallyl-rUTP (Ambion, Austin, TX, USA) were used. The transcription reaction was incubated at room temperature for 2 h and afterwards possible DNA present was digested with the Qiagen RNAse-free DNAse kit (Qiagen, Hilden,

Germany). RNA was purified (RNeasy Mini-Elute Kit, Qiagen, Hilden, Germany) and the concentration was quantified with NanoDrop.

RNA samples (modified amino-allyl nucleotides) were coupled with CyDye using the Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK) dissolved in DMSO (84 μl). For the labeling reaction 20 μl of CyDye were added to 2 μg of pure RNA and 25 mM of sodium bicarbonate buffer (pH 8.7) in a final volume of 40 μl. Samples were incubated during 90 min in the dark and at room temperature. After this time the reaction was stopped by adding 15 μl of 4 M hydroxyl-amine and incubating in the dark for 15 min. RNAse-free water was added to 100 μl and the labeled RNA was quantified.

Microarrays synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) in 8×15 K format were used for hybridization with two samples, labeled with Cy3 and Cy5, respectively. The Cy3/Cy5-labeled target mixtures were fragmented with 10× fragmentation reagent (Ambion). Hybridization on the arrays was performed at 62°C for 16 h in a rotation oven (Agilent). Slides were washed at room temperature in 2× SSC with 0.3% SDS for 10 min, followed by 0.1× SSC with 0.3% SDS at 38°C for 10 min and 0.06× SSPE for 5 min [33]. Data was extracted from microarray images using the Agilent Feature Extraction software, version 9.1 (http://www.agilent.com). Data normalization and further microarray analysis were performed using a set of R based scripts (http://r-project.org) in combination with a custom designed relational database, which runs under the MySQL database management system (http://www.mysql.com). Ward's minimum variance method was used for the generation of hierarchical clustering of probe profiles by calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance – E2) [34].

Statistical analysis

Multivariate analysis was applied for both DGGE fingerprints and PITChip data interpretation. In order to relate changes in total bacterial and *Lactobacillus* community composition to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) [35]. RDA is a multivariate direct gradient analysis method that allows ordering of samples and taxa (i.e. bands) considering that species have linear relationships to environmental variables [36]. Relative abundance (peak areas) of bands (for DGGE) and the sum of signal intensities for 144 genus-level phylogenetic groups (for PITChip) were used as predictors. Treatment classes, day and sex were introduced as nominal environmental variables (dummy variables 0 or 1). The redundancy analysis was performed focusing on inter-samples correlation, and the Monte Carlo permutation test was applied [37] to decide whether treatment and time had a statistically significant influence on the species composition. We used the option unrestricted permutation (since the experiment had a randomized design) that yields completely random permutations [38]. Treatment was considered to affect microbiota composition with p-values < 0.05. Diagrams were plotted as triplots using CanoDraw. Diversity of microbial profiles obtained by PITChip analysis was expressed as Simpson's reciprocal index of diversity (1/D) [39]. This diversity was calculated with the equation $\lambda = 1/\Sigma Pi^2$, where Pi is the proportion of i^{th} taxon. This is the proportion of each probe signal compared to the total signal for each sample. A higher

Simpson's index value indicates a higher degree of diversity.

To perform the statistical analysis of the qPCR results, the number of gene copies was $\log_{10}$ transformed to achieve normal distribution and the mean and standard deviation were calculated. Comparisons between control and SB treatment were calculated with the T-test and were considered statistically significant if $p < 0.05$. In addition, the abundance of *Lactobacillus* group relative to total bacteria gene copy number was calculated for each replicate, and the mean, standard deviation and statistical significance were determined.

Results

The piglets used in the present work and supplemented with SB or not (controls) grew equally well and remained healthy throughout the experiment.

In order to evaluate the effect of pre-weaning treatment of piglets with SB on the GI tract microbiota before and after weaning, a complementary set of 16S rRNA gene-targeted molecular approaches for the profiling and quantification of GI tract microbiota was used. A total of 12 animals were selected from control- and SB-treatment groups, respectively. From each piglet, samples from the stomach, jejunum, ileum, caecum and colon were obtained and microbial DNA was isolated and processed for a variety of total bacterial and group-specific profiling methods, including DGGE analysis of 16S rRNA gene amplicons, qPCR of bacteria and lactobacilli, and global profiling using the PITChip, a pig-specific phylogenetic microarray.

Redundancy analysis of total bacterial and Lactobacillus DGGE profiles

To obtain a first overview of potential effects of the SB treatment on microbiota composition, DGGE profiling of 16S rRNA gene fragments of total bacteria was performed as well as of *Lactobacillus* spp., which were PCR-amplified from ileal, caecal and fecal samples of randomly selected animals from the control and SB-treatment group. Redundancy analysis of the relative abundance of DGGE bands of total bacterial communities showed that SB supplementation during the suckling period had a significant effect in the caecum ($p = 0.034$; Fig. 1A) and in the ileum ($p = 0.028$; Fig. 1B). Specific changes in the microbial profiles were observed in piglets sacrificed at the end of the suckling period (day 29) but not in piglets sacrificed after the post-weaning period (day 40) where the microbial shifts were not significant ($p > 0.05$). Eigenvalues of the first and second RDA axes constrained to the treatment classes were 0.26 and 0.05, respectively, for the analysis of the caecum, and 0.17 and 0.096, respectively, for the ileum analysis. RDA analysis of DGGE profiles of the total bacterial community present in the feces showed as well a significant effect ($p = 0.012$) of the SB supplementation on the microbiota composition (data not shown). Specific analysis of the *Lactobacillus* group showed that SB supplementation during the suckling period had a significant effect in the stomach at 29 days shifting the microbial profile ($p = 0.018$; Fig. 1C), but no effect was observed for any of the other sites. For the *Lactobacillus* analysis in the stomach, the axes accounted for 57.8% of the total variance in the species (Fig. 1C).

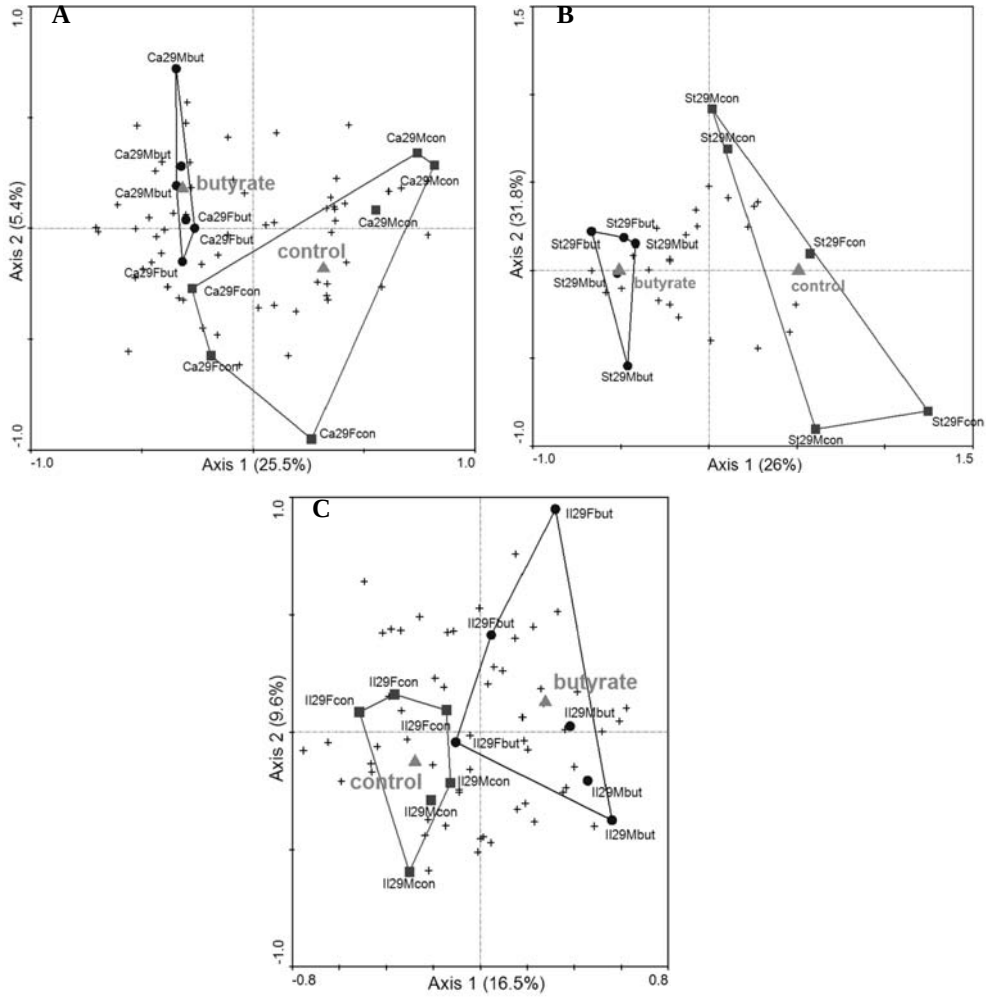

Figure 1. Ordination triplots for RDA analysis of total bacterial (**A** and **B**) and *Lactobacillus* (**C**) community profiles. Species (DGGE bands representing total bacterial community) are represented by crosses (+). Samples are indicated by full squares and full circles as indicated below. Nominal environmental variables are represented by triangles (▲). Samples are grouped by treatment: SB, ●; control, ■. **(A)** Total bacterial DGGE bands analysis of caecum samples collected at day 29; p = 0.034. **(B)** Total bacterial DGGE bands analysis of ileum samples collected at day 29; p = 0.028. (**C**) *Lactobacillus* spp. DGGE bands analysis of stomach samples collected at day 29; p = 0.018. Samples coding: sampling site (Ca = caecum, Il = ileum, St = stomach), day 29, sex (M = male and F = female), and treatment (con = control, but = sodium butyrate).

Changes in the total microbial concentrations

In addition to qualitative community profiling by DGGE, we also quantified the total bacterial load along the gastrointestinal tract using qPCR. The mean values of the amount of bacteria

in the group receiving SB at day 40, expressed as log of gene copies/g of digesta, showed a significant decrease ($p < 0.01$) from the stomach to the jejunum (from 8.58 ± 0.42 to 7.52 ± 0.62 copies/g of digesta) (Fig. 2A). The difference in total bacterial DNA between stomach and ileum was only significant in the control group ($p < 0.01$) at day 29, showing an increase of 1 log unit both before weaning (from 8.6 ± 0.59 to 9.7 ± 0.37 log copies/g of digesta) and after the post-weaning period (from 8.5 ± 0.23 to 9.4 ± 0.92 log copies/g of digesta). As expected, the highest amount of bacterial DNA was found in the caecum and colon both before and after weaning (10.6 ± 0.56 and 10.8 ± 0.58 log copies/g of caecal digesta, and 10.8 ± 0.32 and 11.2 ± 0.22 log copies/g of colonic digesta, respectively). This indicated that the change in bacterial numbers from the stomach and the SI to the caecum and colon was significant before and after weaning ($p < 0.01$), both in the control group and in the SB-treatment group. In contrast to these marked differences along the GI tract, no significant differences were found in bacterial 16S rRNA gene copy numbers in relation to experimental diets in any of the analyzed samples (Fig. 2A).

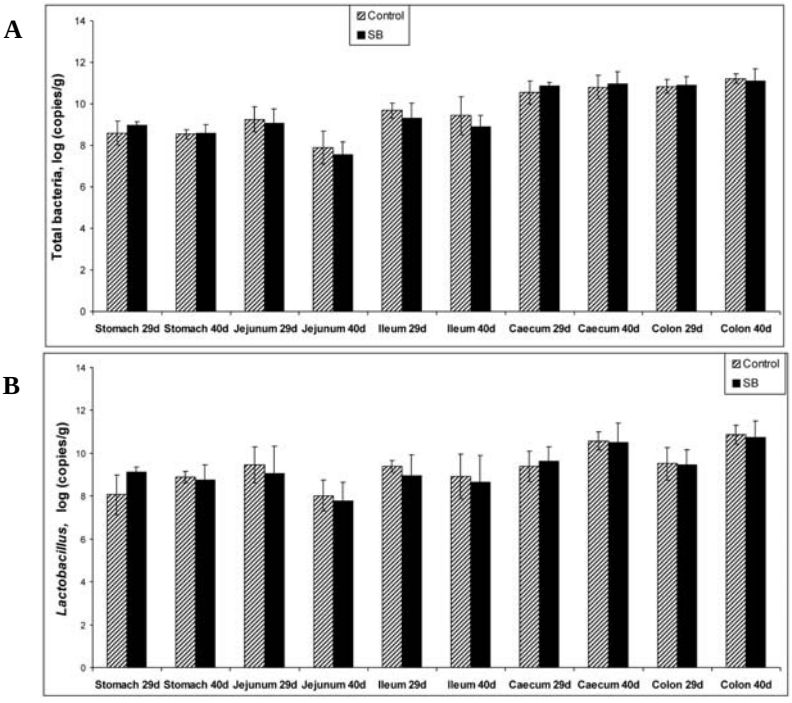

Figure 2. (**A**) Total bacterial loads and (**B**) *Lactobacillus* loads, in the stomach, jejunum, ileum, caecum and colon digesta, measured by quantitative PCR (log gene copies/g of digesta) in pre-weaned (29d) and post-weaned pigs (40d) that received a control diet or the same diet supplemented with 0.3% SB during the suckling period.
∗ indicates that the difference between treatment and control is significant with $p < 0.05$. Different lowercase and uppercase letters indicate significant differences ($p < 0.05$) comparing samples from different locations along the GI tract, from animals in the control and SB-treatment group, respectively, separately for day 29 and day 40. Bars represent means and SEM.

The mean values of *Lactobacillus* DNA (expressed as log of gene copies/g) increased in the control group significantly from the stomach along the GI tract, from 8.07 ± 0.92 to 9.46 ± 0.83 copies/g of digesta in the jejunum ($p = 0.028$), to 9.39 ± 0.28 copies/g in the ileum ($p = 0.041$), to 9.39 ± 0.72 in the caecum ($p = 0.025$) and to 9.51 ± 0.75 ($p = 0.019$) in the colon. However, these changes were not observed in the treatment group (Fig. 2B). At day 40, in both the control group and the SB-treatment group, there was a decrease in the *Lactobacillus* DNA copy numbers from the stomach to the jejunum (significant in the control group; $p = 0.02$) and from there the numbers increased becoming significant between the stomach and the LI ($p < 0.001$). Copy numbers increased from 8.75 ± 0.78 copies/g in the stomach to 10.51 ± 0.98 and 10.74 ± 0.78 copies/g in the caecum and colon, respectively (in the SB group) and from 8.89 ± 0.27 copies/g in the stomach to 10.58 ± 0.43 and 10.87 ± 0.44 copies/g in the caecum and colon, respectively (in the control group). A significant variation in time was only observed in the jejunum of the control group and in the colon of both treatment and control.

Contrary to what was observed for total bacteria, a significant increase ($p = 0.038$) in the amount of *Lactobacillus* DNA between the control group and the piglets receiving SB was observed in the stomach at day 29 (Fig. 2B).

Microarray analysis of microbiota composition

To confirm the results obtained by DGGE and qPCR, and to provide further information at high phylogenetic resolution about the microbiota composition at the different intestinal sites, 59 of the selected samples were analyzed using the PITChip, a recently developed pig-specific phylogenetic microarray. A multivariate analysis (RDA) was performed to visualize the microarray data. This showed that the addition of SB had a significant effect on the microbial distribution in the ileum after weaning (Fig. 3; $p = 0.042$) (data from day 29 is not shown because there were no significant changes). Interestingly, the plot showed a clear difference between the 2 treatments groups and the species that explained at least 90% on the variation of the data were found to correlate negatively with the SB treatment. Moreover, the samples from piglets receiving SB formed a more homogeneous group than the samples from group that received the control diet. To identify the groups that differentiated the microbiota of piglets fed with SB or with the control diet, a Student's T-test was performed on the sum of hybridization signals of each of 144 genus-level phylogenetic groups (Table 3). This analysis showed significant changes in some of the groups' intensities in the ileum samples, both before and after weaning. On day 29, in samples from the SB group, we could find an increase of *Clostridium perfringens*-like species ($p = 0.006$) with respect to the control. After weaning (day 40) there was a significant decrease in the intensity of several taxa, in the SB supplemented group, including *Dorea*-like species, *Eubacterium plexicaudatum-, E.ventriosum*- and *Roseburia intestinalis*-like species belonging to *Clostridium* cluster XIVa, uncultured *Deltaproteobacteria* ($p = 0.006$), *Fibrobacteres succinogenes*-like species (*Fibrobacteres*) and *Treponema*-like species belonging to the *Spirochaetes.* This was confirmed by a bacterial diversity analysis of the different GI tract sections that showed that the microbiota diversity in the ileum of SB fed piglets after weaning was significantly lower than that in the control ($p = 0.02$; Fig. 4). Moreover, this also confirmed the multivariate analysis that indicated a significant effect in the ileum at day 40, with mainly a decrease in the abundance of several phylogenetic groups (Fig. 3, Table 3).

In contrast, microbiota profiles from stomach and jejunum samples were not influenced by the addition of SB. As to the large intestine, student´s T-test demonstrated that in the caecum of pre-weaned piglets there were several genus-level bacterial groups that were significantly affected ($p < 0.05$) by the SB treatment (Table 3).

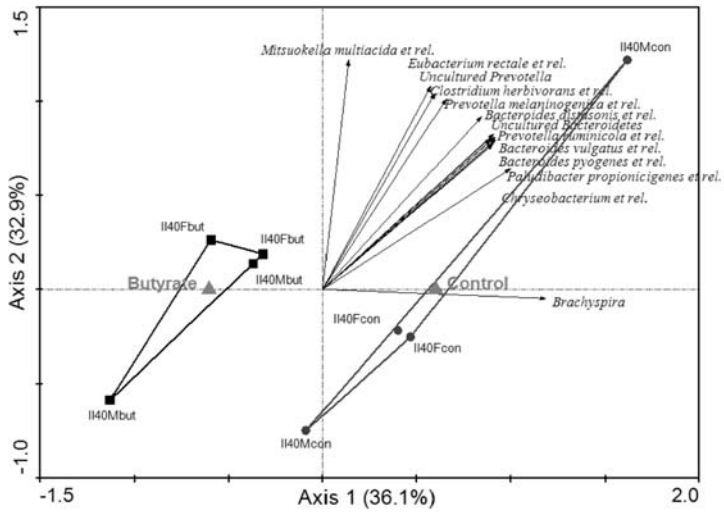

Figure 3. RDA triplot of the microbiota composition in ileal digesta samples of piglets 40 days after weaning, receiving a control diet (●) and piglets receiving a supplementation of SB during the suckling period (■), expressed as the summed hybridization signal of 144 phylogenetic groups. Both treatments are treated as nominal variables and represented by centroids (▲). Microbial groups contributing at least 90% to the explanatory axes are represented as vectors. Percentage values at the axes indicate contribution of the principal components to the explanation of the total variance of the species in the dataset. Monte Carlo permutation test indicated that butyrate supplementation had a significant effect on the variation of microbiota composition with a p-value of 0.042. 29; p = 0.018. Samples coding: sampling site (Il = ileum), day 40, sex (M = male and F = female), and treatment (con = control, but = sodium butyrate).

Compared to the control, there was a decrease of the intensity of *Sphingomonas*-like species within the *Alphaproteobacteria*, and an increase of *Treponema*-like species (*Spirochaetes*). However, pre-weaning treatment with SB did not affect caecal microbial groups after weaning (day 40). Colon microbiota before weaning was affected as well by the addition of SB to the diet. There was a significant decrease of the intensity of several genus-level bacterial groups: *Bacteroides pyogenes*-like species, *Myroides odoratus*-like species, *Prevotella melaninogenica*-like species, uncultured *Bacteroidetes*, uncultured *Porphyromonadeceae* and uncultured *Prevotella* (Table 3).

Table 3. SSU rRNA-based genus-level phylogenetic groups for which PITChip hybridization signals were found to be significantly different between samples from piglets that were fed SB during the suckling period and samples from control piglets. The p-values were calculated comparing to the control and using the sum of intensities (hybridization signals) of 144 phylogenetic groups. The direction of the effect caused on the bacterial group intensity is indicated as up (increase) and down (decrease). Relative abundance is given as an average of control piglets

Site	Higher taxonomic group	Group	Effect	p-value	Relative abundance (%)
Caecum 29d	*Alphaproteobacteria*	*Sphingomonas*-like	down	0.017	0.08
	Spirochaetes	*Treponema*-like	up	0.028	0.04
Colon 29d	*Bacteroidetes*	*Bacteroides pyogenes*-like	down	0.039	0.80
		Prevotella melaninogenica-like	down	0.025	0.48
		Uncultured *Bacteroidetes*	down	0.02	1.0
		Uncultured *Porphyromonadaceae*	down	0.042	0.74
		Uncultured *Prevotella*	down	0.013	3.07
	Flavobacteria	*Myroides odoratus*-like	down	0.038	0.49
Ileum 29d	*Clostridium* cluster I	*Clostridium perfringens*-like	up	0.006	2.13
Ileum 40d	*Alphaproteobacteria*	*Labrys methylaminiphilus*-like	down	0.037	0.45
	Clostridium cluster XIVa	*Dorea*-like	down	0.03	0.83
		Eubacterium plexicaudatum-like	down	0.041	0.37
		Eubacterium ventriosum-like	down	0.043	0.37
		Roseburia intestinalis-like	down	0.037	1.21
	Deltaproteobacteria	Uncultured *Deltaproteobacteria*	down	0.006	0.10
	Fibrobacteres	*Fibrobacter succinogenes*-like	down	0.013	0.66
	Spirochaetes	*Treponema*-like	down	0.025	0.05

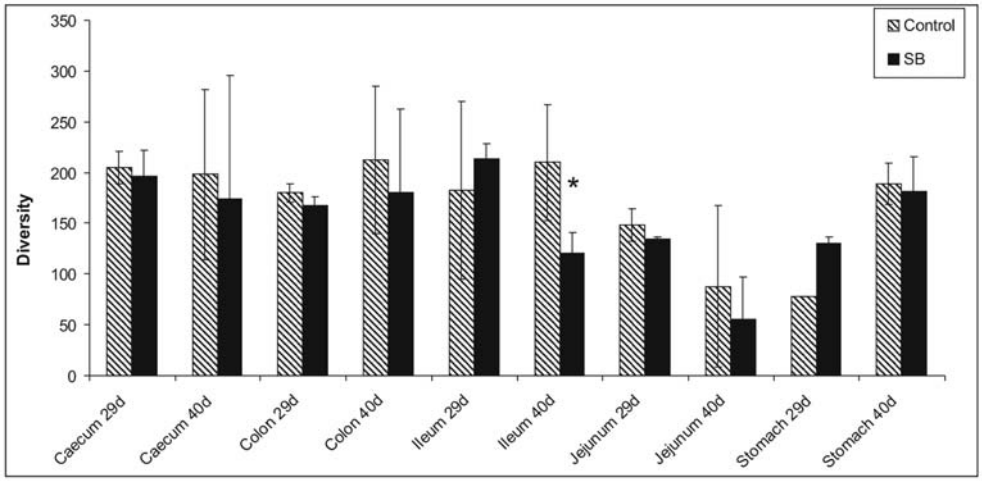

Figure 4. Comparison between Simpson's indexes (diversity) of PITChip profiles obtained for samples from control piglets and samples from piglets treated with SB. Samples were taken from five different GI tract sites at two different time points, before weaning (29d) and after the post-weaning period (40d). * indicates that the difference with the control is significant with $p < 0.05$. Bars represent means and SEM.

Discussion

The main objective of this study was to determine the effect of oral supplementation of SB to piglets, during the suckling period, on the GI tract microbiota at the end of the suckling period and of the post-weaning period. A limited number of studies have previously addressed the effect of feeding SB to piglets, both before and after weaning. One study found that when the organic acid was fed to early weaned pigs at a feed level of 0.3% of the diet, it increased feed efficiency [12], whereas in another study, supplementing milk formula-fed neonatal piglets with SB increased body weight gain [40]. In contrast, it was recently reported that different concentrations of SB, administered after weaning, had no effect on growth performance of weaned pigs [41]. Two additional studies found that SB, especially when provided early after birth, had a positive effect on growth performance and on feed intake in young pigs [21, 43]. However, in the present study, no difference in body weight gain was observed between treatments in pigs slaughtered at 29 or at 40 days of age (T. Pellet and J.-P. Lallès, personal communication).

No effect of SB supplementation was observed on fermentation end-product formation in the different segments of the GI tract of the pigs (W. Pellikaan, personal communication). In contrast to the lack of effect of SB on BW and fermentation profiles, there was a clear effect of SB supplementation on the microbiota dynamics. The various molecular analyses that were performed confirmed that most of the significant differences in the microbiota of piglets that received either SB or the control treatment were observed directly after the suckling period. Significant differences in the microbial composition after weaning (day 40) could only be found in the ileum, indicating that large SB-induced changes in microbiota composition are not sustained beyond weaning once the treatment is finished.

The fact that butyrate can be absorbed rapidly through stomach tissue and thus may not arrive intact to the lower digestive tract has been demonstrated before [42]. Another recent study reported that young pigs fed SB at 0.3% of feed, showed an increase in butyrate levels in the stomach but not in the jejunum [12]. It was observed that feeding growing pigs a fat-coated butyrate product increased the small intestinal plica area [43]. Others found no effect of feeding SB on intestinal morphology, confirming that the organic acid may be absorbed in the upper GI tract and therefore may not directly affect more distal regions of the intestine [41]. In agreement with these results, in the present study, the proportion of butyrate was significantly higher, compared to the control, in the stomach and mid jejunum at the day of weaning in the SB treated group, whereas no significant differences were found in ileum and caecum (Pellikaan et al., in preparation). This is in contrast to the fact that microbial changes were found mainly in the ileum and in the LI, indicating a polar effect of proximal differences in butyrate concentrations on more distally occurring changes in microbiota composition. The only significant change in the stomach was detected with qPCR and was an increase in the total number of *Lactobacillus* spp. copy numbers during the weaning period, after the SB treatment. An increase in the amount of *Lactobacillus* DNA was observed as well by Gong and co-workers in the ileum when adding SB to the diet of piglets [44]. A reason for only finding significant changes on the total abundance of *Lactobacillus* spp. 16S rRNA gene copies, whereas based on PITChip profiling, no significant changes in individual *Lactobacillus*-like species were obsreved in the

stomach, could be that SB acts by reducing the total size of the *Lactobacillus* population and not by promoting the selection of particular bacteria, as it was suggested before [18]. The opposite was observed for the ileum, caecum and colon, where we did not find significant changes for the total bacterial counts but where there were changes in the relative abundance of specific bacterial groups. A similar result was reported before, when piglets receiving butyrate did not have any significant change in total microbial numbers [17]. These authors concluded that it is possible that butyrate could cause changes in the microbial species but not significant changes in bacterial colonization. This could be the case as well in the present work. The values for the total bacteria and *Lactobacillus* in the stomach, jejunum, caecum and colon estimated by qPCR were similar to those described by other authors for cultivable bacteria in pigs [45, 46].

In general, the significant changes observed both in the small and large intestine of piglets fed with SB were related to a decrease of PITChip signal intensities mainly of populations belonging to *Clostridium* cluster XIVa after the post-weaning period and to the *Bacteroidetes* group at weaning. This does not completely agree with another study of piglets weaned at 28 days where SB did not have any effect on clostridia counts along the intestine [41]. The different techniques used for the analysis of the microbiota in both studies, in addition to the different age at which animals were sacrificed and to environmental and dietary differences between the studies, might explain the different effect of SB on intestinal microbiota.

Diversity is a measure of the number and relative abundance of different microorganisms detected, and it has been proposed as an indicator of stability of the intestinal microbiota [47]. It has been proposed that a higher diversity could produce a beneficial effect on animal performance because it prevents the excessive proliferation of a single bacterial group and a likely disturbance of the GI tract microbiota [48]. To this end, our results showed that SB in general did not affect bacterial diversity throughout the GI tract, with the exception of a significant decrease in SB-treated animals in the ileum after 40 days. This change in diversity was concomitant with an overall decrease of the relative abundance of organisms present in the ileum. This antimicrobial effect of an organic acid has been seen in another trial described in this thesis (Chapter 7), and it agrees with previous reports [14, 49].

Weaned piglets are characterized by a more heterogeneous microbial profile than suckling piglets [52, 53], caused by the stress suffered at weaning and the changes that the animal experiences during this period. This suggests that in the present study SB has a positive effect on piglets because samples from different piglets receiving SB had more homogeneous microbial profiles.

In conclusion, feeding pigs SB during the suckling period did affect neither piglets' performance nor total bacterial counts but rather relative abundance of individual groups as detected by PITChip analysis, reinforcing the value of such high resolution phylogenetic microarrays in dissecting microbiota dynamics in complex ecosystems such as the mammalian GI tract. Future studies at the systems level aiming at elucidation of the interactive network of host, microbiota and metabolites such as butyrate will be needed to clearly understand the mechanisms underlying the effects observed when SB is fed to piglets.

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References

1. **Pluske, J. R., Le Dividich, J., and Verstegen, M. W. A.** (2003) Weaning the Pig: Concepts and Consequences. Wageningen Academic Publishers, Wageningen, The Netherlands.
2. **Bosi, P., Casini, L., Finamore, A., Cremokolini, C., Merialdi, G., Trevisi, P., Nobili, F., and Mengheri, E.** (2004) Spray-dried plasma improves growth performance and reduces inflammatory status of weaned pigs challenged with enterotoxigenic *Escherichia coli* K88. *J. Anim. Sci.* 82: 1764-72.
3. **Boudry, G., Peron, V., Le Huerou-Luron, I., Lalles, J. P., and Seve, B.** (2004) Weaning induces both transient and long-lasting modifications of absorptive, secretory, and barrier properties of piglet intestine. *J. Nutr.* 134: 2256-2262.
4. **Barnett, K. L., Kornegay, E. T., Risley, C. R., Lindemann, M. D., and Schurig, G. G.** (1989) Characterization of creep feed consumption and its subsequent effects on immune response, scouring index and performance of weanling pigs. *J. Anim. Sci.* 67: 2698-708.
5. **Van Kessel, A., Shirkey, T. W., Siggers, R. H., Drew, M. D., and Laarveld, B.** (2004) Commensal bacteria and intestinal development. Studies using gnotobiotic pigs., in *Interfacing Immunity, Gut Health and Performance*. Tucker, L. A. and Taylor-Pickard, J. A. (ed.). Nottingham University Press, Nottingham, UK.
6. **Phillips, I., Casewell, M., Cox, T., De Groot, B., Friis, C., Jones, R., Nightingale, C., Preston, R., and Waddell, J.** (2004) Does the use of antibiotics in food animals pose a risk to human health? A critical review of published data. *J. Antimicrob. Chemother.* 53: 28-52.
7. **Kamel, C.** (2001) Natural plant extracts: Classical remedies bring modern animal production solutions. *Cahiers Options Méditerranéennes*. 3: 31-38.
8. **Roselli, M., Finamore, A., Britti, M. S., Bosi, P., Oswald, I., and Mengheri, E.** (2005) Alternatives to in-feed antibiotics in pigs: Evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of in vitro and in vivo results. *Anim. Res.* 54: 203-218.
9. **Partanen, K. H.** (2001) Organic acids - Their efficacy and modes of action in pigs, in *Gut Environment of Pigs*. Piva, A., Bach Knudsen, K. E., and Lindberg, J. E. (ed.). p. 201-217. Nottingham University Press, Nottingham.
10. **Bannink, A., Dijkstra, J., Koopmans, S. J., and Mroz, Z.** (2006) Physiology, regulation and multifunctional activity of the gut wall: a rationale for multicompartmental modelling. *Nutr. Res. Rev.* 19: 227-253.
11. **Martín-Peláez, S., Costabile, A., Hoyles, L., Rastall, R. A., Gibson, G. R., La Ragione, R. M., Woodward, M. J., Mateu, E., and Martín-Orúe, S. M.** (2010) Evaluation of the inclusion of a mixture of organic acids or lactulose into the feed of pigs experimentally challenged with *Salmonella Typhimurium*. *Vet. Microbiol.* 142: 337-345.
12. **Manzanilla, E. G., Nofrarias, M., Anguita, M., Castillo, M., Perez, J. F., Martin-Orue, S. M., Kamel, C., and Gasa, J.** (2006) Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* 84: 2743-51.
13. **Van Deun, K., Haesebrouck, F., Van Immerseel, F., Ducatelle, R., and Pasmans, F.** (2008) Short-chain fatty acids and L-lactate as feed additives to control *Campylobacter jejuni* infections in broilers. *Avian Pathol.* 37: 379-383.
14. **Partanen, K. H. and Mroz, Z.** (1999) Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12: 117-145.
15. **Cummings, J. H. and Macfarlane, G. T.** (1991) The control and consequences of bacterial fermentation in the human colon. *J. Appl. Bacteriol.* 70: 443-459.
16. **Galfi, P. and Bokori, J.** (1990) Feeding trial in pigs with a diet containing sodium n-butyrate. *Acta Vet. Hung.* 38: 3-17.
17. **Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., and Gasa, J.** (2006) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *J. Anim. Sci.* 84: 2725-34.

18. **Sun, C. Q., O'Connor, C. J., and Roberton, A. M.** (2002) The antimicrobial properties of milkfat after partial hydrolysis by calf pregastric lipase. *Chem. Biol. Interact.* 140: 185-98.
19. **Roediger, W. E.** (1980) The colonic epithelium in ulcerative colitis: an energy-deficiency disease? *Lancet*. 2: 712-5.
20. **Le Gall, M., Gallois, M., Seve, B., Louveau, I., Holst, J. J., Oswald, I. P., Lalles, J. P., and Guilloteau, P.** (2009) Comparative effect of orally administered sodium butyrate before or after weaning on growth and several indices of gastrointestinal biology of piglets. *Br. J. Nutr.* 102: 1285-96.
21. **Noblet, J. and Etienne, M.** (1989) Estimation of sow milk nutrient output. *J. Anim. Sci.* 67: 3352-3359.
22. **InraPorc** (2009) InraPorc® - a tool to evaluate nutritional strategies in pigs.
23. **Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H.** (1996) Sequence heterogeneities of genes encoding 16S rRNA in *Paenibacillus polymixa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178: 5636-5643.
24. **Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., and Hammes, W. P.** (2001) Detection of *Lactobacillus*, *Pediococcus*, *Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67: 2578-2585.
25. **Byun, R., Nadkarni, M. A., Chhour, K. L., Martin, F. E., Jacques, N. A., and Hunter, N.** (2004) Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *J. Clin. Microbiol.* 42: 3128-3136.
26. **Suzuki, M. T., Taylor, L. T., and DeLong, E. F.** (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* 66: 4605-4614.
27. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
28. **Muyzer, G., de Waal, E. C., and Uitterlinden, G. A.** (1993) Profiling of complex populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695-700.
29. **Sanguinetti, C. J., Dias Neto, E., and Simpson, A. J. G.** (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques*. 17: 915-919.
30. **Martin, R., Heilig, G. H. J., Zoetendal, E. G., Smidt, H., and Rodriguez, J. M.** (2007) Diversity of the *Lactobacillus* group in breast milk and vagina of healthy women and potential role in the colonization of the infant gut. *J. Appl. Microbiol.* 103: 2638-2644.
31. **Shannon, C. E. and Weaver, W.** (1963) The mathematical theory of communication. University of Illinois Press, Urbana, IL.
32. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
33. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) Calculating melting temperatures for perfectly matched hybrids between oligonucleotides and their target sequences. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
34. **Carter, R. L.** (1989) On the partitioning of squared Euclidean distance and its application in cluster analysis. *Phsychometrika*. 54: 9-23.
35. **Lepš, J. and Šmilauer, P.** (2003) Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
36. **Jongman, R. H. G., Ter Braak, C. J. F., and van Tongeren, O. F. R.** (1995) Data Analysis in Community and Landscape Ecology. Cambridge University Press, New York.
37. **Gittins, R.** (1985) Canonical Analysis. A Review with Applications in Ecology. Springer-Verlag, Berlin.
38. **Ter Braak, C. J. F.** (1990) Update notes: CANOCO version 3.1. GLW-Agricultural Mathematics Group, Wageningen.
39. **Simpson, E. H.** (1949) Measurements of diversity. *Nature*. 163: 688.
40. **Kotunia, A., Wolinski, J., Laubitz, D., Jurkowska, M., Rome, V., Guilloteau, P., and Zabielski, R.**

(2004) Effect of sodium butyrate on the small intestine development in neonatal piglets fed by artificial sow. *J. Physiol. Pharmacol.* 55: 59-68.

41. **Biagi, G., Piva, A., Moschini, M., Vezzali, E., and Roth, F. X.** (2007) Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. *J. Anim. Sci.* 85: 1184-91.
42. **Bugaut, M.** (1987) Occurrence, absorption and metabolism of short chain fatty acids in the digestive tract of mammals. *Comp. Biochem. Physiol. B.* 86: 439-72.
43. **Claus, R., Gunthner, D., and Letzgub, H.** (2006) Effects of feeding fat-coated butyrate on mucosal morphology and function in the small intestine of the pig. *J. Anim. Physiol. Anim. Nutr.* 91: 312-318.
44. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
45. **Jensen, B. B. and Jorgensen, H.** (1994) Effect of dietary fiber on microbial activity and microbial gas production in various regions of the gastrointestinal tract of pigs. *Appl. Environ. Microbiol.* 60: 1897-904.
46. **Krause, D. O., Easter, R. A., White, B. A., and Mackie, R. I.** (1995) Effect of weaning diet on the ecology of adherent lactobacilli in the gastrointestinal tract of the pig. *J. Anim. Sci.* 73: 2347-54.
47. **Zoetendal, E. G., Collier, C. T., Koike, S., Mackie, R. I., and Gaskins, H. R.** (2004) Molecular ecological analysis of the gastrointestinal microbiota: A review. *J. Nutr.* 134: 465-472.
48. **Jensen, B. B., Hojberg, O., Mikkelsen, L. L., Hedemann, M. S., and Canibe, N.** (2003) Enhancing intestinal function to treat and prevent intestinal disease., in *9th International Symposium on Digestible Physiology in Pigs.* Ball, R., O. (ed.). p. 103-121. University of Alberta, Canada, Banff, AB, Canada.
49. **Blank, R., Sauer, W. C., Mosenthin, R., Zentek, J., Huang, S., and Roth, S.** (2001) Effect of fumaric acid supplementation and dietary buffering capacity on the concentration of microbial metabolites in ileal digesta of young pigs. *Can. J. Anim. Sci.* 81: 345-353.


Chapter
7

Piglet Gut Microbial Response to the Addition of an Acidifier and an Antibiotic to the Diet

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Manuscript in preparation

Abstract

An experiment was designed to evaluate the effect of supplementation of an organic acid blend and Oxytetracycline (OTC) on the jejunum mucosa-associated and fecal microbiota of piglets during the first 4 weeks post-weaning. Piglets weaned at around 4 weeks of age were distributed in 16 pens, 4 piglets per pen, and assigned to 4 different treatments, three of which were compared in this study: plain feed/plain water (control group, A), plain feed/0.15 % organic acid mixture in water (organic acid group, B), 400 ppm OTC in feed/plain water (antibiotic group, C). DNA was extracted from jejunum mucosa and fecal content, and microbial profiles were analyzed by 16S ribosomal RNA (rRNA) gene-targeted DGGE (Denaturant Gradient Gel Electrophoresis), qPCR (quantitative PCR) and PITChip (Pig Intestinal Tract Chip)**.** This revealed a shift of the microbial profiles in time, as well as an effect of both additives on the microbial structure, albeit non-significant. Quantification of 16S rRNA gene copy numbers of total bacteria, lactobacilli and enterobacteria showed that only OTC caused a significant increase in the lactobacilli numbers. Overall, the organic acid blend as well as OTC had a significant effect on weaned piglet gut microbiota.

Introduction

It is common practice that pig producers wean piglets at an early age (3 to 4 weeks) in order to diminish economic losses. The drawback is that early weaned pigs suffer several stressful factors related to the introduction of a new diet, a new environment and new social interactions. These stressors trigger a mal-absorption syndrome known as non-infectious diarrhea (NID). Opportunistic pathogens take advantage of this unfavorable situation [1], mainly enterotoxinogenic *Escherichia coli* strains (ETEC), that can then cause the post-weaning diarrhea syndrome (PWDS) [2].

To prevent gastrointestinal (GI) disorders and to improve post-weaning growth rates, feed-grade antibiotics have been used regularly during many years. Nevertheless, although the therapeutic benefits of antibiotics via microbial inhibition are well known [3, 4], and beneficial effects of antibiotics on feed efficiency and growth rate have been demonstrated before [5, 6], the mechanism of action is not clear. Four modes of action have been suggested: inhibition of subclinical infections, reduction of growth-depressing microbial metabolites, reduction of microbial use of nutrients, and enhanced uptake of nutrients through a thinner intestinal wall [3, 7]. However, it should be noted that changes in microbiota composition or metabolic activities have not always been observed following supplementation with antimicrobial growth promoters (AGP), despite observed improvements in animal performance. It was therefore recently hypothesized that AGP act primarily through their anti-inflammatory properties, and that reduced inflammation in GI tract tissues due to AGP supplementation may contribute to observed improvements in animal performance [8].

Although AGPs have a positive effect on weaning piglets, the possible influence on the development of antibiotic resistance in pathogenic microorganisms caused the full ban of these antibiotics as feed additives in the European Union as from 2006 and prompted the research of new alternatives. It has been proposed that acidification could provide a prophylactic effect similar to that provided by feed antibiotics [9, 10], making organic acids a promising alternative to antibiotics [11-14]. Moreover, it has been suggested that while antibiotics inhibit microbial growth in general, acidifiers cause beneficial rather than harmful microorganisms to dominate in the gastrointestinal tract [10]. Furthermore, organic acid supplementation is also applicable in the growing-finishing period and not only in the weaning phase.

In order to provide a sound microbiological basis for above-mentioned hypotheses, in this study we evaluated the potential effects of supplementing piglets' diet with either a commonly used antibiotic, Oxytetracycline (OTC), or with acidified water, applying a complementary set of cultivation-independent biomolecular approaches for the comprehensive characterization of porcine intestinal microbiota. Oxytetracycline is a broad spectrum antibiotic that targets several bacteria among which are chlamydia, mycoplasma, clostridia and spirochaetes. Some strains have developed resistance to this antibiotic. The mode of action consists in the inhibition of key protein production by the microbiota, stopping their proliferation and the infection.

Materials and Methods

Animals, treatments and sampling

A total of 64 Hypor piglets, mixed males and females, were used in this trial. They were allocated in departments at the Halfweg Swine Research Centre (Nutreco Agriculture R&D, Boxmeer, The Netherlands) with 16 pens each and 4 piglets per pen, with a light schedule of 16 hours light and 8 hours dark. No creep feeding was provided during the lactation period. Piglets were weaned at around 4 weeks of age, with an average weight of 6.8 kg, and assigned to 4 different treatments: plain feed/plain water (control group, A), plain feed/organic acid mix in water (organic acid group, B), medicated feed/plain water (antibiotic group, C) and medicated feed/organic acid mix in water (these samples were not analyzed). The length of the study was 4 weeks, during which piglets in the organic acid group received water supplemented with 0.15 % organic acid mixture POC 131 (Selko) and piglets in the antibiotic group were fed a diet supplemented with 400 ppm OTC during the first week after weaning. Feed and water, via nipple, were provided *ad libitum*. Growth, feed use and feed efficiency were measured weekly per pen, diarrhea scores were observed daily per pen and water usage was measured per half department. Per treatment group, 4 piglets were sacrificed after 2 and 4 weeks, respectively, and samples were taken of jejunum, ileum and rectum contents and of jejunum and ileum mucosa for microbiological analysis. Culturing was performed directly while other samples were kept at -20ºC till the molecular analyses were performed.

Commercial Start feeds (non-medicated and medicated with 400 ppm Oxytetracycline) were fed the first week after weaning. Thereafter, commercial Link feed was fed to all piglets.

DNA isolation and PCR-DGGE (Denaturant Gradient Gel Electrophoresis)

In total 48 samples (jejunum mucosa and feces) from 24 piglets that had either a control diet (plain feed/plain water) or a supplemented diet (plain feed/acidified water and medicated feed/plain water) were selected for DNA extraction. Samples (stored at -20ºC) were defrosted and DNA was extracted from 300 mg of digesta using the Fast DNA Spin Kit for Soil (Qbiogene, Inc, Carlsbad, CA, USA). Jejunum mucosa samples were pre-treated with 500 µL of PBS (Sodium Phosphate Buffer), 50 µL of SDS (10%) and 10 µL of proteinase K (20 mg/ml). Electrophoresis in agarose gels 1.2% (w/v) containing ethidium bromide was used to check the quality of DNA. To study the total microbial profiles of the samples by DGGE, the V6-V8 variable region of the bacterial 16S rRNA (ribosomal RNA) gene was amplified using the universal primers set S-D-Bact-0968-a-S-GC/S-D-Bact-1401-a-A-17 (5´-CGCCCGGGGCGCGCCCCGGGCGGGGCGGGGGCACGGGGGGAACGCGAAGAA CCTTAC-3´, 5´-CGGTGTGTACAAGACCC-3´) [15]. To study *Lactobacillus*-specific GI tract community, primers Lac1/Lac2 (5´-AGCAGTAGGGAATCTTCA-3´, 5´-CGCCCGGG GCGCGCCCCGGGCGGCCCGGGGGCACCGGGGATTYCACCGCTACACATG-3´) [16] were used to amplify the V3 region of the bacterial 16S rRNA gene. PCRs were performed using a GoTaq® DNA Polymerase kit from Promega (Madison, WI, USA.). Each PCR mixture (50 µl) contained 1.25 U of GoTaq® DNA polymerase, Green GoTaq® reaction buffer containing 1.5 mM $MgCl_2$, 0.2 mM of each deoxynucleoside triphosphate, 0.2 µM of the primers, 1 µl of DNA solution (~1 ng/µl) and UV-sterilized water. The samples were amplified in a thermocycler T1

(Whatman Biometra, Göttingen, Germany) using the following program: pre-denaturation at 94°C for 5 min, 35 cycles of denaturation at 94°C for 30 sec, annealing at 56°C for 20 sec, extension at 68°C for 40 sec and final extension at 68°C for 7 min. The size of the PCR products was checked by electrophoresis on a 1% (w/v) agarose gel containing ethidium bromide. DNA concentration was determined with a NanoDrop ND-1000 spectrophotometer (NanoDrop® Technologies, Wilmington, DE, USA).

The V6-V8 PCR amplicons were separated by DGGE [17] using a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Electrophoresis was performed in an 8% (v/v) polyacrylamide gel (37.5:1 acrylamide-bisacrylamide and dimensions: 200 by 200 by 1 mm) and 0.5× Tris-acetate-EDTA (TAE) (pH 8) buffer. Gels with a different gradient (30-60% and 30-55% for total bacterial and *Lactobacillus*-group specific amplicons respectively) were prepared using a gradient maker and a pump (Econopump; Bio-Rad Laboratories). The gels were electrophoresed for 16 h at 85 V in 0.5× TAE buffer at a constant temperature of 60°C and stained with $AgNO_3$ [18]. Bionumerics software package version 4.5 (Applied MathS, Kortrijk, Belgium) was used to normalize and analyze the gels.

Phylogenetic microarray analysis

The PITChip (Chapter 3) was used for simultaneous high-resolution profiling and phylogenetic analysis of the samples. The protocols for hybridization and analysis of the data were used essentially as described before [19, 20].

RNA production: The 16S rRNA gene from the DNA samples was amplified using primers T7prom-Bact-27-for/Uni-1492-rev (5´-TGAATTGTAATACGACTCACTATAGGGGTTTGA TCCTGGCTCAG-3´, 5´-CGGCTACCTTGTTACGAC-3´) [21]. The PCR reaction was performed with 10 ng of DNA in a final volume of 50 µl and the conditions were as follows: 94°C for 2 min followed by 35 cycles of 94°C (30 sec), 52°C (40 sec) and 72°C (90 sec), and a final extension at 72°C for 7 min. PCR amplicons were purified (NucleoSpin Extract II kit, Macherey-Nagel, Düren, Germany) and the concentrations were measured using a NanoDrop ND-1000 spectrophotometer.

In vitro transcription of the 16S rRNA genes carrying the T7-promoter was performed using the Riboprobe System (Promega, La Jolla, USA). The reaction mix contained 500 ng of the PCR amplicon together with rATP, rGTP, rCTP and a 1:1 mix of rUTP and aminoallyl-rUTP (Ambion, Austin, TX, USA). This transcription reaction was incubated at room temperature for 2 h followed by a DNAse treatment with the Qiagen RNAse-free DNAse kit (Qiagen, Hilden, Germany). Produced RNA was purified (RNeasy Mini-Elute Kit, Qiagen, Hilden, Germany) and the concentration was quantified with a NanoDrop.

Labeling of samples and hybridization: The Post-Labeling Reactive Dye (Amersham Biosciences, Little Chalfont, UK) dissolved in 84 µl of DMSO was used to couple the RNA samples with CyDye. The reaction was performed with 20 µl of CyDye, 2 µg of pure RNA and 25 mM of sodium bicarbonate buffer (pH 8.7) in a final volume of 40 µl. Samples were incubated during 90 min in the dark and at room temperature. The reaction was stopped by adding 15 µl of 4 M hydroxyl-amine and incubating in the dark for 15 min. RNAse-free water

was added to 100 µl and the labeled RNA was quantified.

From each sample two labeled fractions, with Cy3 and Cy5 respectively, were hybridized to the microarrays synthesized by Agilent Technologies (Agilent Technologies, Palo Alto, CA, USA) in 8×15 K format. For that purpose, the Cy3/Cy5-labeled target mixtures were treated with 10× fragmentation reagent (Ambion, Austin, TX, USA). Hybridization on the arrays was performed at 62.5°C for 16 h in a rotation oven (Agilent). Slides were washed at room temperature in 2× SSC with 0.3% SDS for 10 min, followed by 0.1× SSC with 0.3% SDS at 38°C for 10 min and 0.06× SSPE for 5 min [22].

PITChip data analysis: Microarray slides were scanned and the data was extracted from the images using the Agilent Feature Extraction software, version 9.1 (http://www.agilent.com). For the data normalization and further analysis a set of R based scripts (http://r-project.org) were used in combination with a custom designed relational database [19, 20], which runs under the MySQL database management system (http://www.mysql.com).

Quantitative PCR (qPCR)

Quantification of total bacteria, lactobacilli and enterobacteria groups was done by qPCR using an iCycler IQ real-time detection system associated with the iCycler optical system interface software version 2.3 (Bio-Rad, Veenendaal, The Netherlands). The reaction mixture (25 µL) consisted of 12.5 µL of IQ SYBR Green Supermix (Bio-Rad), 0.2 µM of each primer and 5 µL of template DNA diluted in water (10, 100 or 1000 times) to avoid PCR inhibition. The standard curves were generated using serially diluted rRNA gene amplicons obtained from pure *Lactobacillus* and *E.coli* strains. Universal primers, Bact 1369 (5´-CGGTGAATACGTTCYCGG-3´) and Prok 1492 (5´-GGWTACCTTGTTACGACTT-3´) [23] were used to estimate the total number of copies of the bacterial 16S rRNA gene in each sample by triplicate. The qPCR reaction was performed with an initial denaturation step of 95ºC for 3 min, followed by 40 cycles of 95ºC for 15 sec, 56ºC for 30 sec and 72ºC for 30 sec, and two final steps of 95ºC and 60ºC for 1 min each. For the amplification of lactobacilli 16S rRNA genes, primers Lacto F (5´-TGGAAACAGRTGCTAATACCG-3´) and Lacto R (5´-GTCCATTGTGGAAGATTCCC- 3´) [24] were used and the reaction steps were: 95ºC for 10 min, followed by 40 cycles of 95ºC for 15 sec and 62ºC for 1 min, and two final steps of 95ºC and 60ºC for 1 min each. In order to amplify a fragment of the 23S rRNA gene of enterobacteria present in the samples primers En-lsu3f (5´-TGCCGTAACTTCGGGAGAAGGCA-3´) and En-lsu3r (5´-TCAAGGCTCAATGTTCAGTGTC-3´) [25] were used and the following reaction was performed: initial step of 50ºC for 2 min, 95ºC for 10 min followed by 40 cycles of 95ºC for 15 sec and 60ºC for 1 min, and two final steps of 95ºC and 60ºC for 1 min each. Melting curve analysis of amplicons was done after all amplifications.

Statistical analysis

Statistical comparison of the DGGE profiles based on UPGMA clustering (Unweighed Pair Group Method, based on Arithmetic mean), and Pearson's correlation was performed to calculate similarity indexes as implemented in Bionumerics.

Ward's minimum variance method was used for the generation of hierarchical clustering

of probe profiles generated by the PITChip, by calculating a distance matrix between the samples based on the squared difference between each pair of profiles (squared Euclidian distance – E2) [26]

Multivariate analysis, and more specifically redundancy analysis (RDA), was applied for PITChip data interpretation in order to relate changes in microbial community composition to environmental variables, as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) [27]. RDA is a multivariate direct gradient analysis method that allows ordering of samples and taxa (i.e. taxonomic groups) considering that the latter have linear relationships to environmental variables. The sums of signal intensities for 144 genus-like phylogenetic groups were used as predictors. Treatment classes and time points were introduced as nominal environmental variables by defining them as 0 or 1 (dummy variables). RDA was performed focusing on inter-sample correlation and the Monte Carlo permutation test was applied [28] using the option "unrestricted permutation" (because the experiment had a randomized design) to decide whether treatment and time had a statistically significant influence on the species composition. The environmental variables were considered to influence significantly the microbiota composition at p-values < 0.05. Diagrams were plotted as triplots using CanoDraw for Windows.

Diversity of microbial profiles obtained by DGGE and PITChip analysis was expressed as Simpson's reciprocal index of diversity (1/D) [29]. This diversity was calculated with the equation $\lambda = 1/\Sigma Pi^2$, where Pi is the proportion of i^{th} bands or taxon (proportion of each band density or probe signal compared to the total density or signal for each sample). A higher Simpson's index value indicates a higher degree of diversity.

To statistically analyze the qPCR results, the number of gene copies was $\log_{10}$ transformed to achieve normal distribution, and the mean and standard deviation were calculated. In order to asses statistical significance of the changes among the treatment groups and between time points, Student's T-test was performed (two tailed). P-values were statistically significant when lower than 0.05 and highly significant when lower than 0.01.

Results

In the present study, a complementary set of molecular approaches targeting the bacterial 16S rRNA gene was applied towards the evaluation of potential changes in microbiota composition in different compartments of the porcine gastrointestinal tract after dietary treatment of weaning piglets with either a commonly used antibiotic, Oxytetracycline, or alternatively a blend of organic acids. To this end, fecal and jejunum mucosa samples were used as representative for different locations along the GI tract.

In vivo data correlated with the microbiological results (data not shown). It was found that antibiotic supplementation to the diet significantly reduced the incidence of diarrhea in the 4 weeks period following weaning, supporting the observation that the effects of OTC were seen at week 4. Similarly, the supplementation of 0.15% organic acid blend also significantly reduced the incidence of diarrhea.

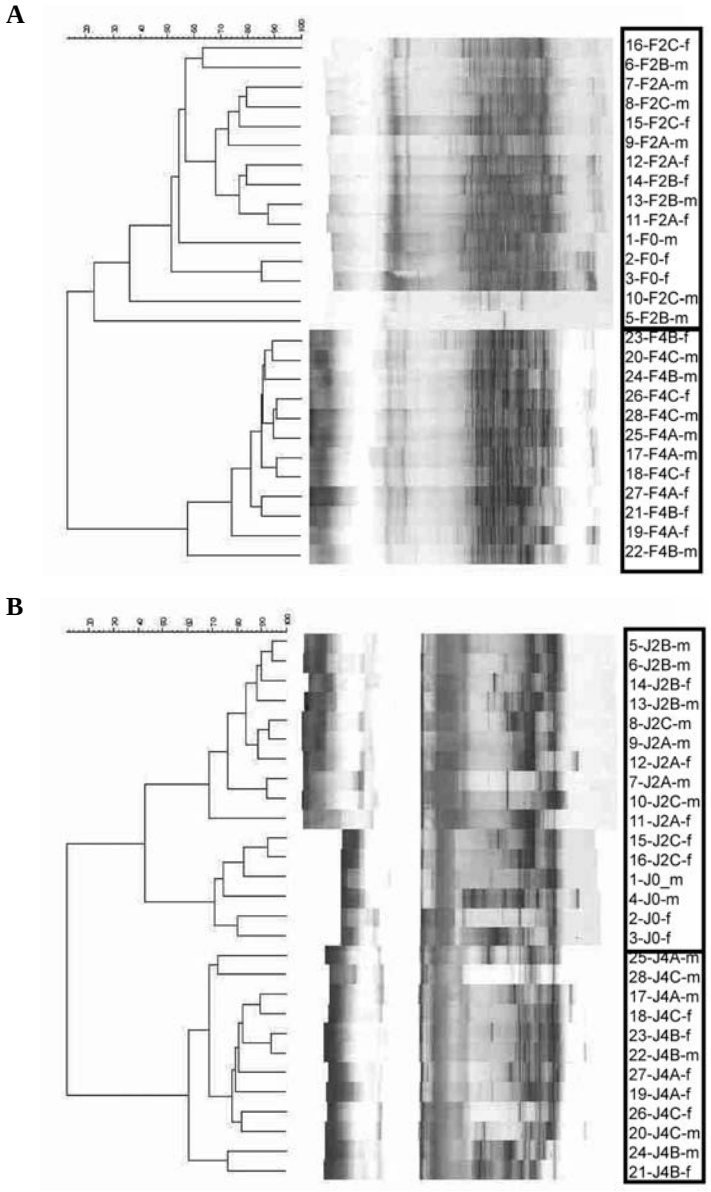

Figure 1. DGGE profiles of total bacterial groups from piglets receiving either a control diet, a diet supplemented with an organic acid blend or a diet supplemented with oxytetracycline. Samples were from fecal material (**A**) and jejunum mucosa (**B**) and were collected at week 2 and week 4 after weaning. Samples coding: pig number followed by sampling site (F = feces, J = jejunum mucosa), week (0, 2 or 4), treatment (A = control, B = organic acid blend, C = antibiotic) and sex (m = male and f = female). The two clusters are marked with different boxes.

DGGE profiling

In order to provide an overview of potential effects of treatment as well as time of sampling on porcine fecal and jejunum mucosa-associated microbiota, the similarities of the corresponding total bacterial and lactobacilli-specific DGGE profiles were compared based on Pearson's correlation coefficients, which were subsequently used for the construction of UPGMA dendograms. Cluster analysis of the two time points (week 2 with week 4) for total bacteria profiles of each set of samples (feces and jejunum) showed two separate clusters, one with samples from week 2 and the other one with week 4 samples (Fig. 1) indicating the development of the microbiota in time. The same was observed for lactobacilli profiles (data not shown).

Furthermore, to assess whether time after weaning and/or treatment affected bacterial diversity, Simpson's reciprocal index of diversity was calculated based on number and relative intensity of DGGE bands. The diversity was higher in feces samples than in jejunum, as expected. The total bacteria community was significantly less diverse at week 4 than at week 2 for feces in control pigs (p-value = 0.008; Table 2). A significant difference in microbial composition related to treatment was observed in the lactobacilli community, the diversity of which decreased in the jejunum mucosa at week 2 when OA was supplemented to the diet, compared to the control. Moreover, in the lactobacilli-specific DGGE of week 2 with feces samples, two clusters were observed, one corresponding to samples from pigs receiving the dietary treatment A (plain feed/plain water) and another cluster comprising samples from dietary treatment B (plain feed/acidified water) and C (in-feed antibiotic/plain water) (data not shown). More specifically, there was one band that was clearly present in samples from piglets receiving the control diet (plain feed/plain water) but was inhibited in piglets receiving the antibiotic or the organic acid blend, at week 2. At week 4 profiles from piglets from the three treatments showed the band again (Fig. 2).

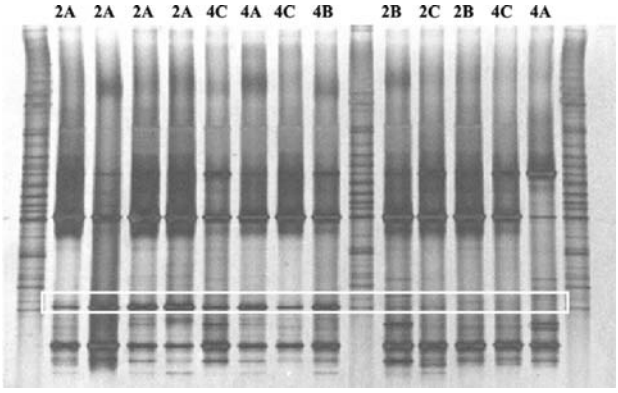

Figure 2. DGGE profiles of lactobacilli species present in feces samples from piglets receiving either a control diet (A), a diet with acidified water (B) or a diet supplemented with an antibiotic (OTC; C). Samples were taken at week 2 and week 4 after weaning. The white box marks a band corresponding to a lactobacilli species that is inhibited by both the organic acid treatment and the antibiotic treatment at week 2.

Detailed insight into fecal microbial profiles

Fecal samples were selected for a more detailed assessment of their microbial composition using the PITChip, a diagnostic microarray custom-designed for the comprehensive profiling of porcine intestinal microbiota. To this end, the hybridization signal intensities of probes corresponding to 144 SSU (small subunit) rRNA-based genus-level phylogenetic groups were used.

A multivariate analysis (RDA) of the hybridization signals for each sample showed that there was no significant effect, neither of any of the treatments nor of time. However, the analysis also indicated that one of the samples taken from one of the animals fed diet B (22-F4B-m) was a strong outlier (data not shown). When this sample was removed from the data set and re-analyzed we could observe a statistically significant change ($p = 0.05$) of the profiles in the presence of the organic acid blend (Fig. 3). For this reason we did not include that sample in the statistical analysis of relative abundance of different phylogenetic groups.

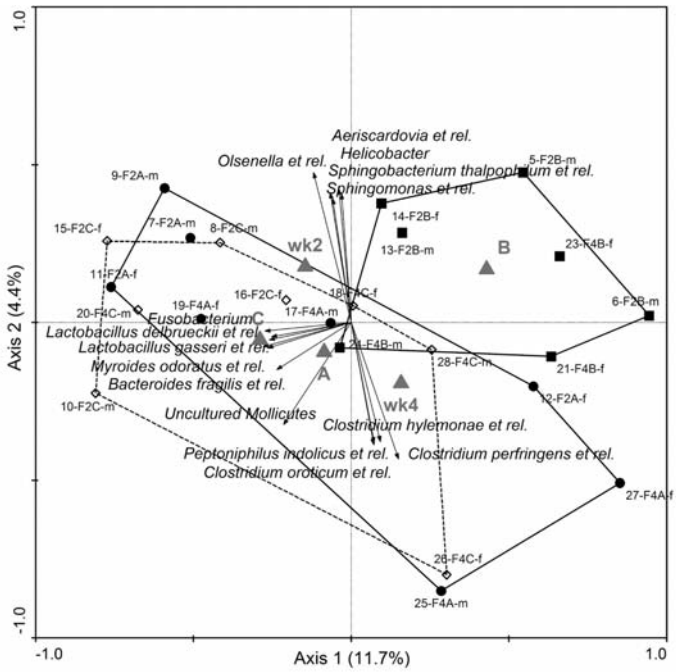

Figure 3. RDA triplot of the microbial profiles of weaned piglets, expressed as the summed hybridization signals of 144 phylogenetic groups. Samples were from piglets that received either a control diet (A; ●), a diet with acidified water (B; ■) or a diet with an in-feed antibiotic (C; ◊). Microbial groups that contributed at least 22% to the explanatory axis are represented as vectors and the environmental variables, diet A, B and C, and time (week 2/4) as centroids of the plot. Both axes explain together 16.1% of the total variance in the dataset. Samples from treatment B were significantly different ($p = 0.05$). Samples coding: pig number followed by sampling site (F = feces, J = jejunum mucosa), week (2 or 4), treatment (A = control, B = organic acid blend, C = antibiotic) and sex (m = male and f = female).

Microbial changes related to diet: RDA analysis of the microbial profiles considering the environmental variables treatment (control, organic acid blend and antibiotic) and time (week 2 and week 4) showed that there was a significant effect of the acidifier on the microbial composition ($p = 0.05$; Fig. 3). When analyzing samples from the two time points separately, RDA revealed that this treatment effect was observed only two weeks but not four weeks after weaning (data not shown). Among the phylogenetic groups that explained at least 22% of the distribution, there were three *Lactobacillus gasseri*-, *Lactobacillus paracasei*- and *Lactobacillus delbruckii*-like species, as well as populations related to *Clostridium perfringens*, *Clostridium oroticum* and *Clostridium hylemonae*. These *Clostridium* species were more abundant in piglets receiving a control or antibiotic supplemented diet. It was also observed that the control and the antibiotic-treatment groups had a more similar microbial composition than the organic acid group, because both centroids of the plot representing the two environmental variables (control and antibiotic treatment) clustered on the left lower quartile of the triplot. Moreover, the analysis showed that the microbiota of piglets treated with the organic acid blend had a more uniform composition than the piglets from the control and antibiotic treatment group.

Table 1 lists the phylogenetic groups with the highest relative abundance (> 2%) for each treatment group for both sampling points.

Table 1. Phylogenetic groups with relative abundance of at least 2% in the 3 treatment groups at week 2 (upper table) and at week 4 (lower table).

Week 2

Control		Organic acid		Antibiotic	
Group	**%**	**Group**	**%**	**Group**	**%**
Sporobacter termitidis et rel.	2.94	*Sporobacter termitidis* et rel.	3.08	*Sporobacter termitidis* et rel.	2.65
Lachnospira pectinoschiza et rel.	2.92	*Lachnospira pectinoschiza* et rel.	3.06	*Lachnospira pectinoschiza* et rel.	2.62
Uncultured Clostridia XIVb	2.36	*Lactobacillus plantarum* et rel.	2.42	*Lactobacillus plantarum* et rel.	2.25
Lactobacillus plantarum et rel.	2.31	Uncultured Clostridia XIVb	2.36	Uncultured Clostridia XIVb	2.20
Bryantella et rel.	2.16	*Ruminococcus callidus* et rel	2.29	*Ruminococcus callidus* et rel	2.11
Uncultured Clostridia IV	2.11	*Bryantella* et rel.	2.23	*Lactobacillus salivarius* et rel.	2.07
Ruminococcus callidus et rel	2.10	Uncultured Clostridia XIVa	2.17	Uncultured *Prevotella*	2.05
Uncultured Clostridia XIVa	2.05	*Lactobacillus salivarius* et rel.	2.10		
		Uncultured Clostridia IV	2.00		

Week 4

Control		Organic acid		Antibiotic	
Group	**%**	**Group**	**%**	**Group**	**%**
Lachnospira pectinoschiza et rel.	2.98	*Sporobacter termitidis* et rel.	3.16	*Sporobacter termitidis* et rel.	3.11
Sporobacter termitidis et rel.	2.63	*Lachnospira pectinoschiza* et rel.	3.02	*Lachnospira pectinoschiza* et rel.	2.74
Lactobacillus plantarum et rel.	2.32	Uncultured Clostridia XIVb	2.57	*Lactobacillus plantarum* et rel.	2.49
Uncultured Clostridia XIVb	2.25	*Clostridium perfringens* et rel.	2.38	Uncultured Clostridia XIVb	2.31
Clostridium perfringens et rel.	2.20	*Bryantella* et rel.	2.29	*Clostridium perfringens* et rel.	2.20
Uncultured Clostridia XIVa	2.14	*Ruminococcus callidus* et rel	2.28	*Lactobacillus salivarius* et rel.	2.12
Bryantella et rel.	2.13	Uncultured Clostridia XIVa	2.21		
Ruminococcus callidus et rel	2.13	Uncultured Clostridia IV	2.21		
Lactobacillus salivarius et rel.	2.10	*Bacillus* et rel.	2.13		
Uncultured Clostridia IV	2.00	*Lactobacillus plantarum* et rel.	2.05		

This showed that the microbiota composition was quite similar among treatments with mainly members of the *Clostridium* clusters I, IV, XIVa and XIVb, *Bacilli* and *Bacteroidetes* species-like groups. In both time points, a decrease was observed in the number of groups above 2%, in the group receiving the antibiotic, indicating a reduction in diversity at the genus-like group level. At week 4 after weaning, *Clostridium perfringens*-like species appeared as one of the abundant groups.

Differences in Simpson's reciprocal index of diversity of the microbial profiles obtained by PITChip were not significant, showing a lower diversity in samples from piglets that received the organic acid blend compared to the control (204.7 ± 26.5 versus 218.4 ± 42.3 respectively) and even lower in samples from piglets treated with the antibiotic (188.1 ± 41.1), at week 2 after weaning. Four weeks after weaning, the diversity of the microbial profiles was lower than two weeks earlier (diversity of the control was 167.3 ± 60.8). The group treated with the organic acid mixture had again a lower diversity than the control (126.9 ± 87.1 versus 167.3 ± 60.8) but piglets treated with the antibiotic had a higher microbial diversity compared to the control group (210.4 ± 49.0 versus 167.3 ± 60.8), which was, however, not significant (Table 2).

Table 2. Simpson's diversity index ± standard deviation, calculated for PITChip (upper table) and DGGE (lower table) profiles for both time points (week 2 and week 4), both sample types (jejunum mucosa and feces), the 3 diets and in DGGE for total bacterial and lactobacilli communities and for diet. Significant differences between week 2 and week 4 are marked as ** for $p < 0.01$; Significant differences between treatments are marked with letters.

PITCHip

	Diet A	Diet B	Diet C
week 2	218.4 ± 42.35	204.7 ± 26.49	188.1 ± 41.12
week 4	167.3 ± 60.80	126.9 ± 87.06	210.4 ± 48.99

DGGE

Lactobacillus

	Mucosa			Feces		
	Diet A	**Diet B**	**Diet C**	**Diet A**	**Diet B**	**Diet C**
week 2	4.80 ± 0.89[a]	3.65 ± 0.10[b]	3.66 ± 0.77[a]	8.96 ± 1.24	8.61 ± 2.38	9.24 ± 2.50
week 4	6.94 ± 5.16	5.50 ± 3.31	8.10 ± 3.95	12.19 ± 0.67	10.48 ± 1.30	11.56 ± 1.62

Total bacteria

	Mucosa			Feces		
	Diet A	**Diet B**	**Diet C**	**Diet A**	**Diet B**	**Diet C**
week 2	11.37 ± 1.98	14.96 ± 3.50	15.58 ± 3.85	34.20 ± 4.09	22.80± 13.22	25.6 ± 9.63
week 4	11.69 ± 0.94	12.78 ± 2.61	9.41 ± 3.01	23.79 ± 1.12**	25.81 ± 3.13	22.67 ± 0.66

In order to identify those phylogenetic groups for which the relative abundance was significantly different between the three treatments, Student's T-tests were performed on the summed hybridization signals of each of the 144 phylogenetic groups. Piglets fed with a diet supplemented with the organic acid blend had a significant increase of several bacterial groups two weeks after weaning (Table 3).

Table 3. List of phylogenetic groups that changed significantly 2 weeks after weaning, when an organic acid blend was supplemented to the water (treatment B). P-values are indicated as well as the change observed, decrease or increase of the relative abundance when compared to the control. Abundance is given as average of values observed for animals in the control group.

Higher taxonomic group	Group	p-value	Change	Abundance (%)
Actinobacteria	*Actinomyces* et rel.	0.044	Increase	0.53
	Leucobacter	0.033	Increase	0.40
	Microbacterium	0.042	Increase	0.66
	Micrococcus et rel.	0.045	Increase	0.65
	Tonsillophilus	0.039	Increase	0.56
Bacilli	Uncultured *Bacilli*	0.034	Increase	1.05
Clostridium cluster IV	*Subdoligramulum* et rel.	0.012	Increase	0.85
Clostridium cluster XIVa	*Eubacterium plexicaudatum* et rel.	0.041	Increase	0.38
	Lachnobacterium bovis et rel.	0.034	Increase	1.21
Clostridium cluster XVI	*Eubacterium biforme* et rel.	0.030	Increase	0.56
Deltaproteobacteria	Uncultured *Deltaproteobacteria*	0.046	Decrease	0.08
Flavobacteria	*Myroides odoratus* et rel.	0.037	Decrease	0.37
Gammaproteobacteria	*Actinobacillus* et rel.	0.049	Increase	0.43
	Avibacterium	0.030	Increase	0.32
	Pasteurella	0.033	Increase	0.32
	Ruminobacter et rel.	0.033	Increase	0.31
	Xanthomonas et rel.	0.013	Increase	0.89
Mollicutes	*Acholeplasma* et rel.	0.031	Increase	0.54
	Bulleidia moorei et rel.	0.036	Increase	0.26
	Uncultured *Mollicutes*	0.022	Decrease	0.001

In contrast, samples from piglets that received the antibiotic had hardly any significant change compared to the control diet, agreeing with what was observed with the multivariate analysis. There was only a decrease of Uncultured *Clostridia* XIVa ($p = 0.021$) and an increase of *Enterococcus*-like species ($p = 0.046$). At week 4 after weaning, the microbiota of the group receiving the acidified water was not much different from the control group, with only a few groups that showed a significant change, *Turneriella*- and *Xanthomonas*-like species decreased significantly ($p = 0.033$ and $p = 0.044$ respectively; Table 4).

Temporal development of the fecal microbiota after weaning: Whereas cluster analysis of DGGE profiles showed separate clusters depending on the time of sampling, multivariate analysis of corresponding PITChip fingerprints indicated that this difference between sampling days was not statistically significant ($p = 0.404$; data not shown).

Microbiota composition of piglets receiving the control diet was quite stable over time, with similar relative abundance of the different phylogenetic groups present in feces. Overall, only a decrease in *Erysipelothrix*- ($p = 0.012$) and *Solobacterium moorei*-like species ($p = 0.042$), both members of the *Mollicutes*, and an increase of *Streptococcus suis*-like species ($p = 0.019$) belonging to the *Bacilli*, were observed. In the treatment groups, the shifts in population between week 2 and week 4 were more pronounced, especially in the organic acid treatment group. Based on the PITChip data, the acidifier inhibited mainly the growth of species belonging to *Clostridium* cluster XIVa, *Gammaproteobacteria* and *Bacilli* (data not shown). Within the antibiotic treatment group the significant changes in time were scarcer and comprised mostly an

increase in relative abundance of groups belonging to the *Clostridium* clusters I, IV, IX, XIVa and XV, and *Spirochaetes*. These groups included: *Clostridium perfringens*-, *Faecalibacterium*-, *Veillonella*-, *Roseburia intestinalis*-, *Eubacterium*- and *Leptospira*-like species.

Table 4. List of phylogenetic groups that changed significantly 4 weeks after weaning, when OTC (antibiotic) was supplemented to the feed. P-values are indicated as well as the change observed, decrease or increase of the relative abundance when compared to the control. Abundance is given as average of values observed for animals in the control group.

Higher taxonomic group	Group	p-value	Effect	Abundance (%)
Actinobacteria	*Leucobacter*	0.029	Increase	0.40
	Microbacterium	0.013	Increase	0.66
	Micrococcus et rel.	0.012	Increase	0.65
	Uncultured *Actinobacteria*	0.020	Increase	0.58
Bacilli	*Turicibacter* et rel.	0.047	Increase	0.39
	Weissella et rel.	0.021	Increase	0.49
Clostridium cluster IV	*Faecalibacterium* et rel.	0.011	Increase	1.38
Clostridium cluster IX	*Phascolarctobacterium faecium* et rel.	0.031	Increase	0.80
	Veillonella	0.012	Increase	0.52
Clostridium cluster XI	*Anaerovorax* et rel.	0.016	Decrease	1.28
Clostridium cluster XV	*Eubacterium* et rel.	0.013	Increase	0.52
Clostridium cluster XVI	*Eubacterium biforme* et rel.	0.016	Increase	0.56
Mollicutes	*Acholeplasma* et rel.	0.013	Increase	0.54
	Bulleidia moorei et rel.	0.026	Increase	0.26
	Erysipelothrix	0.010	Increase	0.64
	Solobacterium moorei et rel.	0.015	Increase	0.45
Spirochaetes	*Leptospira*	0.038	Increase	0.46

Analysis of lactobacilli in the feces of weaned piglets: As mentioned above, DGGE indicated a change in the lactobacilli profiles related to treatment, in samples from 2 weeks after weaning. In order to evaluate whether populations corresponding to these changes could be identified using PITChip analysis data, a multivariate analysis (RDA) was performed using the summed hybridization signals of all lactobacilli groups (Fig. 4). Although this analysis showed that there was no significant effect of the treatment on the *Lactobacillus* species distribution, a clear shift with respect to the different lactobacilli groups was observed (Fig. 4). The RDA triplot showed a higher number of lactobacilli species having a higher positive correlation with treatment C than with B. Moreover, it indicated that the DGGE profile band that was disappearing at week 2 after weaning, with treatment C and in lesser degree with treatment B, most likely represented a *Lactobacillus paracasei*-like organism.

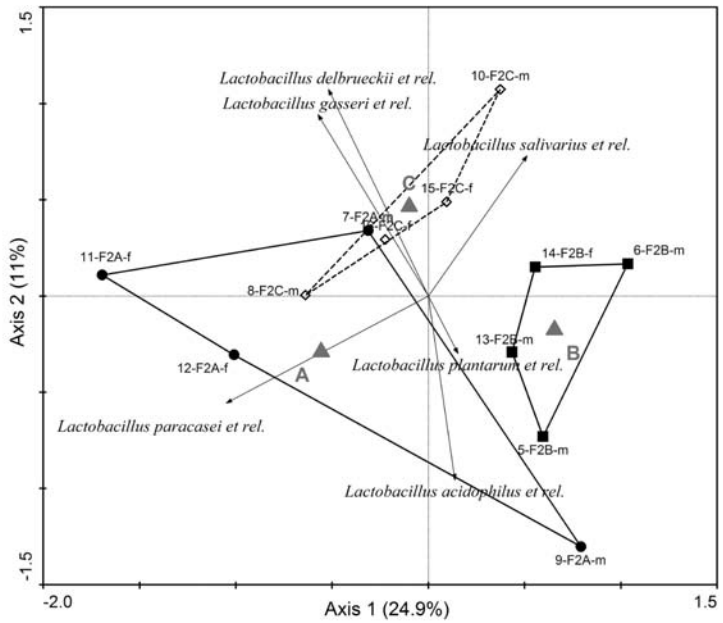

Figure 4. RDA triplot of lactobacilli profiles of samples taken 2 weeks after weaning, expressed as summed hybridization signals of 144 phylogenetic groups. Samples are classified according to treatment. A (●) corresponds to the control diet, B (■) to the acidified water diet and C (◊) to the antibiotic supplemented diet. All lactobacilli groups that contributed to the explanatory axis are represented as vectors, and the environmental variables, diet A, B and C, as centroids of the plot. The axes explain together 35.9% of the total variance in the dataset.

Abundance of total bacteria, lactobacilli and enterobacteria in fecal samples

Quantitative PCR was used to determine the abundance of total bacteria, total lactobacilli and total enterobacteria present in fecal samples of piglets at two and four weeks after weaning. Total bacterial numbers 2 weeks after weaning (Table 5) were found to be almost significantly lower ($p = 0.058$) in piglets fed with the organic acid blend supplemented diet than in piglets receiving the control diet. This result was in agreement with the PITChip data that showed a significant change of the profiles at week 2 only with the organic acid treatment. At week 4 the bacterial counts were higher than two weeks earlier and very similar among treatments. Total lactobacilli counts were very similar among treatments at week 2 but were significantly higher ($p = 0.011$) at week 4 in piglets that received the antibiotic, compared to piglets in the acidified water treatment group. Enterobacteria counts were also comparable after the first two post-weaning weeks and increased more than 1 log unit in the following two weeks in the control group and in the group treated with the antibiotic, however, the counts were lower at week 4 for piglets treated with the acidified water. These changes, though, were not statistically significant.

The ratio between enterobacteria and lactobacilli, that has been used often as an indicator of gut health, increased in time in piglets fed with the control diet and did not change in piglets with the antibiotic supplemented diet (0.59 versus 0.69 and 0.66 versus 0.67, correspondingly)

but was decreased in piglets receiving the acidified diet (0.65 versus 0.55), albeit not significantly (Table 5).

Table 5. Quantitative PCR counts of total bacteria, lactobacilli and enterobacteria in fecal samples of piglets receiving a control diet (A), a diet supplemented with an organic acid blend (B) and a diet supplemented with OTC (C). Samples were collected at week 2 and week 4 after weaning. Counts are expressed as mean of log copies/g FM ± SD.

Species	Week 2			Week 4		
	Diet A	Diet B	Diet C	Diet A	Diet B	Diet C
Bacteria	9.73 ± 0.51	8.97 ± 0.40	9.36 ± 1.03	10.18 ± 0.27	10.0 ± 0.15	10.12 ± 0.04
Lactobacilli	8.93 ± 0.92	8.10 ± 0.69	8.39 ± 1.93	9.44 ± 0.64	8.75 ± 0.45	9.72 ± 0.19
Enterobacteria	5.33 ± 1.05	5.25 ± 1.27	5.39 ± 1.35	6.52 ± 1.83	4.85 ± 0.59	6.50 ± 1.49
Entero:lacto	0.54	0.65	0.66	0.69	0.55	0.67

Discussion

The effects of addition of organic acids and antibiotics on intestinal microbiota and pig performance have been addressed by several studies in the past [30-32], but less often a blend of organic acids has been used.

In the present work the effects of both additives were studied *in vivo* using a complementary set of molecular approaches for the qualitative and quantitative analysis of pig GI tract microbiota. We observed a higher diversity in feces as compared to jejunum mucosa-associated microbiota, as expected [33], and a shift in the microbial profiles from week 2 to week 4 after weaning. Such microbial shifts during the first weeks after weaning have been reported before [33-38]. More specifically, the higher lactobacilli diversity at week 4 could indicate a recovery of this community after the decrease observed by several studies directly after weaning [35, 39, 40]. In addition, the significant decrease in total bacteria in feces at week 4 only in the control group could indicate a stabilizing effect of the treatments on the microbiota.

Significant changes in diversity caused by the treatments did not correlate between DGGE and PITChip data. DGGE fingerprint clustering indicated a significant change of the profiles in time but when changes in the microbial composition were followed with the PITChip, the difference between the 2 time points was not significant. This could be due to the limitations encountered when comparing results from a high-throughput technique as is the phylogenentic PITChip microarray with DGGE and other similar profiling techniques that have limited resolution when analyzing complex ecosystems such as the piglet GI tract, where bands can represent mixtures of phylotypes [41-43].

Oxytetracycline is expected to act against Gram-positive bacteria, as many other growth promoting antibiotics [44, 45]. Therefore a decrease in total bacterial numbers or lactobacilli could be expected in the group receiving this antibiotic. However, total bacterial counts were only almost significantly lower in the presence of the acidifier at week 2 and surprisingly lactobacilli counts were significantly higher in the antibiotic group when compared to the group

receiving the organic acid, at week 4. Both observations have been described before. Castillo et al. [31] reported the lack of effect of the antibiotic avilamycin or the organic acid butyrate on total bacteria and lactobacilli numbers, as it was seen in this study at week 2. Furthermore, other groups have reported the increase in lactobacilli numbers when an antimicrobial was supplemented to piglets [5, 30, 46]. The effect of OTC at week 4, incrementing lactobacilli and some pathogens and decreasing the abundance of species above 2%, but not at week 2, could indicate a long term effect of the antibiotic, in line with previous observations that a single dose of parenteral long-acting amoxicillin reduced bacterial diversity in neonatal piglets even 5 weeks after administration [47].

The fact that total bacteria, lactobacilli and enterobacteria numbers, both at week 2 and 4, were lower (even if not significant) in the presence of the organic acid blend, could indicate a general antimicrobial effect as it has been reported previously [12, 48, 49]. Canibe et al. [48] showed that when potassium diformate was added to a starter diet for piglets, there was a decrease of total anaerobic bacteria, lactic acid bacteria and coliforms. This was also proven by the lower ratio enterobacteria:lactobacilli that has been used often as an indicator of gut health [50]. Nevertheless, the fact that none of these effects were significant when compared to the control suggested that the treatment (acidifier and OTC) effects were not related to a decrease in bacterial counts or diversity but to changes in specific groups (microbial structure). This was confirmed by the fact that when the microbial profiles were analyzed with a much more sensitive technique as the PITChip, which allows the semi-quantitative assessment of changes in individual phylogenetic groups, the effect of the addition of organic acid blend to the diet of piglets was found to be significant at week 2, with mainly a significant increase in bacterial groups, in contrast to the lower total counts found. The lack of effect of treatment at week 4 could be because, as it has been described before [35, 37], gut microbiota stabilizes 3 to 4 weeks after weaning and therefore, the effect that the treatment could have had is not as outspoken as it was at week 2 when the microbiota was unbalanced because of the weaning transition. Furthermore, it should be kept in mind that the OTC treatment was in fact only exerted during the first week after weaning

The observed fact that a population related to *Lactobacillus paracasei* could be corresponding to the DGGE band that disappears at week 2 in the presence of antibiotic and has lower abundance in the presence of the acidifier but appeared again at week 4, could indicate that a population related to *L. paracasei* became resistant to OTC. The gain of resistance of several strains of this *Lactobacillus* specie has been observed before with tetracycline [51].

In conclusion, we showed that both treatments, the organic acid blend and OTC, had an overall non-significant effect on pig performance; however, both treatments, and most significantly that with the organic acid blend, induced significant changes in the microbial structure.

Acknowledgments

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References

1. **Wallgren, P. and Melin, L.** (2001) Weaning systems in relation to disease, in *The Weaner Pig*. Varley, M. A. and Wiseman, J. (ed.). CABI Publishing, UK.
2. **Kyriakis, S. C.** (1989) New aspects of the prevention and/or treatment of the major stress induced diseases of the early weaned piglet. *Pig News and Information*. 2: 177-181.
3. **Visek, W. J.** (1978) Mode of Growth Promotion by Antibiotics. *J. Anim. Sci.* 46: 1447-1469.
4. **Tollefson, L., Altekruse, S. F., and Potter, M. E.** (1997) Therapeutic antibiotics in animal feeds and antibiotic resistance. *Rev. Sci. Tech. Oie*. 16: 709-715.
5. **Collier, C. T., Smiricky-Tjardes, M. R., Albin, D. M., Wubben, J. E., Gabert, V. M., Deplancke, B., Bane, D., Anderson, D. B., and Gaskins, H. R.** (2003) Molecular ecological analysis of porcine ileal microbiota responses to antimicrobial growth promoters. *J. Anim. Sci.* 81: 3035-3045.
6. **Hays, V. W.** (1991) Effects of antibiotics, in *Growth Regulation in Farm Animals. Advances in Meat Research*. Pearson, A. M. and Dutson, T. R. (ed.). Elsevier Applied Science, New York.
7. **Anderson, D. B., McCracken, V. J., Aminov, R. I., Simpson, J. M., Mackie, R. I., Verstegen, M. W. A., and Gaskins, H. R.** (1999) Gut microbiology and growth-promoting antibiotics in swine. *Nutr. Abstr. Rev.* 70: 101-108.
8. **Niewold, T. A.** (2007) The nonantibiotic anti-inflammatory effect of antimicrobial growth promoters, the real mode of action? A hypothesis. *Poult. Sci.* 86: 605-609.
9. **Scipioni, R., Zaghini, G., and Biavati, B.** (1978) Researches on the Use of Acidified Diets for Early Weaning of Piglets. *Zootec. Nutr. Anim.* 4: 201-218.
10. **Mathew, A. G., Sutton, A. L., Scheidt, A. B., Forsyth, D. M., Patterson, J. A., and Kelly, D. T.** (1991) Effects of a Propionic-Acid Containing Feed Additive on Performance and Intestinal Microbial Fermentation of the Weanling Pig, in *Digestive Physiology in Pigs*. Verstegen, M. W. A., Huisman, J., and Denhartog, L. A. (ed.). p. 464-469.
11. **Partanen, K. H.** (2001) Organic acids - Their efficacy and modes of action in pigs, in *Gut Environment of Pigs*. Piva, A., Bach Knudsen, K. E., and Lindberg, J. E. (ed.). p. 201-217. Nottingham University Press, Nottingham.
12. **Partanen, K. H. and Mroz, Z.** (1999) Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12: 117-145.
13. **Roth, F. X. and Kirchgessner, M.** (1998) Organic acids as feed additives for young pigs: Nutritional and gastrointestinal effects. *J. Anim. Feed Sci.* 7: 25-33.
14. **Manzanilla, E. G., Nofrarias, M., Anguita, M., Castillo, M., Perez, J. F., Martin-Orue, S. M., Kamel, C., and Gasa, J.** (2006) Effects of butyrate, avilamycin, and a plant extract combination on the intestinal equilibrium of early-weaned pigs. *J. Anim. Sci.* 84: 2743-51.
15. **Nübel, U., Engelen, B., Felske, A., Snaidr, J., Wieshuber, A., Amann, R. I., Ludwig, W., and Backhaus, H.** (1996) Sequence heterogeneities of genes encoding 16S rRNA in *Paenibacillus polymixa* detected by temperature gradient gel electrophoresis. *J. Bacteriol.* 178: 5636-5643.
16. **Walter, J., Hertel, C., Tannock, G. W., Lis, C. M., Munro, K., and Hammes, W. P.** (2001) Detection of *Lactobacillus, Pediococcus, Leuconostoc*, and *Weissella* species in human feces by using group-specific PCR primers and denaturing gradient gel electrophoresis. *Appl. Environ. Microbiol.* 67: 2578-2585.
17. **Muyzer, G., de Waal, E. C., and Uitterlinden, G. A.** (1993) Profiling of complex populations by denaturing gradient gel electrophoresis analysis of polymerase chain reaction-amplified genes coding for 16S rRNA. *Appl. Environ. Microbiol.* 59: 695-700.
18. **Sanguinetti, C. J., Dias Neto, E., and Simpson, A. J. G.** (1994) Rapid silver staining and recovery of PCR products separated on polyacrylamide gels. *BioTechniques*. 17: 915-919.
19. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.

20. **Rajilić-Stojanović, M.** (2007) Diversity of the human gastrointestinal microbiota: novel perspectives from high throughput analyses, in *Laboratory of Microbiology*. Wageningen University, Wageningen, The Netherlands.
21. **Lane, D. J.** (1991) 16S/23S rRNA sequencing., in *Nucleic acid techniques in bacterial systematics*. Stackebrandt, E. and Goodfellow, M. (ed.). p. 115–175. J. Wiley & Sons, Chichester, United Kindom.
22. **Sambrook, J., Fritsch, E. F., and Maniatis, T.** (1989) Calculating melting temperatures for perfectly matched hybrids between oligonucleotides and their target sequences. Molecular cloning: a laboratory manual. Vol. 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.
23. **Suzuki, M. T., Taylor, L. T., and DeLong, E. F.** (2000) Quantitative analysis of small-subunit rRNA genes in mixed microbial populations via 5 '-nuclease assays. *Appl. Environ. Microbiol.* 66: 4605-4614.
24. **Byun, R., Nadkarni, M. A., Chhour, K. L., Martin, F. E., Jacques, N. A., and Hunter, N.** (2004) Quantitative analysis of diverse *Lactobacillus* species present in advanced dental caries. *J. Clin. Microbiol.* 42: 3128-3136.
25. **Matsuda, K., Tsuji, H., Asahara, T., Kado, Y., and Nomoto, K.** (2007) Sensitive Quantitative Detection of Commensal Bacteria by rRNA-Targeted Transcription-PCR. *Appl. Environ. Microbiol.* 73: 32-39.
26. **Carter, R. L.** (1989) On the partitioning of squared Euclidean distance and its application in cluster analysis. *Phsychometrika*. 54: 9-23.
27. **Lepš, J. and Šmilauer, P.** (2003) Multivariate analysis of ecological data using CANOCO. University Press, Cambridge.
28. **Gittins, R.** (1985) Canonical Analysis. A Review with Applications in Ecology. Springer-Verlag, Berlin.
29. **Simpson, E. H.** (1949) Measurements of diversity. *Nature*. 163: 688.
30. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
31. **Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., and Gasa, J.** (2006) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *J. Anim. Sci.* 84: 2725-34.
32. **Tsiloyiannis, V. K., Kyriakis, S. C., Vlemmas, J., and Sarris, K.** (2001) The effect of organic acids on the control of porcine post-weaning diarrhoea. *Res. Vet. Sci.* 70: 287-293.
33. **Hayashi, H., Takahashi, R., Nishi, T., Sakamoto, M., and Benno, Y.** (2005) Molecular analysis of jejunal, ileal, caecal and recto-sigmoidal human colonic microbiota using 16S rRNA gene libraries and terminal restriction fragment length polymorphism. *J. Med. Microbiol.* 54: 1093-1101.
34. **Janczyk, P., Pieper, R., Smidt, H., and Souffrant, W. B.** (2007) Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis. *FEMS Microbiol. Ecol.* 61: 132-140.
35. **Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8: 1191-1199.
36. **Inoue, R., Tsukahara, T., Nakanishi, N., and Ushida, K.** (2005) Development of the intestinal microbiota in the piglet. *J. Gen. Appl. Microbiol.* 51: 257-265.
37. **Jensen, B. B.** (1998) The impact of feed additives on the microbial ecology of the gut in young pigs. *J. Anim. Feed Sci.* 7: 45-64.
38. **Su, Y., Yao, W., Perez-Gutierrez, O. N., Smidt, H., and Zhu, W. Y.** (2008) Changes in abundance of Lactobacillus spp. and *Streptococcus suis* in the stomach, jejunum and ileum of piglets after weaning. *FEMS Microbiol. Ecol.* 66: 546-555.
39. **Pieper, R., Janczyk, P., Schumann, R., and Souffrant, W. B.** (2006) The intestinal microflora of piglets around weaning - with emphasis on lactobacilli. *Archiva Zootechnica*. 9: 28-40.
40. **Pieper, R., Janczyk, P., Zeyner, A., Smidt, H., Guiard, V., and Souffrant, W. B.** (2008) Ecophysiology of the developing total bacterial and Lactobacillus communities in the terminal small intestine of weaning piglets. *Microb. Ecol.* 56: 474-483.

41. **BuchholzCleven, B. E. E., Rattunde, B., and Straub, K. L.** (1997) Screening for genetic diversity of isolates of anaerobic Fe(II)-oxidizing bacteria using DGGE and whole-cell hybridization. *Syst. Appl. Microbiol.* 20: 301-309.
42. **Vallaeys, T., Topp, E., Muyzer, G., Macheret, V., Laguerre, G., Rigaud, A., and Soulas, G.** (1997) Evaluation of denaturing gradient gel electrophoresis in the detection of 16S rDNA sequence variation in rhizobia and methanotrophs. *FEMS Microbiol. Ecol.* 24: 279-285.
43. **Simpson, J. M., McCracken, V. J., White, B. A., Gaskins, H. R., and Mackie, R. I.** (1999) Application of denaturant gradient gel electrophoresis for the analysis of the porcine gastrointestinal microbiota. *J. Microbiol. Methods*. 36: 167-79.
44. **Engberg, R. M., Hedemann, M. S., Leser, T. D., and Jensen, B. B.** (2000) Effect of zinc bacitracin and salinomycin on intestinal microflora and performance of broilers. *Poult. Sci.* 79: 1311-1319.
45. **Knarreborg, A., Simon, M. A., Engberg, R. M., Jensen, B. B., and Tannock, G. W.** (2002) Effects of Dietary Fat Source and Subtherapeutic Levels of Antibiotic on the Bacterial Community in the Ileum of Broiler Chickens at Various Ages. *Appl. Environ. Microbiol.* 68: 5918-5924.
46. **Dumonceaux, T. J., Hill, J. E., Hemmingsen, S. M., and Van Kessel, A. G.** (2006) Characterization of intestinal microbiota and response to dietary virginiamycin supplementation in the broiler chicken. *Appl. Environ. Microbiol.* 72: 2815-23.
47. **Janzcyk, P., Pieper, R., Souffrant, W. B., Bimczok, D., Rothkötter, H. J., and Smidt, H.** (2007) Parenteral long-acting amoxicillin reduces intestinal bacterial community diversity in piglets even 5 weeks after the administration *ISME Journal*. 1: 180-183.
48. **Canibe, N., Steien, S. H., Overland, M., and Jensen, B. B.** (2001) Effect of K-diformate in starter diets on acidity, microbiota, and the amount of organic acids in the digestive tract of piglets, and on gastric alterations. *J. Anim. Sci.* 79: 2123-2133.
49. **Russell, J. B. and DiezGonzalez, F.** (1998) The effects of fermentation acids on bacterial growth, in *Adv. Microb. Physiol*.p. 205-234.
50. **Castillo, M., Martin-Orue, S. M., Nofrarias, M., Manzanilla, E. G., and Gasa, J.** (2007) Changes in caecal microbiota and mucosal morphology of weaned pigs. *Vet. Microbiol.* 124: 239-247.
51. **Comunian, R., Daga, E., Dupré, I., Paba, A., Devirgiliis, C., Piccioni, V., Perozzi, G., Zonenschain, D., Rebecchi, A., Morelli, L., De Lorentiis, A., and Giraffa, G.** (2010) Susceptibility to tetracycline and erythromycin of *Lactobacillus paracasei* strains isolated from traditional Italian fermented foods. *Int. J. Food Microbiol.* 138: 151-156.

Chapter

General Discussion and Concluding Remarks

Microbial changes at weaning

The main aim of the research described in this thesis was to provide new insights into the response of piglet's gastrointestinal (GI) tract microbiota to in-feed supplementation with possible alternatives to antibiotics used as growth promoters. Pig gut microbiota is a complex ecosystem that co-evolves with the pig from birth and that has a very important role in animal health. Host, diet and microbiota itself influence the dynamics of this microbiota (**Chapter 1**). During the suckling period the microbial community is fairly stable [1] but this changes during weaning, when dramatic disturbances can occur in this ecosystem unbalancing the microbial levels and leading to post-weaning GI tract disorders that can cause important economic losses in the pig industry [2, 3]. To overcome these problems, in-feed antibiotics have been used during many years in starter diets with the goal of lowering the presence of opportunistic pathogens and giving the commensal microbiota of the piglet the opportunity of recovering and establishing a natural barrier against these pathogens (colonization resistance) [4, 5]. However, the increasing number of cases of cross-resistance in humans provoked the ban of in-feed antimicrobials in animal production in January 2006 [6]. During the last years, many studies have been reported on the effect that weaning has on the piglet and its microbiota with the emphasis very often on the changes of *Lactobacillus* spp. populations [7-9]. This focus on lactobacilli is due to the probiotic effects that they are considered to have on the host, such as modulation of the GI tract ecosystem, antagonism against pathogenic bacteria and maintaining the intestinal mucosal barrier [10-14]. Another microorganism that is often associated with pigs is *Streptococcus suis* [15], considered to be the cause of several infections in pigs, causing diseases such as meningitis, pneumonia, septicemia and arthritis. Furthermore, it has been described as a human pathogen [16, 17]. **Chapter 2** describes the effect of weaning on bacterial community composition with a special attention to *Lactobacillus* spp. and *Streptococcus suis*. DGGE fingerprinting of PCR-amplified 16S rRNA gene fragments indicated that piglets suffered dramatic changes in the gastrointestinal bacterial community composition during the weaning transition, most probably due to dietary changes (from maternal milk to a solid feed), confirming previous published studies. For example, Konstantinov et al. described how the porcine microbiota develops during the weaning period, providing evidence for the appearance of potential pathogens when there is a microbial instability such as the one caused by weaning [18]. These results are supported by the strong increase in abundance of *S. suis* as reported in **Chapter 2**, detected both by DGGE and a qPCR assay developed in this work, to be able to quantify *S. suis* in the stomach, ileum and jejunum of piglets. Moreover a significant decrease of *Lactobacillus* spp. numbers was observed, again indicating the negative effect of the weaning process on a beneficial microbial group like lactobacilli, and in line with previous observations [7, 8, 18]. A dramatic decrease in lactobacilli counts was also found in **Chapter 5** that describes the dynamics of piglet ileum microbiota from 3 days before weaning until 15 days after weaning. Major changes in the *Lactobacillus* spp. numbers were observed during the weaning transition, reaching the lowest value 2 days after weaning.

Dietary modulation of the GI tract microbiota – A need for high-throughput diagnostic tools

The ban on the use of in-feed antibiotic growth promoters (AGP) has triggered the urge to search for alternatives that can improve animal health without having negative consequences on it, or in a later stage on humans, through the development and spread of bacterial populations resistant to one or multiple antibiotics. Nevertheless, this search has proven to be a difficult and time consuming task due to the broad range of causes and pathogens responsible for targeted intestinal disorders in addition to a lack of knowledge on the mechanism of action and minimum doses of alternative substances needed to have a positive effect. The common trait of all the additives tested until now is their potential to protect the gastrointestinal tract against the growth of pathogens. In general, these alternatives include substances such as amino acids, several mineral and organic acids (OA), prebiotics, probiotics and substances of plant origin including essential oils (EO) [19, 20].

In this thesis, microbial dynamics related to the addition to the diet of different potential alternative substances, representative of above-mentioned categories, has been studied both *in vitro* (**Chapter 4**) and *in vivo* (**Chapter 3**, **5**, **6** and **7**). In order to provide an overview of which PENS (plants and natural substances) and which minimum required doses have an effect on animal performance and microbiota dynamics, the *in vitro* trial described in **Chapter 4** was performed, screening a large number of different PENS with respect to their effect on microbial composition and fermentative activity.

Because of the high complexity of the gut microbiota, the inter-individual variation and large amount of samples, the identification of microbial changes related to dietary treatments requires the availability of high-throughput techniques. In **Chapter 3**, the development of a high-resolution, semi-quantitative and high-throughput tool was described with the aim of gaining novel insight in composition and dynamics of porcine GI tract microbiota. This tool is a phylogenetic microarray specific for the pig intestinal microbiota (PITChip), which was designed based on the SSU rRNA (small subunit ribosomal RNA) gene. For its development a non-redundant pig gastrointestinal tract microbiota database with in total 627 unique operational taxonomic units (OTU) was built. The OTUs were defined as groups of sequences with less than 98% identity with other SSU rRNA sequences in the database [21, 22]. Different phylogenetic microarrays based on the SSU rRNA gene, targeting different specific ecosystems, have been designed in the last years [23], such as the HITChip (targeting the human GI tract) that was used as a reference for the development of the PITChip [24]. Other examples are the MITChip (for mouse GI tract microbiota) (Derrien et al. in preparation), as well as diagnostic microarrays targeting sulfate-reducing bacteria [25] and microbes from the human oral cavity [26]. In line with data previously described for the HITChip, also the PITChip could be shown to provide high-resolution fingerprints as well as information on phylogenetic composition of porcine GI tract microbiota with superior technical reproducibility. In order to further validate the PITChip, data were compared with those previously published by us and other research groups on relative abundance of different taxonomic groups that constitute the porcine GI tract microbiota. Furthermore, to provide additional benchmarking, PITChip profiles were compared for several colon digesta samples with compositional data generated by 454 pyrosequencing

and quantitative PCR, as described in **Chapter 3.** It has previously been shown that microbiota compositional data generated by the HITChip and pyrosequencing of different variable regions of the 16S rRNA gene are in good agreement, especially at higher taxonomic levels above the family rank [27]. As expected, similar results were obtained here for the PITChip. Even though there were some differences observed between the results of the three analytical tools, such as differences in relative abundance of some groups, all the values agreed with those commonly found in the pig GI tract. As an example, the abundance of *Bacteroidetes* as observed by PITChip analysis was consistent to what was found in other pig studies [28, 29] but lower than in humans [30] and mice [31].

Organic acids

Organic acids are known to reduce the growth of some undesired bacteria such as coliforms and *Salmonella typhimurium* [32-34]. It has been hypothesized that organic acids can have the same role as the endogenous production of lactic acid, reducing growth depression during weaning when supplemented immediately post-weaning [35]. In addition sodium butyrate (SB) has been reported to modulate the gut microbiota [36].

In this thesis, several chapters describe the effect of organic acids on the gut microbiota. The effect of pre-weaning sodium butyrate administration was explored in **Chapter 6** while 3 different kinds of organic acid blends were addressed in **Chapter 3** and **Chapter 7**. Moreover **Chapter 7** compared the effect of the organic acid with a commonly used antibiotic (oxytetracycline). An increase in average daily gain was observed in previous studies in piglets receiving formic acid and potassium diformate [32, 34, 37]. Although one of the organic acid blends in **Chapter 3** contained formic acid, no effect on daily gain was observed, neither in pigs treated with sodium butyrate. Whereas the blend of organic acids described in **Chapter 7** did not significantly change average daily gain, it significantly reduced the incidence of diarrhea. Piglets treated with sodium butyrate experienced an increase of body weight, albeit not significant, in line with previous reports providing evidence that sodium butyrate can increase piglet's body weight [38, 39]. It could be the case that pigs have been kept under good hygienic conditions and in this case less effects of the organic acid can be expected [40]

The integration of the data of microbial analysis performed in above-mentioned chapters revealed a clear effect on microbial dynamics through the addition of different organic acids to the diet of weaned (**Chapter 3** and **7**) or unweaned piglets (**Chapter 6**). Changes observed occurred mainly just after the suckling period. Because in the different experiments the length of the supplementation of the organic acid was different, during the suckling period in **Chapter 6** and during the full experimental period in the other 2 trials, it cannot be concluded that the diminishing effects during some days after weaning was due to the end of the acid supplementation. Le Gall et al. investigated the effect of the period when sodium butyrate was supplemented, showing that when piglets received sodium butyrate during the suckling period only, the positive effect on growth rate was observed till the end of the experiment, confirming results described here [39]. Moreover, 2 of the 3 experiments indicated a higher effect of the organic acid in the small intestine than in the large intestine. This was observed with SB and with the 2 blends of acids described in **Chapter 3**. It is likely that this effect could be because

volatile fatty acids (VFA) are absorbed by the mucosal surfaces in the proximal GI tract at a fast speed, provoking a decrease of the organic acid from the stomach to the duodenum [41, 42]. Furthermore, Biagi et al. reported a lack of effect of sodium butyrate on intestinal morphology, confirming that the organic acid may be absorbed in the upper GI tract not having a strong effect on the distal intestine [43]. However, in **Chapter 7** the samples analyzed corresponded to fecal samples indicating that there was an effect as well on the large intestine. Another conclusion from integrating the data of **Chapter 6** and **Chapter 7** is that only sodium butyrate had a stimulating effect on the lactobacilli group. An increased number of *Lactobacillus* spp. was detected in the stomach and ileum of the weaned piglets after they were fed sodium butyrate, a finding that corroborates previous reports [44]. However, several other studies showed a decrease in lactobacilli when they used other acidifiers in the diet, such as a combination of lactic and formic acids [45], and benzoic acid [45, 46].

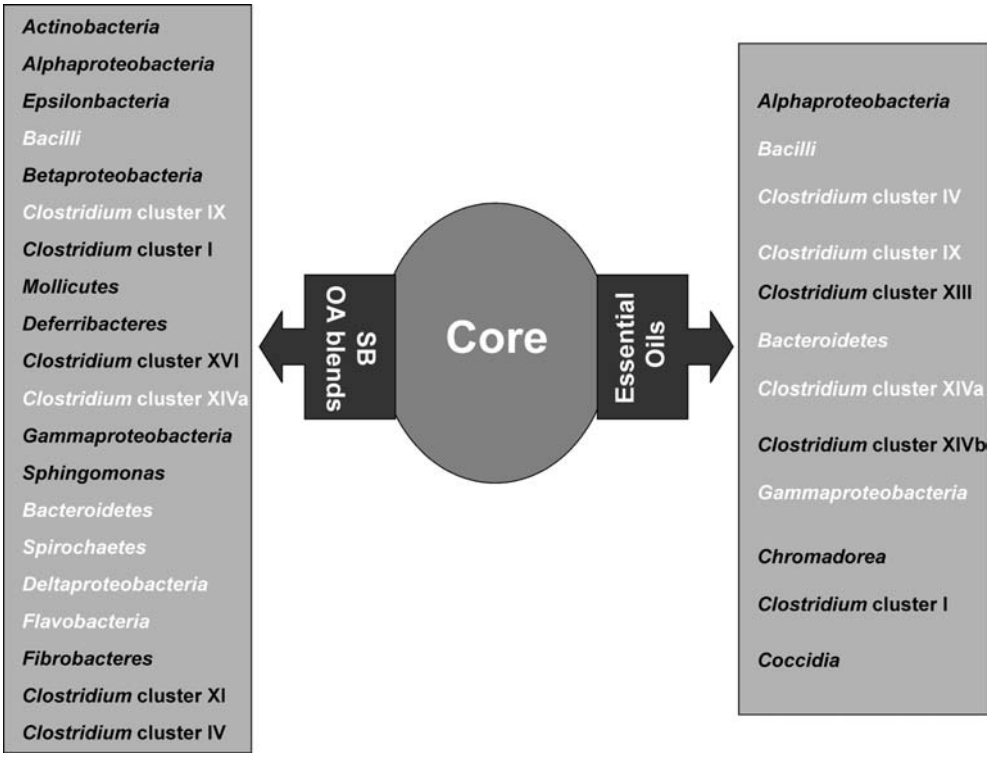

Figure 1. Diagram showing the bacterial groups affected significantly by the additives studied in this thesis. On the left, phylogenetic classes that increased or decreased with the addition of sodium butyrate or a blend of organic acids. On the right, phylogenetic classes that increased or decreased with the addition of essential oils (oregano oil or the active component carvacrol). Names in white are the groups affected at the same time by 2 or 3 additives of each class (organic acids and essential oils).

A generally observed effect of all the organic acids was a stabilization of the microbiota. This could indicate the beneficial properties of the organic acids as supplementation might be able to counteract microbiota instability specifically occurring around the time of weaning [1, 18, 47].

PITChip analysis allowed for the identification of the main microbial groups affected by the addition of organic acids to the diet of the piglets (Fig. 1). Different organic acids had a different effect on the microbial composition. Sodium butyrate caused a general decrease in abundance, i.e. corresponding probe hybridization signals, of those species that changed significantly. On the other hand, observed hybridization signals were significantly higher in animals receiving any of the 2 blends of organic acids used in **Chapter 3** and the same effect was observed for the organic acid blend of **Chapter 7**, indicating an increase in abundance of specific microbial groups. It could be hypothesized that while sodium butyrate has an antimicrobial effect on several of the microbial groups present in the pig GI tract [32, 34], the blends of organic acids seem to have a promoting effect on beneficial bacteria. This is in agreement with data presented by Gong et al. that reported an increase in *Lactobacillus* spp. when sodium butyrate was added to the diet [44]. It should be noted, however, that in the group of organisms that changed with the diet, we could identify both possible beneficial strains, like *Lactobacillus* [7], as well as potential pathogens.

Essential oils

Many essential oils are being studied as feed additives due to their antimicrobial properties [48-50]. To this end, **Chapter 4** describes the *in vitro* screening of several PENS, comprising different organic acids, essential oils, plant extracts and prebiotics. The only additive, however, that seemed to have a significant effect on the microbial composition at the concentration levels tested was an aqueous solution of oregano oil at a much higher concentration than levels used in the *in vivo* trials. **Chapter 5** reports the effect of carvacrol (active component of oregano oil) on ileal microbiota, when it was supplemented to piglets immediately post-weaning. Samples were taken at different time points from 3 days pre-weaning and during 2 weeks. A drawback from *in vivo* experiments with animals is the inherent limitation with respect to the follow up of the same animal in time due to the necessity to sacrifice them in order to collect intestinal samples beyond fecal material. In the experiment described in **Chapter 5** this problem was circumvented using cannulated animals, allowing the sampling of ileal content during certain periods. This, furthermore, allowed minimizing the effect of inter-individual variability. Essential oils proved to have an effect on the microbial composition, mainly reducing an important number of phylogenetic groups in their abundance, although for some groups also an increase in relative abundance could be observed. The main changes were observed mainly for groups belonging to the *Bacilli*, *Bacteroidetes* and several *Clostridium* clusters (I, IV, IX, XIII, XIVa and XIVb). This observation was in line with previous reports, stating that essential oils have an antimicrobial effect on both Gram-positive and Gram-negative bacteria [20]. Yet another study showed that Gram-positives are more sensitive to essential oils [51].

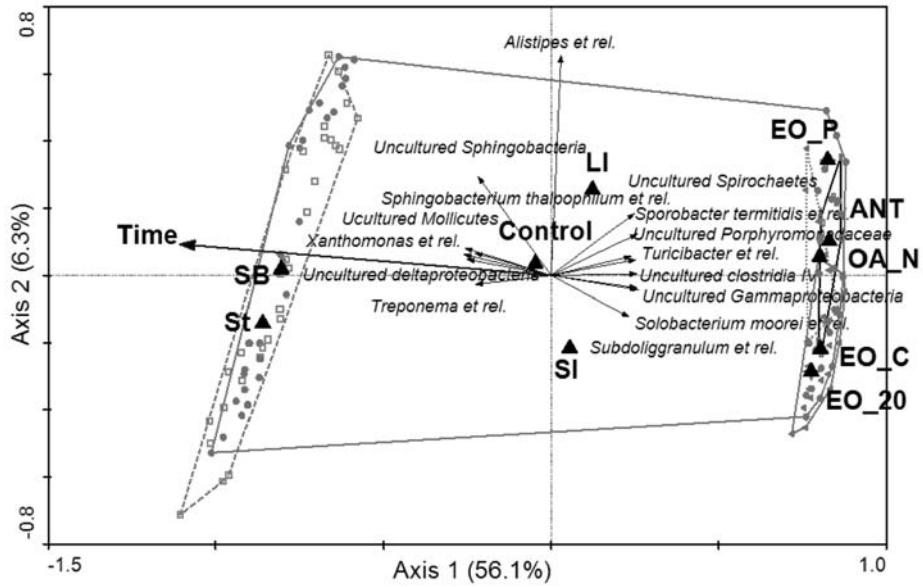

Figure 2. RDA triplot representing the distribution of treatments depending on the effect that they have on the microbial groups residing in the porcine GI tract. Summed hybridization signals of 144 probes were used. The percentages indicate the contribution of the axes to the explanation of the variance. Phylogenetic groups that appear, explained at least 60% of the variation and are represented as vectors. Environmental variables are centroids of the plot and time (quantitative variable is a vector). The different nominal variables that appear in the plot correspond to: SI (small intestine), LI (large intestine), St (stomach), Control (control), SB (sodium butyrate; **Chapter 6**), OA_N (organic acids blend; **Chapter 7**), ANT (oxytetracycline; **Chapter 7**), EO_C (carvacrol; **Chapter 5**), EO_20 (water-soluble oregano oil; **Chapter 4**), EO_P (pure oregano oil).

The kinetic parameters that characterized the *in vitro* fermentation assays described in **Chapter 4** further reinforced the antimicrobial effect of oregano oil. To this end, a lower cumulative gas production (OMCV) and organic matter loss (OMloss) pointed to a significant reduction of microbial activity; ammonia and VFA concentration were also decreased. Furthermore, carvacrol had a positive effect on *Lactobacillus* spp., increasing their numbers after weaning at an earlier time than in the control. Although the active component of the essential oil in both chapters was the same, the microbial groups shifted not exactly in the same way. This could be due to differences in experimental design, the main one that one experiment was *in vitro* while the other one was *in vivo*. Moreover, it should be noted that the inoculum used for the *in vitro* fermentation batch experiments described in **Chapter 4** was from feces collected 2 weeks after weaning, while in **Chapter 5** samples were from ileum and the supplementation started immediately after weaning. To this end, it is important to realize that time of sampling, especially in a dramatically dynamic period as that one around weaning, has an important effect on observed microbial shifts [1]. When comparing the results of carvacrol and the water solution of oregano oil, it appeared that although there were many changes on

microbial groups due to the addition of carvacrol, the overall effect was not significant, while in **Chapter 3**, the reported effect was stronger. This could be related with the inclusion levels of the two essential oils in the trial, being the concentration reported in **Chapter 3** much higher than in **Chapter 5** and than in previous studies [52].

When comparing those phylogenetic groups most significantly affected by the addition of either organic acids or essential oils, it appeared that these groups were in many cases members of the same class.

In **Chapters 5**, **6** and **7**, the effect of time on the development of piglet gut microbiota was studied as well, finding always a significant difference in time, with a common decrease on bacterial communities around weaning and a subsequent increase in time. This is in line with many studies, showing that especially around the time of weaning porcine intestinal microbiota is a highly dynamic ecosystem that can undergo extensive shifts [1, 7, 9, 53].

A final comparison of all the treatments together except the one described in **Chapter 3**, using Redundancy Analysis (RDA), showed a significant difference between the organic acid blends and sodium butyrate, confirming the effect mentioned above of the decrease in phylogenetic groups caused by sodium butyrate in contrast to the increase caused by the blend of acids (Fig. 2). These differences could be due to a synergistic effect of the acids. Also the effect of the blend was more similar to the effect of the antibiotic and the effect of the oregano oil, indicating a similar antimicrobial effect.

Future perspectives

In future studies, a new innovative approach is needed to be able to find new alternatives for in feed-antimicrobials. Up to now, we studied the effect of the additives on the host as well as on microbiota composition. A complex ecosystem like the GI tract can be viewed as an immense resource of functional genes directly involved in the maturation of intestinal tissues, in providing defenses against pathogens and in nutritional functions [54, 55]. Thus, the next step would be to study these genes and corresponding pathways involved in the process of pathogen recognition by the microbes and host response, in order to know more about their mode of action and be able to modulate intestinal homeostasis and immune function.

In order to have a good insight into this interplay between host and microbes, functional genomic approaches are needed. Pig DNA microarrays are available, making possible gene expression profiling providing a wealth of information on the molecular cross-talk between intestinal epithelial cells and microbes. Metagenomic sequencing has recently been described at massive scale by Qin et al. [56] together with the construction of a gene catalogue that allows studying associations between microbiota genes and mammalian host phenotypes. Another approach to study the functionality of individual members of the gut microbiota and their interactions with the host and among each other is Stable Isotope Probing [57-59].

Recent technological as well as conceptual developments, some of which truly revolutionizing the life sciences, now for the first time allow for the sound and comprehensive understanding of the development of GI tract function and its relation with the colonization of the GI tract by commensal and potentially pathogenic microbiota. This thesis provides a step towards the implementation of these new approaches, generating important information towards

sound design of sustainable dietary strategies aiming at the replacement of in feed antibiotics.

References

1. **Inoue, R., Tsukahara, T., Nakanishi, N., and Ushida, K.** (2005) Development of the intestinal microbiota in the piglet. *J. Gen. Appl. Microbiol.* 51: 257-265.
2. **Lallès, J. P., Bosi, P., Smidt, H., and Stokes, C. R.** (2007) Weaning - a challenge to gut physiologists. *Livest. Sci.* 108: 82-93.
3. **Lallès, J. P., Boudry, G., Favier, C., Le Floc'h, N., Lurona, I., Montagne, L., Oswald, I. P., Pie, S., Piel, C., and Seve, B.** (2004) Gut function and dysfunction in young pigs: physiology. *Anim. Res.* 53: 301-316.
4. **Sekirov, I. and Finlay, B. B.** (2009) The role of the intestinal microbiota in enteric infection. *J. Physiol. (Lond).* 587: 4159-4167.
5. **Dibner, J. J. and Richards, J. D.** (2005) Antibiotic growth promoters in agriculture: history and mode of action. *Poult. Sci.* 84: 634-643.
6. **Wegener, H. C.** (2003) Antibiotics in animal feed and their role in resistance development. *Curr. Opin. Microbiol.* 6: 439-445.
7. **Pieper, R., Janczyk, P., Schumann, R., and Souffrant, W. B.** (2006) The intestinal microflora of piglets around weaning - with emphasis on lactobacilli. *Archiva Zootechnica*. 9: 28-40.
8. **Pieper, R., Janczyk, P., Zeyner, A., Smidt, H., Guiard, V., and Souffrant, W. B.** (2008) Ecophysiology of the developing total bacterial and Lactobacillus communities in the terminal small intestine of weaning piglets. *Microb. Ecol.* 56: 474-483.
9. **Janczyk, P., Pieper, R., Smidt, H., and Souffrant, W. B.** (2007) Changes in the diversity of pig ileal lactobacilli around weaning determined by means of 16S rRNA gene amplification and denaturing gradient gel electrophoresis. *FEMS Microbiol. Ecol.* 61: 132-140.
10. **Konstantinov, S. R., Smidt, H., Akkermans, A. D. L., Casini, L., Trevisi, P., Mazzoni, M., Filippi, S. D., Bosi, P., and de Vos, W. M.** Abundance of *Lactobacillus sobrius* reduces *Escherichia coli* K88 levels in the gut and promotes growth of challenged piglets. *Submitted for publication*.
11. **Gupta, V. and Garg, R.** (2009) Probiotics. *Indian J. Med. Microbiol.* 27: 202-209.
12. **Martin, R., Delgado, S., Maldonado, A., Jimenez, E., Olivares, M., Fernandez, L., Sobrino, O. J., and Rodriguez, J. M.** (2009) Isolation of lactobacilli from sow milk and evaluation of their probiotic potential. *J. Dairy Res.* 76: 418-425.
13. **Roselli, M., Finamore, A., Britti, M. S., Konstantinov, S. R., Smidt, H., de Vos, W. M., and Mengheri, E.** (2007) The novel porcine *Lactobacillus sobrius* strain protects intestinal cells from enterotoxigenic *Escherichia coli* K88 infection and prevents membrane barrier damage. *J. Nutr.* 137: 2709-2716.
14. **Konstantinov, S. R., Smidt, H., Akkermans, A. D. L., Casini, L., Trevisi, P., Mazzoni, M., De Filippi, S., Bosi, P., and de Vos, W. M.** (2008) Feeding of *Lactobacillus sobrius* reduces Escherichia coli F4 levels in the gut and promotes growth of infected piglets. *FEMS Microbiol. Ecol.* 66: 599-607.
15. **Su, Y., Yao, W., Perez-Gutierrez, O., Smidt, H., and Zhu, W. Y.** (2008) 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets after oral administration of *Lactobacillus sobrius* S1. *Anaerobe*. 14: 78-86.
16. **Wertheim, H. F. L., Nguyen, H. N., Taylor, W., Lien, T. T. M., Ngo, H. T., Nguyen, T. Q., Nguyen, B. N. T., Nguyen, H. H., Nguyen, H. M., Nguyen, C. T., Dao, T. T., Nguyen, T. V., Fox, A., Farrar, J., Schultsz, C., Nguyen, H. D., Van Nguyen, K., and Horby, P.** (2009) Streptococcus suis, an Important Cause of Adult Bacterial Meningitis in Northern Vietnam. *PLoS ONE*. 4.
17. **Wertheim, H. F. L., Nghia, H. D. T., Taylor, W., and Schultsz, C.** (2009) Streptococcus suis: An Emerging Human Pathogen. *Clin. Infect. Dis.* 48: 617-625.
18. **Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., Akkermans, A. D. L., Smidt, H., and de Vos, W. M.** (2006) Post-natal development of the porcine microbiota composition and activities. *Environ. Microbiol.* 8: 1191-1199.
19. **Roselli, M., Britti, M. S., Le Huerou-Luron, I., Marfaing, H., Zhu, W. Y., and Mengheri, E.** (2007) Effect of different plant extracts and natural substances (PENS) against membrane damage induced by

enterotoxigenic Escherichia coli K88 in pig intestinal cells. *Toxicol. In Vitro*. 21: 224-9.

20. **Lalles, J. P., Bosi, P., Janczyk, P., Koopmans, S. J., and Torrallardona, D.** (2009) Impact of bioactive substances on the gastrointestinal tract and performance of weaned piglets: a review. *Animal*. 3: 1625-1643.
21. **Eckburg, P. B., Bik, E. M., Bernstein, C. N., Purdom, E., Dethlefsen, L., Sargent, M., Gill, S. R., Nelson, K. E., and Relman, D. A.** (2005) Diversity of the human intestinal microbial flora. *Science*. 308: 1635-1638.
22. **Gill, S. R., Pop, M., DeBoy, R. T., Eckburg, P. B., Turnbaugh, P. J., Samuel, B. S., Gordon, J. I., Relman, D. A., Fraser-Liggett, C. M., and Nelson, K. E.** (2006) Metagenomic analysis of the human distal gut microbiome. *Science*. 312: 1355-1359.
23. **Zoetendal, E. G., Rajilić-Stojanović, M., and de Vos, W. M.** (2008) High throughput diversity und functionality analysis of the gastrointestinal tract microbiota. *Gut*. 57: 1605-1615.
24. **Rajilic-Stojanovic, M., Heilig, H. G., Molenaar, D., Kajander, K., Surakka, A., Smidt, H., and de Vos, W. M.** (2009) Development and application of the human intestinal tract chip, a phylogenetic microarray: analysis of universally conserved phylotypes in the abundant microbiota of young and elderly adults. *Environ. Microbiol.* 11: 1736-1751.
25. **Loy, A., Lehner, A., Lee, N., Adamczyk, J., Meier, H., Ernst, J., Schleifer, K.-H., and Wagner, M.** (2002) Oligonucleotide microarray for 16S rRNA gene-based detection of all recognized lineages of sulfate-reducing prokaryotes in the environment. *Appl. Environ. Microbiol.* 68: 5064-5081.
26. **Smoot, L. M., Smoot, J. C., Smidt, H., Noble, P. A., Konneke, M., McMurry, Z. A., and Stahl, D. A.** (2005) DNA microarrays as salivary diagnostic tools for characterizing the oral cavity's microbial community. *Adv. Dent. Res*. 18: 6-11.
27. **Claesson, M. J., O'Sullivan, O., Wang, Q., Nikkila, J., Marchesi, J. R., Smidt, H., de Vos, W. M., Ross, R. P., and O'Toole, P. W.** (2009) Comparative Analysis of Pyrosequencing and a Phylogenetic Microarray for Exploring Microbial Community Structures in the Human Distal Intestine. *PLoS ONE*. 4: e6669.
28. **Guo, X., Xia, X., Tang, R., Zhou, J., Zhao, H., and Wang, K.** (2008) Development of a real-time PCR method for Firmicutes and Bacteroidetes in faeces and its application to quantify intestinal population of obese and lean pigs. *Lett. Appl. Microbiol.* 47: 367-373.
29. **Leser, T. D., Amenuvor, J. Z., Jensen, T. K., Lindecrona, R. H., Boye, M., and Moller, K.** (2002) Culture-Independent Analysis of Gut Bacteria: the Pig Gastrointestinal Tract Microbiota Revisited. *Appl. Environ. Microbiol.* 68: 673-690.
30. **Ley, R. E., Backhed, F., Turnbaugh, P., Lozupone, C. A., Knight, R. D., and Gordon, J. I.** (2005) Obesity alters gut microbial ecology. *Proc. Natl. Acad. Sci. U. S. A.* 102: 11070-11075.
31. **Ley, R. E., Turnbaugh, P. J., Klein, S., and Gordon, J. I.** (2006) Microbial ecology: Human gut microbes associated with obesity. *Nature*. 444: 1022-1023.
32. **Roselli, M., Finamore, A., Britti, M. S., Bosi, P., Oswald, I., and Mengheri, E.** (2005) Alternatives to in-feed antibiotics in pigs: Evaluation of probiotics, zinc or organic acids as protective agents for the intestinal mucosa. A comparison of in vitro and in vivo results. *Anim. Res.* 54: 203-218.
33. **Partanen, K. H.** (2001) Organic acids - Their efficacy and modes of action in pigs, in *Gut Environment of Pigs*. Piva, A., Bach Knudsen, K. E., and Lindberg, J. E. (ed.). p. 201-217. Nottingham University Press, Nottingham.
34. **Bannink, A., Dijkstra, J., Koopmans, S. J., and Mroz, Z.** (2006) Physiology, regulation and multifunctional activity of the gut wall: a rationale for multicompartmental modelling. *Nutr. Res. Rev.* 19: 227-253.
35. **Partanen, K. H. and Mroz, Z.** (1999) Organic acids for performance enhancement in pig diets. *Nutr. Res. Rev.* 12: 117-145.
36. **Castillo, M., Martin-Orue, S. M., Roca, M., Manzanilla, E. G., Badiola, I., Perez, J. F., and Gasa, J.** (2006) The response of gastrointestinal microbiota to avilamycin, butyrate, and plant extracts in early-weaned pigs. *J. Anim. Sci.* 84: 2725-34.

37. **Mroz, Z., Koopmans, S. J., Bannink, A., Partanen, A. K., Krasucki, W., Overland, M., and Radcliffe, S.** (2006) Carboxylin acids as bioregulators and gut growth promoters in non-ruminants, in *Biology of Nutrition in Growing Animals*. Mosenthin, R., Zentek, J., and Zebrowska, T. (ed.). p. 81-133. Elsevier Science, Amsterdam, The Netherlands.
38. **Mazzoni, M., Le Gall, M., De Filippi, S., Minieri, L., Trevisi, P., Wolinski, J., Lalatta-Costerbosa, G., Lalles, J. P., Guilloteau, P., and Bosi, P.** (2008) Supplemental sodium butyrate stimulates different gastric cells in weaned pigs. *J. Nutr.* 138: 1426-31.
39. **Le Gall, M., Gallois, M., Seve, B., Louveau, I., Holst, J. J., Oswald, I. P., Lalles, J. P., and Guilloteau, P.** (2009) Comparative effect of orally administered sodium butyrate before or after weaning on growth and several indices of gastrointestinal biology of piglets. *Br. J. Nutr.* 102: 1285-96.
40. **Houdijk, J.** (1998) Effect of non-digestible oligosaccharides in young pig diets, in *Animal nutrition*. Wageningen University, Wageningen, The Netherlands.
41. **Canibe, N., Hojberg, O., Hojsgaard, S., and Jensen, B. B.** (2005) Feed physical form and formic acid addition to the feed affect the gastrointestinal ecology and growth performance of growing pigs. *J. Anim. Sci.* 83: 1287-302.
42. **Hanzlik, R. P., Fowler, S. C., and Eells, J. T.** (2005) Absorption and elimination of formate following oral administration of calcium formate in female human subjects. *Drug Metab. Dispos.* 33: 282-286.
43. **Biagi, G., Piva, A., Moschini, M., Vezzali, E., and Roth, F. X.** (2007) Performance, intestinal microflora, and wall morphology of weanling pigs fed sodium butyrate. *J. Anim. Sci.* 85: 1184-91.
44. **Gong, J., Yu, H., Liu, T., Li, M., Si, W., De Lange, C. F. M., and Dewey, C.** (2008) Characterization of ileal bacterial microbiota in newly-weaned pigs in response to feeding lincomycin, organic acids or herbal extract. *Livest. Sci.* 116: 318-322.
45. **Guggenbuhl, P., Seon, A., Quintana, A. P., and Nunes, C. S.** (2007) Effects of dietary supplementation with benzoic acid (VevoVitall (R)) on the zootechnical performance, the gastrointestinal microflora and the ileal digestibility of the young pig. *Livest. Sci.* 108: 218-221.
46. **Kluge, H., Broz, J., and Eder, K.** (2006) Effect of benzoic acid on growth performance, nutrient digestibility, nitrogen balance, gastrointestinal microflora and parameters of microbial metabolism in piglets. *J. Anim. Physiol. Anim. Nutr.* 90: 316-324.
47. **Castillo, M., Martin-Orue, S. M., Nofrarias, M., Manzanilla, E. G., and Gasa, J.** (2007) Changes in caecal microbiota and mucosal morphology of weaned pigs. *Vet. Microbiol.* 124: 239-247.
48. **Janczyk, P., Pieper, R., Urubschurov, V., Wendler, K. R., and Souffrant, W. B.** (2009) Investigations on the Effects of Dietary Essential Oils and Different Husbandry Conditions on the Gut Ecology in Piglets after Weaning. *Int. J. Microbiol.*
49. **Janczyk, P., Trevisi, P., Souffrant, W. B., and Bosi, P.** (2008) Effect of thymol on microbial diversity in the porcine jejunum. *Int. J. Food Microbiol.* 126: 258-261.
50. **Peñalver, P., Huerta, B., Borge, C., Astorga, R., Romero, R., and Perea, A.** (2005) Antimicrobial activity of five essential oils against origin strains of the Enterobacteriaceae family. *APMIS*. 113: 1-6.
51. **Bozin, B., Mimica-Dukic, N., Simin, N., and Anackov, G.** (2006) Characterization of the volatile composition of essential oils of some lamiaceae spices and the antimicrobial and antioxidant activities of the entire oils. *J. Agric. Food Chem.* 54: 1822-1828.
52. **Busquet, M., Calsamiglia, S., Ferret, A., and Kamel, C.** (2006) Plant extracts affect in vitro rumen microbial fermentation. *J. Dairy Sci.* 89: 761-71.
53. **Su, Y., Yao, W., Perez-Gutierrez, O. N., Smidt, H., and Zhu, W. Y.** (2008) Changes in abundance of Lactobacillus spp. and *Streptococcus suis* in the stomach, jejunum and ileum of piglets after weaning. *FEMS Microbiol. Ecol.* 66: 546-555.
54. **Bäckhed, F., Ley, R. E., Sonnenburg, J. L., Peterson, D. A., and Gordon, J. I.** (2005) Host-bacterial mutualism in the human intestine. *Science*. 307: 1915-20.
55. **Hooper, L. V., Midtvedt, T., and Gordon, J. I.** (2002) How host-microbial interactions shape the nutrient environment of the mammalian intestine. *Annu. Rev. Nutr.* 22: 283-307.
56. **Qin, J. J., Li, R. Q., Raes, J., Arumugam, M., Burgdorf, K. S., Manichanh, C., Nielsen, T., Pons,**

N., Levenez, F., Yamada, T., Mende, D. R., Li, J. H., Xu, J. M., Li, S. C., Li, D. F., Cao, J. J., Wang, B., Liang, H. Q., Zheng, H. S., Xie, Y. L., Tap, J., Lepage, P., Bertalan, M., Batto, J. M., Hansen, T., Le Paslier, D., Linneberg, A., Nielsen, H. B., Pelletier, E., Renault, P., Sicheritz-Ponten, T., Turner, K., Zhu, H. M., Yu, C., Li, S. T., Jian, M., Zhou, Y., Li, Y. R., Zhang, X. Q., Li, S. G., Qin, N., Yang, H. M., Wang, J., Brunak, S., Dore, J., Guarner, F., Kristiansen, K., Pedersen, O., Parkhill, J., Weissenbach, J., Bork, P., Ehrlich, S. D., and Wang, J. A human gut microbial gene catalogue established by metagenomic sequencing. *Nature*. 464: 59-U70.

57. **Kovatcheva-Datchary, P., Zoetendal, E. G., Venema, K., de, V. W. M., and Smidt, H.** (2009) Review: Tools for the tract: understanding the functionality of the gastrointestinal tract. *Therap. Adv. Gastroenterol*. 4: 9-22.

58. **Egert, M., de Graaf, A. A., Smidt, H., de Vos, W. M., and Venema, K.** (2006) Beyond diversity: functional microbiomics of the human colon. *Trends Microbiol.* 14: 86-91.

59. **Kreuzer-Martin, H. W.** (2007) Stable isotope probing: Linking functional activity to specific members of microbial communities. *Soil Sci. Soc. Am. J.* 71: 611-619.

Appendix

Appendix

Table S1. List of 627 unique OTU's.

Full name (OTU= Level 3)	Level 0 (phylum)	Level 1 (class or *Clostridium* cluster)	Level 2(90% similarity)
Actinomyces suimastitidis	*Actinobacteria*	*Actinobacteria*	*Actinomyces* et rel.
Actinomyces hyovaginalis	*Actinobacteria*	*Actinobacteria*	*Actinomyces* et rel.
Aeriscardovia aeriphila	*Actinobacteria*	*Actinobacteria*	*Aeriscardovia* et rel.
uncultured bacterium	*Actinobacteria*	*Actinobacteria*	*Aeriscardovia* et rel.
Bifidobacterium minimum	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium pseudolongum subsp. *globosum*	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium psychraerophilum	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium sp. group II-3	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium longum subsp. *suis*	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium sp. BBDP69	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium boum	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium thermophilum	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium choerinum	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Bifidobacterium adolescentis ATCC 15703	*Actinobacteria*	*Actinobacteria*	*Bifidobacterium*
Collinsella aerofaciens	*Actinobacteria*	*Actinobacteria*	*Collinsella*
Corynebacterium glucuronolyticum	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Dietzia sp. BBDP49	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Mycobacterium sp. CHNTR34	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Corynebacterium sp. BBDP43	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Corynebacterium sp. BBDP60	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Rhodococcus sp. BBTR50	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Corynebacterium xerosis	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
swine manure bacterium 37-6	*Actinobacteria*	*Actinobacteria*	*Corynebacterium* et rel.
Eggerthella lenta	*Actinobacteria*	*Actinobacteria*	*Eggerthella* et rel.
Leucobacter sp. BBDP56	*Actinobacteria*	*Actinobacteria*	*Leucobacter*
Microbacterium sp. BBCT30	*Actinobacteria*	*Actinobacteria*	*Microbacterium*
Microbacterium sp. BBDP82	*Actinobacteria*	*Actinobacteria*	*Microbacterium*
Microbacterium sp. BBDP58	*Actinobacteria*	*Actinobacteria*	*Microbacterium*
uncultured bacterium	*Actinobacteria*	*Actinobacteria*	*Micrococcus* et rel.
Arthrobacter sp. BBTR33	*Actinobacteria*	*Actinobacteria*	*Micrococcus* et rel.
Brevibacterium sp. CHNDP32	*Actinobacteria*	*Actinobacteria*	*Micrococcus* et rel.
uncultured bacterium	*Actinobacteria*	*Actinobacteria*	*Olsenella* et rel.
Propionibacterium acnes	*Actinobacteria*	*Actinobacteria*	*Propionibacterium*
Propionibacterium granulosum	*Actinobacteria*	*Actinobacteria*	*Propionibacterium*
Tonsillophilus suis	*Actinobacteria*	*Actinobacteria*	*Tonsillophilus*
uncultured bacterium	*Actinobacteria*	*Actinobacteria*	Uncultured *Actinobacteria*
Streptomyces rubrocyanodiastaticus subsp. *piger*	*Actinobacteria*	*Actinobacteria*	Uncultured *Actinobacteria*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Alistipes* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Alistipes* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Alistipes* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Alistipes* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Alistipes* et rel.
Bacteroides coprosuis	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides coprosuis* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides distasonis* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides distasonis* et rel.
bacterium mpn-isolate group 6	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides distasonis* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides distasonis* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides fragilis* et rel.
Bacteroides uniformis	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides fragilis* et rel.
Bacteroides fragilis NCTC 9343	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides fragilis* et rel.
Bacteroides suis	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides pyogenes* et rel.
Bacteroides pyogenes	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides pyogenes* et rel.
bacterium mpn-isolate group 5	*Bacteroidetes*	*Bacteroidetes*	*Bacteroides vulgatus* et rel.
uncultured anaerobic bacterium	*Bacteroidetes*	*Bacteroidetes*	*Paludibacter propionicigenes* et rel.
Porphyromonas asaccharolytica	*Bacteroidetes*	*Bacteroidetes*	*Porphyromonas asaccharolytica* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Prevotella melaninogenica* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Prevotella melaninogenica* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Prevotella melaninogenica* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	*Prevotella melaninogenica* et rel.
Prevotella ruminicola	*Bacteroidetes*	*Bacteroidetes*	*Prevotella ruminicola* et rel.
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Bacteroidetes*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Bacteroidetes*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Bacteroidetes*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Porphyromonadaceae*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Porphyromonadaceae*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Porphyromonadaceae*
bacterium mpn-isolate group 3	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
bacterium mpn-isolate group 2	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*

Full name (OTU= Level 3)	**Level 0 (phylum)**	**Level 1 (class or *Clostridium* cluster)**	**Level 2(90% similarity)**
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
uncultured bacterium	*Bacteroidetes*	*Bacteroidetes*	Uncultured *Prevotella*
Chryseobacterium sp. BBTR48	*Bacteroidetes*	*Flavobacteria*	*Chryseobacterium* et rel.
Chryseobacterium sp. BBCT14	*Bacteroidetes*	*Flavobacteria*	*Chryseobacterium* et rel.
Chryseobacterium sp. CHNTR56	*Bacteroidetes*	*Flavobacteria*	*Chryseobacterium* et rel.
Chryseobacterium sp. BBCT19	*Bacteroidetes*	*Flavobacteria*	*Chryseobacterium* et rel.
bacterium SM3-6	*Bacteroidetes*	*Flavobacteria*	*Flavobacterium cucumis* et rel.
bacterium SM9-19	*Bacteroidetes*	*Flavobacteria*	*Myroides odoratus* et rel.
Bacteroidetes bacterium CHNCT12	*Bacteroidetes*	*Sphingobacteria*	*Sphingobacterium thalpophilum* et rel.
cilia-associated respiratory bacterium 96-1590	*Bacteroidetes*	*Sphingobacteria*	Uncultured *Sphingobacteria*
soil bacterium CHNTR54	*Bacteroidetes*	*Sphingobacteria*	Uncultured *Sphingobacteria*
Chlamydia suis	*Chlamydiae*	*Chlamydiae*	*Chlamydia*
uncultured bacterium	*Deferribacteres*	*Deferribacteres*	*Mucispirillum schaedleri* et rel.
Fibrobacter succinogenes	*Fibrobacteres*	*Fibrobacteres*	*Fibrobacter succinogenes* et rel.
Fibrobacter intestinalis	*Fibrobacteres*	*Fibrobacteres*	*Fibrobacter succinogenes* et rel.
Allofustis seminis	*Firmicutes*	*Bacilli*	*Allofustis*
Atopostipes suicloacalis	*Firmicutes*	*Bacilli*	*Atopostipes*
Bacillus pumilus	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
uncultured *Bacillus* sp.	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
Bacillus gelatini	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
swine effluent bacterium BBDP25	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
Bacillus sp. CHNTR52	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
Bacillus subtilis subsp. *subtilis*	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
Bacillus sp. KPU 0013	*Firmicutes*	*Bacilli*	*Bacillus* et rel.
Enterococcus durans	*Firmicutes*	*Bacilli*	*Enterococcus*
Enterococcus hirae	*Firmicutes*	*Bacilli*	*Enterococcus*
Enterococcus villorum	*Firmicutes*	*Bacilli*	*Enterococcus*
Enterococcus avium	*Firmicutes*	*Bacilli*	*Enterococcus*
Enterococcus sp. BBDP31	*Firmicutes*	*Bacilli*	*Enterococcus*
swine manure pit bacterium PPC9	*Firmicutes*	*Bacilli*	*Enterococcus*
Enterococcus faecium	*Firmicutes*	*Bacilli*	*Enterococcus*
uncultured bacterium	*Firmicutes*	*Bacilli*	*Gemella haemolysans* et rel.
Gemella haemolysans	*Firmicutes*	*Bacilli*	*Gemella haemolysans* et rel.
Lactobacillus johnsonii	*Firmicutes*	*Bacilli*	*Lactobacillus acidophilus* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus delbrueckii* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus delbrueckii* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus delbrueckii* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus delbrueckii* et rel.
Lactobacillus delbrueckii subsp. *bulgaricus* ATCC 1	*Firmicutes*	*Bacilli*	*Lactobacillus delbrueckii* et rel.
Lactobacillus amylovorus	*Firmicutes*	*Bacilli*	*Lactobacillus gasseri* et rel.
Lactobacillus crispatus	*Firmicutes*	*Bacilli*	*Lactobacillus gasseri* et rel.
Lactobacillus sobrius	*Firmicutes*	*Bacilli*	*Lactobacillus gasseri* et rel.
Lactobacillus acidophilus NCFM	*Firmicutes*	*Bacilli*	*Lactobacillus gasseri* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus paracasei* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus paracasei* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus paracasei* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Lactobacillus reuteri	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Pediococcus pentosaceus	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Lactobacillus mucosae	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Lactobacillus fermentum	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Lactobacillus brevis ATCC 367	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
Lactobacillus plantarum WCFS1	*Firmicutes*	*Bacilli*	*Lactobacillus plantarum* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
Lactobacillus agilis	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
Lactobacillus sp. BBDP73	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
Lactobacillus salivarius	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
Lactobacillus sp. 123B	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
Lactobacillus saerimneri	*Firmicutes*	*Bacilli*	*Lactobacillus salivarius* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Staphylococcus aureus* et rel.
Staphylococcus sp. CHNDP33	*Firmicutes*	*Bacilli*	*Staphylococcus aureus* et rel.
Staphylococcus sp. CHNDP23	*Firmicutes*	*Bacilli*	*Staphylococcus aureus* et rel.

Full name (OTU= Level 3)	**Level 0 (phylum)**	**Level 1 (class or *Clostridium* cluster)**	**Level 2(90% similarity)**
swine manure bacterium RT-22	*Firmicutes*	*Bacilli*	*Staphylococcus aureus* et rel.
Streptococcus equi subsp. *equi*	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus hyointestinalis	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus agalactiae	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
bacterium mpn-isolate group 12	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus pyogenes	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus ferus	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus uberis	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus porcinus	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus canis	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus equinus	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus dysgalactiae subsp. *dysgalactiae*	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
swine manure bacterium RT-18A	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus orisuis	*Firmicutes*	*Bacilli*	*Streptococcus bovis* et rel.
Streptococcus pneumoniae	*Firmicutes*	*Bacilli*	*Streptococcus intermedius* et rel.
Streptococcus intermedius	*Firmicutes*	*Bacilli*	*Streptococcus intermedius* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Streptococcus salivarius* et rel.
Streptococcus salivarius	*Firmicutes*	*Bacilli*	*Streptococcus salivarius* et rel.
Streptococcus alactolyticus	*Firmicutes*	*Bacilli*	*Streptococcus suis* et rel.
Streptococcus suis	*Firmicutes*	*Bacilli*	*Streptococcus suis* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Streptococcus suis* et rel.
swine manure bacterium RT-6B	*Firmicutes*	*Bacilli*	*Streptococcus suis* et rel.
Streptococcus thoraltensis	*Firmicutes*	*Bacilli*	*Streptococcus thoraltensis* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	*Streptococcus thoraltensis* et rel.
Tetragenococcus halophilus	*Firmicutes*	*Bacilli*	*Tetragenococcus*
uncultured bacterium	*Firmicutes*	*Bacilli*	*Turicibacter* et rel.
uncultured bacterium	*Firmicutes*	*Bacilli*	Uncultured *Bacilli*
swine effluent bacterium BBDP62	*Firmicutes*	*Bacilli*	Uncultured *Bacilli*
Carnobacterium sp. BBDP54	*Firmicutes*	*Bacilli*	Uncultured *Bacilli*
uncultured bacterium	*Firmicutes*	*Bacilli*	*Weissella* et rel.
Clostridium perfringens	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
Clostridium botulinum	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
swine manure bacterium RT-8B	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
swine manure pit bacterium PPC78A	*Firmicutes*	*Clostridium* cluster I	*Clostridium perfringens* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Anaerotruncus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium leptum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Clostridium leptum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Eubacterium cellulosi* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Eubacterium siraeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Eubacterium siraeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium prausnitzii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium prausnitzii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium prausnitzii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Faecalibacterium prausnitzii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Papillibacter cinnamivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Papillibacter cinnamivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Papillibacter cinnamivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus bromii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus bromii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.

Full name (OTU= Level 3)	Level 0 (phylum)	Level 1 (class or *Clostridium* cluster)	Level 2(90% similarity)
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
Ruminococcus flavefaciens	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
Ruminococcus flavefaciens	*Firmicutes*	*Clostridium* cluster IV	*Ruminococcus callidus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
Bacteroides capillosus	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Sporobacter termitidis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Subdoligramulum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	*Subdoligramulum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IV	Uncultured *Clostridia* IV
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Dialister* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Dialister* et rel.
Megasphaera elsdenii	*Firmicutes*	*Clostridium* cluster IX	*Megasphaera elsdenii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Megasphaera elsdenii* et rel.
bacterium mpn-isolate group 9	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
Mitsuokella multiacida	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
Anaerovibrio lipolyticus	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
Selenomonas ruminantium	*Firmicutes*	*Clostridium* cluster IX	*Mitsuokella multiacida* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Peptococcus niger* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Phascolarctobacterium faecium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Phascolarctobacterium faecium* et rel.
Acidaminococcus fermentans	*Firmicutes*	*Clostridium* cluster IX	*Phascolarctobacterium faecium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Phascolarctobacterium faecium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster IX	*Veillonella*
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
swine manure bacterium 37-2	*Firmicutes*	*Clostridium* cluster XI	*Anaerovorax* et rel.
Eubacterium tenue	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.
Peptostreptococcus anaerobius	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.

Full name (OTU= Level 3)	**Level 0 (phylum)**	**Level 1 (class or *Clostridium* cluster)**	**Level 2(90% similarity)**
Clostridium sp. zx7	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.
swine manure bacterium RT-8A	*Firmicutes*	*Clostridium* cluster XI	*Clostridium difficile* et rel.
swine effluent bacterium CHNDP11	*Firmicutes*	*Clostridium* cluster XIII	*Peptoniphilus indolicus* et rel.
swine effluent bacterium CHNDP1	*Firmicutes*	*Clostridium* cluster XIII	*Peptoniphilus indolicus* et rel.
swine manure bacterium 37-5	*Firmicutes*	*Clostridium* cluster XIII	*Peptoniphilus indolicus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Bryantella* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Butyrivibrio crossotus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Butyrivibrio crossotus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Butyrivibrio crossotus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Butyrivibrio crossotus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Butyrivibrio crossotus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
Clostridium herbivorans	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium herbivorans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium hylemonae* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium lactifermentans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium lactifermentans* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium nexile* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium nexile* et rel.
Hespellia porcina	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium oroticum* et rel.
Hespellia stercorisuis	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium oroticum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium sphenoides* et rel.
Clostridium hathewayi	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium sphenoides* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium symbosium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium symbosium* et rel.
Clostridium symbiosum	*Firmicutes*	*Clostridium* cluster XIVa	*Clostridium symbosium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Coprococcus eutactus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Coprococcus eutactus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Coprococcus eutactus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Coprococcus eutactus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Coprococcus eutactus* et rel.
bacterium mpn-isolate group 16	*Firmicutes*	*Clostridium* cluster XIVa	*Dorea* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Dorea* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Dorea* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium hallii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium hallii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium hallii* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium plexicaudatum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium plexicaudatum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium plexicaudatum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
Butyrivibrio fibrisolvens	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
Eubacterium rectale	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium rectale* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium ventriosum* et rel.
Eubacterium ventriosum	*Firmicutes*	*Clostridium* cluster XIVa	*Eubacterium ventriosum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnobacillus bovis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnobacillus bovis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnobacillus bovis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnobacillus bovis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.

Full name (OTU= Level 3)	Level 0 (phylum)	Level 1 (class or *Clostridium* cluster)	Level 2(90% similarity)
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
Eubacterium eligens	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
Lachnospira pectinoschiza	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Lachnospira pectinoschiza* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Roseburia intestinalis* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus ganvus* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus ganvus* et rel.
bacterium mpn-isolate group 18	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
bacterium mpn-isolate group 19	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
Ruminococcus productus	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
Clostridium sp. CCUG 52336	*Firmicutes*	*Clostridium* cluster XIVa	*Ruminococcus obeum* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVa	Uncultured *Clostridia* XIVa
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XIVb	Uncultured *Clostridia* XIVb
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XV	*Eubacterium* et rel.
Eubacterium sp. BBDP67	*Firmicutes*	*Clostridium* cluster XV	*Eubacterium* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVI	*Eubacterium biforme* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVI	*Eubacterium biforme* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVI	*Eubacterium biforme* et rel.
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVII	*Catenibacterium*
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVII	*Catenibacterium*

Appendix

Full name (OTU= Level 3)	Level 0 (phylum)	Level 1 (class or *Clostridium* cluster)	Level 2(90% similarity)
uncultured bacterium	*Firmicutes*	*Clostridium* cluster XVII	*Catenibacterium*
Acholeplasma granularum	*Firmicutes*	*Mollicutes*	*Acholeplasma* et rel.
Acholeplasma cavigenitalium	*Firmicutes*	*Mollicutes*	*Acholeplasma* et rel.
uncultured bacterium	*Firmicutes*	*Mollicutes*	*Bulleidia moorei* et rel.
Erysipelothrix rhusiopathiae	*Firmicutes*	*Mollicutes*	*Erysipelothrix*
Erysipelothrix sp. Oita0548	*Firmicutes*	*Mollicutes*	*Erysipelothrix*
Erysipelothrix sp. Chiba9393	*Firmicutes*	*Mollicutes*	*Erysipelothrix*
Mycoplasma hyopharyngis	*Firmicutes*	*Mollicutes*	*Mycoplasma*
Mycoplasma hyosynoviae	*Firmicutes*	*Mollicutes*	*Mycoplasma*
Mycoplasma flocculare	*Firmicutes*	*Mollicutes*	*Mycoplasma*
Mycoplasma synoviae 53	*Firmicutes*	*Mollicutes*	*Mycoplasma*
Mycoplasma hyopneumoniae J	*Firmicutes*	*Mollicutes*	*Mycoplasma*
uncultured bacterium	*Firmicutes*	*Mollicutes*	*Solobacterium moorei* et rel.
uncultured bacterium	*Firmicutes*	*Mollicutes*	*Solobacterium moorei* et rel.
uncultured bacterium	*Firmicutes*	*Mollicutes*	Uncultured *Mollicutes*
Fusobacterium necrophorum	*Fusobacteria*	*Fusobacteria*	*Fusobacterium*
uncultured bacterium	*Planctomycetes*	*Planctomycetacia*	Uncultured *Planctomycetacia*
Brevundimonas sp. BBDP1024	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacter* et rel.
Brevundimonas sp. CHNTR43	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacter* et rel.
Caulobacter sp. BBCT11	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacter* et rel.
Caulobacter sp. BBCT22	*Proteobacteria*	*Alphaproteobacteria*	*Caulobacter* et rel.
uncultured bacterium	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Rhizobium sp. CHNTR53	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Ochrobactrum sp. CHNTR29	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Rhizobium sp. BBTR4	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Pseudaminobacter sp. CHNTR41	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Sinorhizobium sp. BBCT64	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
alpha proteobacterium BBTR41	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Labrys methylaminiphilus	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Rhizobium sp. CHNTR26	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
Kaistia sp. BBTR58	*Proteobacteria*	*Alphaproteobacteria*	*Labrys methylaminiphilus* et rel.
uncultured bacterium	*Proteobacteria*	*Alphaproteobacteria*	*Oceanospirillum* et rel.
uncultured anaerobic bacterium	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacter* et rel.
Rhodobacter sp. TUT3733	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacter* et rel.
Paracoccus sp. BBTR62	*Proteobacteria*	*Alphaproteobacteria*	*Rhodobacter* et rel.
Sphingomonas sp. CHNTR37	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonas* et rel.
Sphingomonas sp. BBCT20	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonas* et rel.
Sphingomonas sp. BBCT69	*Proteobacteria*	*Alphaproteobacteria*	*Sphingomonas* et rel.
uncultured bacterium	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Burkholderia fungorum	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Psendomonas sp. BBCT8	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Denitrobacter sp. CHNCT17	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Herbaspirillum sp. CHNTR44	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Burkholderia sp. CHNCT3	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Variovorax sp. BBCT26	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
bacterium SM2-6	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Alcaligenes sp. BBTR16	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Bordetella bronchiseptica	*Proteobacteria*	*Betaproteobacteria*	*Bordetella* et rel.
Uruburuella suis	*Proteobacteria*	*Betaproteobacteria*	*Neisseria* et rel.
Oxalobacteraceae bacterium CHNTR40	*Proteobacteria*	*Betaproteobacteria*	*Oxalobacter* et rel.
uncultured bacterium	*Proteobacteria*	*Betaproteobacteria*	*Sutterella wadsorthia* et rel.
uncultured bacterium	*Proteobacteria*	*Betaproteobacteria*	*Sutterella wadsorthia* et rel.
uncultured bacterium	*Proteobacteria*	*Betaproteobacteria*	Uncultured *Betaproteobacteria*
uncultured bacterium	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrio* et rel.
Desulfovibrio piger	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrio* et rel.
Lawsonia intracellularis	*Proteobacteria*	*Deltaproteobacteria*	*Desulfovibrio* et rel.
uncultured bacterium	*Proteobacteria*	*Deltaproteobacteria*	Uncultured *Deltaproteobacteria*
Arcobacter sp. 16389	*Proteobacteria*	*Epsilonproteobacteria*	*Arcobacter*
Arcobacter cryaerophilus	*Proteobacteria*	*Epsilonproteobacteria*	*Arcobacter*
Campylobacter hyointestinalis subsp. *lawsonii*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
Campylobacter mucosalis	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
uncultured bacterium	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
Campylobacter lanienae	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
Campylobacter coli	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
Campylobacter hyointestinalis subsp. *hyointestinal*	*Proteobacteria*	*Epsilonproteobacteria*	*Campylobacter*
Candidatus Helicobacter suis	*Proteobacteria*	*Epsilonproteobacteria*	*Candidatus* Helicobacter
Candidatus Helicobacter heilmannii	*Proteobacteria*	*Epsilonproteobacteria*	*Candidatus* Helicobacter
Helicobacter canadensis	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
uncultured bacterium	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
Helicobacter sp.	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
Helicobacter rappini	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
Helicobacter rappini	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
Helicobacter sp. 'B52D Seymour'	*Proteobacteria*	*Epsilonproteobacteria*	*Helicobacter*
bacterium SM4-6	*Proteobacteria*	*Gammaproteobacteria*	*Acitenobacter* et rel.
Actinobacillus minor	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus minor	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus porcinus	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus minor	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus rossii	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus indolicus	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Mannheimia sp. BA597/9	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus equuli subsp. *equuli*	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.

Full name (OTU= Level 3)	Level 0 (phylum)	Level 1 (class or *Clostridium* cluster)	Level 2(90% similarity)
Actinobacillus porcinus	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus porcitonsillarum	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Pasteurella caballi	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus indolicus	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Actinobacillus rossii	*Proteobacteria*	*Gammaproteobacteria*	*Actinobacillus* et rel.
Avibacterium gallinarum	*Proteobacteria*	*Gammaproteobacteria*	*Avibacterium*
Bisgaard Taxon 10	*Proteobacteria*	*Gammaproteobacteria*	Bisgaard
Yersinia aleksiciae	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
uncultured bacterium	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
Escherichia sp. BBDP20	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
Shigella sp. BBDP15	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
Serratia sp. BBTR54	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
swine manure bacterium RT-2D	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
swine manure bacterium 37-11	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
Klebsiella pneumoniae	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
swine manure bacterium RT-5C	*Proteobacteria*	*Gammaproteobacteria*	*Escherichia coli* et rel.
Pasteurella aerogenes	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurella*
Pasteurella multocida subsp. *gallicida*	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurella*
Pasteurella multocida subsp. *multocida*	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurella*
Pasteurella mairii	*Proteobacteria*	*Gammaproteobacteria*	*Pasteurella*
Pseudomonas sp. CHNTR36	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonas* et rel.
Pseudomonas sp. BBTR5	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonas* et rel.
Pseudomonas sp. CHNCT24	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonas* et rel.
Pseudomonas sp. BBTR25	*Proteobacteria*	*Gammaproteobacteria*	*Pseudomonas* et rel.
Psychrobacter sp. CHNDP34	*Proteobacteria*	*Gammaproteobacteria*	*Psychrobacter* et rel.
uncultured bacterium	*Proteobacteria*	*Gammaproteobacteria*	*Psychrobacter* et rel.
Ruminobacter amylophilus	*Proteobacteria*	*Gammaproteobacteria*	*Ruminobacter* et rel.
uncultured bacterium	*Proteobacteria*	*Gammaproteobacteria*	Uncultured *Gammaproteobacteria*
uncultured bacterium	*Proteobacteria*	*Gammaproteobacteria*	Uncultured *Gammaproteobacteria*
uncultured *Chromatiaceae* bacterium	*Proteobacteria*	*Gammaproteobacteria*	Uncultured *Gammaproteobacteria*
Vibrio furnissii	*Proteobacteria*	*Gammaproteobacteria*	*Vibrio* et rel.
Stenotrophomonas sp. BBTR57	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Ignatzschineria sp. CHNDP40	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
swine effluent bacterium CHNDP41	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Luteimonas sp. CHNTR31	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Dyella sp. CHNCT5	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Xanthomonas retroflexus	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Lysobacter sp. BBCT65	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Pseudoxanthomonas sp. CHNTR38	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Rhodanobacter sp. CHNTR45	*Proteobacteria*	*Gammaproteobacteria*	*Xanthomonas* et rel.
Brachyspira intermedia	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Brachyspira innocens	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Brachyspira murdochii	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Brachyspira suanatina	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Brachyspira pilosicoli	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Brachyspira hyodysenteriae	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
swine intestinal spirochete	*Spirochaetes*	*Spirochaetes*	*Brachyspira*
Leptospira fainei	*Spirochaetes*	*Spirochaetes*	*Leptospira*
Candidatus Treponema suis	*Spirochaetes*	*Spirochaetes*	*Treponema* et rel.
uncultured bacterium	*Spirochaetes*	*Spirochaetes*	*Treponema* et rel.
Treponema succinifaciens	*Spirochaetes*	*Spirochaetes*	*Treponema* et rel.
Turneriella parva	*Spirochaetes*	*Spirochaetes*	*Turneriella*
uncultured bacterium	*Spirochaetes*	*Spirochaetes*	Uncultured *Spirochaetes*

Supplements

Supplements

Summary

The pig gastrointestinal (GI) tract ecosystem is inhabited by a complex, dynamic and diverse microbiota since birth. This microbiota has a key role in host health by forming a barrier against pathogens, supplying the pig with nutrients, stimulating the immune system, modulating gene host expression and contributing to gut morphology. The microbiota colonizes the pig at birth and co-evolves with its host till reaching a dynamic community characteristic for each individual. However, during this process there are events that challenge the integrity of the microbiota, being the most important one the weaning period, leading to an important imbalance in the composition and activity of the GI tract microbial community.

Modulation of the microbial composition and activity is possible through the feed due to the fact that gut microbes use substrates provided by the diet as main source of growth substrates. Thus, in order to prevent and overcome gut disorders caused by weaning, several feeding strategies have been evaluated during several years.

This thesis presents the knowledge acquired during the study of the response of piglet GI tract microbiota to the supplementation with different feed additives, in combination with the development of a high-throughput diagnosis tool instrumental for the comprehensive understanding of microbial dynamics. A wide range of plant extracts and other natural substances are presented, with emphasis on representatives from the categories organic acids (sodium butyrate and blends of different organic acids) and essential oils (oregano oil as well as its active ingredient carvacrol), describing microbial changes related to the supplementation of these additives. The application of cultivation-independent molecular tools together with the developed phylogenetic microarray, PITChip (Pig Intestinal Tract Chip), allowed us to follow microbial changes in time and in response to the dietary supplements, confirming that the studied organic acids and essential oil have an impact on the gut microbiota and concomitant effect on the animal performance in the following days after weaning. Moreover, an overall view is provided of all the microbial changes triggered by the different feeding strategies in order to assess to what extent common and/or different microbial groups were mainly affected, providing important information towards sound design of sustainable dietary strategies aiming at the replacement of in feed antibiotics.

High-throughput approaches are now available for investigating the global impact of exogenous factors on the GI tract microbiota and the consequences, and underlying cellular and molecular mechanisms at the levels of the gut epithelium, immune cells and microbial functionality. This will not only allow assessing compositional and functional dynamics of the developing microbiota, but will contribute to further expanding our current knowledge on the interplay between the pig host, its diet and the GI tract microbiota.

Samenvatting

Het maag-darmstelsel van het varken bevat, al vanaf de geboorte, een complexe, dynamische en gevarieerde microbiota. Deze microbiota speelt een sleutelrol bij de gezondheid van het varken door het vormen van een barriere tegen pathogenen, het geven van nutriënten aan het varken, het stimuleren van het immuunsysteem en het bijdragen aan de morfologie van de darm. De microbiota vormt zich in het varken bij de geboorte en co-evolueert met zijn gastheer tot een dynamische en unieke microbiota. Echter, tijdens dit proces kunnen er gebeurtenissen plaatsvinden die de integriteit van de microbiota op de proef stellen, wat kan leiden tot het uit balans raken van de samenstelling en activiteit van de microbiota van het maag-darmstelsel. De belangrijkste gebeurtenis in het leven van het varken die dit teweeg kan brengen is de periode na het spenen.

Verandering van de microbiële samenstelling en activiteit als gevolg van de voeding is mogelijk vanwege het feit dat darmbacteriën de substraten, die door het voer worden geleverd, gebruiken als belangrijkste bron voor hun groei. Vandaar dat al een aantal jaren verschillende voerstrategiën zijn bestudeerd om te kijken of ze darmstoornissen kunnen voorkomen en ondervangen die veroorzaakt worden door het spenen.

Dit proefschrift presenteert de kennis die verkregen is in de studie naar het effect van verschillende voedingssupplementen op de microbiota van het maag-darmstelsel in biggen, in combinatie met de ontwikkeling van een methode gebaseerd op DNA microarrays voor high-throughput diagnose om een grondig inzicht te krijgen in de microbiële dynamica. Een grote verscheidenheid aan plantenextracten en andere natuurlijke stoffen worden gepresenteerd, met de nadruk op een aantal organische zuren (natrium butyraat en mengsels van diverse organische zuren) en etherische oliën (oregano olie alsmede het actieve ingredient hiervan, carvacrol), waarbij de microbiële veranderingen als gevolg van het toedienen van deze supplementen worden beschreven. De geïntegreerde toepassing van verscheidene moleculaire technieken, waarbij geen kweken gebruikt hoeven te worden, waaronder de ontwikkelde fylogenetische microarray, PITChip (Pig Intestinal Tract Chip), stelde ons in staat om de microbiële veranderingen te volgen in de tijd en als gevolg van de voedingssupplementen. Dit bevestigde de invloed van de bestudeerde organische zuren en etherische oliën op de darm microbiota en het bijkomend effect op de prestaties van het dier in de dagen vlak na het spenen. Bovendien wordt een algemeen beeld gegeven van alle microbiële veranderingen die teweeg zijn gebracht door de verschillende voerstrategiën om vast te kunnen stellen in welke mate algemene en/ of verschillende microbiële groepen voornamelijk beïnvloed werden. Dit geeft belangrijke informatie voor een gedegen ontwerp van duurzame voerstrategieën die gericht zijn op het vervangen van antibiotica in de voeding.

High-throughput benaderingen zijn nu beschikbaar voor het onderzoeken van het algemene effect van exogene factoren op de microbiota van het maag-darmstelsel en de gevolgen en onderliggende cellulaire en moleculaire mechanismen op het niveau van het darm epiteel, immuun cellen en microbiële functionaliteit. Dit stelt ons niet alleen in staat om de compositionele en functionele dynamica vast te stellen van de ontwikkelende microbiota, maar het draagt ook bij aan een verdere ontwikkeling van onze huidige kennis van de interactie tussen het varken als gastheer, zijn dieet en de microbiota van zijn maag-darmstelsel.

Resumen

El tracto gastrointestinal del cerdo es un ecosistema colonizado, desde el nacimiento, por una microbiota compleja, dinámica y diversa. Esta microbiota juega un papel clave en la salud del hospedador, ya que forma una barrera contra organismos patógenos, suplementa al cerdo con nutrientes, estimula el sistema inmune, modula la expresión genética del hospedador y contribuye a la morfología del sistema digestivo. La microbiota coloniza el tracto gastrointestinal del cerdo durante su nacimiento y evoluciona con éste hasta alcanzar una comunidad dinámica que es característica de cada individuo. Sin embargo, durante este proceso hay eventos que ponen en riesgo la integridad de la microbiota intestinal, siendo uno de los más importantes el proceso de destete, el cual provoca un desequilibrio importante en la composición y actividad de la comunidad microbiana del tracto gastrointestinal.

Es posible modular la composición y actividad microbiana mediante la alimentación, ya que las bacterias presentes en el intestino utilizan como fuente principal de sustratos para el crecimiento aquellos presentes en la dieta del hospedador. Por esta razón, y con el fin de prevenir y superar los desarreglos intestinales causados por el destete, se han evaluado en los últimos años diferentes estrategias alimenticias.

Esta tesis describe el estudio del comportamiento de la microbiota del tracto gastrointestinal de los lechones, al suministrar diferentes aditivos en la dieta. Además, explica el desarrollo de una técnica de diagnostico de alta resolución o "high-throughput" utilizada para comprender la dinámica de la composición de la microbiota intestinal. Este estudio utiliza una gran variedad de extractos de plantas y otras substancias naturales, especialmente representantes de las categorías de los ácidos orgánicos (butirato sódico y mezclas de diferentes ácidos orgánicos) y los aceites esenciales (aceite de orégano así como su ingrediente activo, carvacrol). También hacemos una descripción de los cambios microbianos ocurridos por la adición de estos compuestos en la dieta. Estudiamos los cambios microbianos desarrollados a lo largo del tiempo en respuesta a los aditivos alimenticios mediante el uso de técnicas moleculares (cultivo-independientes) y el "microchip" filogenético diseñado en esta tesis (PITChip, siglas del inglés "Pig Intestinal Tract Chip"). Estas técnicas confirmaron que los ácidos orgánicos y aceites esenciales estudiados en esta tesis afectan a la microbiota intestinal, y por lo tanto al rendimiento del animal durante los días posteriores al destete. Asimismo, se presenta un resumen de todos los cambios microbianos provocados por las diferentes estrategias alimenticias, para identificar aquellos grupos principales afectados (comunes o diferentes), proporcionando información esencial para el diseño de nuevas estrategias alimenticias dirigidas a la sustitución de antibióticos utilizados en las dietas porcinas.

Actualmente existen varias técnicas "high-throughput" disponibles para el estudio del efecto que distintos factores externos ejercen en la composición de la microbiota del tracto gastrointestinal, además de los mecanismos moleculares a nivel de epitelio intestinal, células inmunes y función microbiana. Esto nos permitirá evaluar tanto la dinámica en la composición y la función de la microbiota en desarrollo, como la interacción entre el cerdo como hospedador, la dieta y la microbiota que habita su tracto gastrointestinal.

About the author

Odette Pérez Gutiérrez was born in Barcelona (Spain) on 11th June 1975. After finishing her elementary education she started secondary school, orientation towards natural sciences. She studied at the Institut Químic de Sarrià (IQS), Ramón Llull University (URL) in Barcelona, obtaining her Master's degree in Organic Chemistry in 1999 and a Chemical Engineering degree in 2002. During that time, in 2001, she started as a research scientist in the Laboratory of Biochemistry (IQS) and she worked during 6 months in the project "Detection of tartaric acid in wine samples". In 2002, after obtaining her Master's degree, she moved to the Netherlands and started to work as a research scientist for the Laboratory of Microbiology, Wageningen University, with a VLAG fellowship in the project entitled "Optimization of enzyme stability by engineering cyclic Polypeptides (β-glucanase)". A year later, she joined the *CD-CHEF* project (Quantification of Coeliac Disease toxic gluten in foodstuffs using a Chip system with integrated Extraction, Fluidics and biosensoric detection) again as a research scientist, in the same group. At the end of 2004, she started a PhD on microbial changes related to the use of different feed additives in piglets, in the Laboratory of Microbiology, Wageningen University. This project was part of the FEED FOR PIG HEALTH project, from the European Commission. The results of this work are presented in this thesis. Since 2009, Odette is working as a Post-Doc in the same laboratory in a follow up project of her thesis, part of the INTERPLAY project from the European Commission.

List of publications

Faijes, M., Pérez Gutiérrez, X., Pérez, O., Planas, A. (2003) Glycosynthase Activity of *Bacillus licheniformis* 1,3-1,4-β-Glucanase Mutants: Specificity, Kinetics and Mechanism. *Biochemistry*. 42: 13304-13318.

van Lieshout, J. F. T., Pérez Gutiérrez, O., Vroom, W., Koutsopoulos, S., Planas, A., de Vos, W. M., van der Oost, J. Stabilization of an Endo-β-1,3-1,4-Glucanase by Cyclization. *Submitted for publication.*

Su, Y., Yao, W., Pérez Gutiérrez, O., Smidt, H., Zhu, W. Y. (2008) 16S ribosomal RNA-based methods to monitor changes in the hindgut bacterial community of piglets alter oral administration of *Lactobacillus sobrius* S1. *Anaerobe*. 14(2): 78-86.

Su Y., Yao W., Pérez Gutiérrez O., Smidt H., Zhu W. Y. (2008) Changes in abundance of *Lactobacillus* spp. and *Streptococcus suis* in the stomach, jejunum and ileum of piglets after weaning. *FEMS Microbiol. Ecol*. 66(3): 546-555.

Pellikaan, W. F., Pérez Gutiérrez, O., Kluess, J., Bikker, P., Fledderus, J., Smidt, H. (2010). Effect of carvacrol on fermentation characteristics in the ileum of piglets during the process of weaning. *Livest. Sci. Accepted for publication.*

Pérez Gutiérrez, O., van den Bogert, B., Derrien, M., Koopmans, S-J., Molenaar, D., de Vos, W. M., Smidt, H. Design of a high throughput diagnostic microarray for the characterization of pig gastrointestinal tract microbiota. *In preparation*

Pérez Gutiérrez, O., Pellikaan, W. F., Verstegen, M. W. A., de Vos, W. M., Smidt, H. (2010). Activity and composition of piglet's faecal microbiota after *in vitro* fermentation in the presence of plant extracts and bioactive compounds. *Submitted for publication.*

Pérez Gutiérrez, O., de Filippi, S., Akkermans-van Vliet, W., Kluess, J., Bikker, P., Fledderus, J., Pellikaan, W. F., Verstegen, M. W. A., de Vos, W. M., Smidt, H. Dynamics of piglets' ileal microbiota in response to carvacrol. *In preparation.*

Pérez Gutiérrez, O., Fajardo, P., Pellet, T., Lallès, J-P., Pellikaan, W. F., Verstegen, M. W. A., de Vos, W. M., Smidt, H. Dynamics of gastrointestinal microbiota composition of weaning piglets fed with sodium butyrate during the suckling period. *In preparation.*

Pérez Gutiérrez, O., Heshof, R., Smits, C., Meijer, J., Verstegen, M. W. A., de Vos, W. M., Smidt, H. Piglets' gut microbial response to the addition of an acidifier and an antibiotic to the diet. *In preparation.*

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The first time I arrived to the Netherlands, almost 8 years ago (sjongejonge!), I couldn't imagine two things: that in 2010 I would be still in the Netherlands and that I would be defending my thesis. This was mainly because I came only for 9 months and because before arriving to the Netherlands I was sure that I didn't want to do a PhD. Now, after all those years I am very happy that I came and did a PhD and for having this good feeling I have to thank many people, who made living and working in this green and wet country a unique experience.

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Ps: you all know my bad memory…so forgive me if I forgot someone.

Overview of completed training activities

Discipline specific activities

Ecophysiology of the GI-tract, VLAG & WIAS, Wageningen	2005
Metabolomics, VLAG & EPS, Wageningen	2005
Sustainable Animal Health through Eubiosis-Relevance for Man, ETH, Ascona, Switzerland	2006
Darmen Dag, GFHD Foundation, Maastricht (poster presented)	2005
Gut Microbiology: Research to improve Health, Immune Response and Nutrition, INRA-RRI, Aberdeen, UK (poster presented)	2006
Darmen Dag, GFHD Foundation, Utrecht (poster presented)	2006
International Course on whole cell identification of Procaryotes and Eukaryotes (FISH)	2006
ARB – a software environment for sequence data, Ribocon / PRI, Wageningen	2007
Gut Microbiota in Health and Disease – potential role of probiotics, GFHD Foundation, Amsterdam	2007
NVvM meeting, Papendal (poster presented)	2007
Darmen Dag, GFHD Foundation, Wageningen (poster presented)	2007
6th Joint Symposium INRA-RRI, Clermont-Ferrand, France (poster presented)	2008
Darmen Dag, GFHD Foundation, Vlaardingen (poster presented)	2008
HITChip course, Laboratory of Microbiology	2008

General courses

VLAG PhD week, VLAG, Bilthoven	2005
Techniques for writing and presenting a scientific paper, PE&RC, Wageningen	2006
Multivariate Analysis, PE&RC, Wageningen	2009

Other activities

Colloquia of Microbiology, Laboratory of Microbiology	2004-08
Aio/Postdoc meetings, Laboratory of Microbiology	2004-08
The European Commission project meetings, FEED FOR PIG HEALTH (FOOD-CT-2004-506144)	2004-08

Teaching obligations

Advance Food Microbiology	2005
Advanced fermentation science	2006
Advanced fermentation science	2007
Advance Food Microbiology	2008
Advanced course Microbiology	2008
Supervising a MSc students	2005-08

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