Development of gut microbiota in pigs and the effect of diet, antibiotics and other environmental factors

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Abstract

The intestinal tract of humans and animals is colonized by trillions of microorganisms that constitute a community or ecosystem known as the gut microbiota. The gut microbiota undergoes remarkable alterations during early age, reaches a relative stable state in adulthood, and is driven by internal and external factors such as genotype of the host, diet and antibiotics. The objective of this research was to determine the effects of antibiotic treatment, microbial exposure and diet on the development of intestinal microbiota, focusing on the pig as an important production animal as well as a model for human. To achieve this objective, a series of intervention experiments were performed both in piglets and adult pigs.

To determine the impact of antibiotic treatment on the development of intestinal microbiota of piglets, two experiments were performed. The first experiment aimed to determine the effect of perinatal maternal antibiotic treatment on the intestinal microbiota of piglets. In this experiment, the sows received amoxicillin orally around parturition, and their offspring was serially sacrificed up to 42 days of age for analysis of ileal and colonic microbiota. It was observed that amoxicillin treatment drastically impacted the sows' faecal microbiota, and furthermore influenced specific microbial groups in the ileum and colon of the piglets before and after weaning. These findings indicated that maternal amoxicillin treatment may indirectly affect the gut microbiota of offspring through disturbing the maternal microbiota and the transfer of maternal microbiota to the offspring. In a second experiment, we determined the effect of early antibiotic treatment on intestinal microbial colonization and immune development of piglets. Additionally, the effect of stress factors associated with routine farm practice was investigated. Antibiotic treatment affected the composition and diversity of jejunal microbiota, and reduced the expression of a large number of genes involved in immune-related processes. The cumulative effect of management procedures on top of the use of an antibiotic was limited. This study reinforced the notion that the early phase of life is critical for intestinal immune development, also under regular production circumstances.

Apart from antibiotic treatment, the effect of early microbial association on the development of intestinal microbiota and immune system of piglets was also studied in this thesis. One group of caesarean derived piglets was inoculated with a mixture of three microbial species (*Lactobacillus amylovorus*, *Clostridium glycolicum*, and *Parabacteroides* sp. ASF519) at day 1 and 2 after birth (the simple microbial association group), whereas a second group of piglets was inoculated with the above mixture at day 1 and 2 after birth as

well as diluted adult sow faeces at day 3 and 4 after birth as the complex microbial association (CA) group. CA caused an increase of faecal microbial diversity and accelerated the faecal microbiota to develop into a stable and diverse microbiota. CA significantly affected luminal microbial composition and gene expression in jejunal and ileal mucosa, albeit in different ways. In the pig ileum, CA led to an increased relative abundance of microbial groups known to have beneficial effects, whereas it reduced the relative contribution of potential pathobionts. CA also induced the enrichment of immune-related gene sets in the ileal mucosa.

Another research goal of this thesis was to determine the influence of diet on the microbiota in the large intestine of adult pigs. To this end, the effect of resistance starch (RS) was evaluated in two studies. In the first study, pigs were either assigned to an RS diet or a digestible starch (DS) diet for two weeks. Samples from along the intestine were collected for measuring luminal microbiota composition, short chain fatty acid (SCFA) concentrations and the expression of host genes involved in SCFA uptake, SCFA signalling, and satiety regulation in mucosal tissue. In both the caecum and colon, differences in microbiota composition and SCFA concentrations were observed between DS- and RS-fed pigs. Caecal tissue expression of genes encoding monocarboxylate transporter 1 and glucagon was induced by RS. Based on these results, an additional experiment was performed. In this study, ten pigs, fitted with a cannula in the proximal colon for repeated collection of tissue biopsies and luminal content, were fed a DS diet, or a diet high in RS (34%) for two consecutive periods of 14 days in a crossover design. RS increased the relative abundance of several butyrate-producing microbial groups and reduced that of potentially pathogenic members of the genus *Leptospira* and the phylum of Proteobacteria. Concentrations of acetate, propionate and butyrate in carotid plasma were significantly higher after RS consumption. Upon RS feeding, oxidative metabolic pathways, such as TCA cycle and beta-oxidation, were induced, whereas many immune response pathways, including adaptive and innate immune system, as well as cell division were suppressed. The nuclear receptor PPARG was identified as a potential key upstream regulator.

In conclusion, this thesis provides direct evidence that maternal antibiotic treatment, early antibiotic admistration and microbial exposure affect the development of intestinal microbiota of the piglets. Moreover, both early antibiotic admistration and microbial exposure affected piglet mucosal tissue gene expression. These findings reinforce the notion that the early phase of life is critical for the development of intestinal microbiota and immune system. Furthermore, it is proposed that manipulation of the microbial association at early age may be a way of supporting functional gut development. In addition to the above discussed early life envents, a diet with RS can also affect the microbiota in the large intestine of adult pigs. This thesis provides an enhanced understanding of the interaction

between diet, microbiota and host in a number of complementary pig models and revealed the impact of antibiotics in early life microbial colonization. The gained insight is expected to be instrumental in improving sustainable pig management. Moreover, it may also be useful in understanding similar processes in the human gut.

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Chapter 1

General introduction and outline

Development of intestinal microbiota

The intestinal tract of humans and animals is colonized by trillions of microorganisms that constitute a community or ecosystem known as the gut microbiota. This complex gut microbiota develops after birth as newborns are considered to be virtually sterile. During and after birth, microorganisms originating from surrounding environments, including faecal material, rapidly colonize the gut of neonates and young animals [1-3]. However, recent studies reported the presence of microbes in the human intra-uterine environment, suggesting that some microbial colonization prior to the delivery can not be excluded [4-8].

Considerable knowledge has been assembled on the development of the intestinal microbiota in human. Generally, the first colonizers that dominate the human intestinal tract are facultative anaerobes including *Escherichia coli* and other *Enterobacteriaceae*, followed by anaerobic bacteria such as *Bifidobacterium*, *Clostridium*, *Bacteroides*, and *Ruminococcus* [9, 10]. The microbial succession during the first few weeks of life in the intestinal tract of chicken, pigs, and calves follows a similar pattern, and both coliforms and streptococci dominate the microbiota within a few days of birth. Obligate anaerobes appear some time later, even though neonatal animals are exposed to greater numbers of faecal and environmental bacteria than human neonates [11]. For infants it is known that after this initial colonization, the microbiota undergoes consecutive changes in composition and function until a relatively stable climax community is established at around 3 years after birth [12].

It is difficult to define a 'normal' intestinal microbiota for humans and animals at early age. The composition and temporal patterns of the microbial communities have been shown to vary widely from baby to baby [13], and furthermore the infant microbiome displays high interpersonal variability at an early age [14]. Nevertheless, some general trends can be inferred from previous studies. For example, *Bifidobacterium*, which decreases in abundance with age, has been generally reported as a predominant bacterial genus colonizing the early life infant gut [15-17]. The introduction of solid foods can significantly affect the gut microbiota, driving it into an adult type [13, 18]. In a single US baby follow up it was found that ingestion of table foods caused a sustained increase in the abundance of Bacteroidetes, elevated faecal short chain fatty acid (SCFA) levels, resulted in an enrichment of genes associated with carbohydrate utilization, vitamin biosynthesis, and xenobiotic degradation, and produced a more stable community composition, all of which are characteristic of the adult microbiome [19]. In this baby, the earliest microbiome was found to be enriched in genes facilitating lactose utilization, and functional genes involved in plant polysaccharide metabolism were present before the introduction of solid food, priming the infant gut for an adult diet [19]. Another study of Danish infants also showed that cessation of breastfeeding and introduction of a complementary feeding induce

replacement of a microbiota characterized by lactobacilli, bifidobacteria, and *Enterobacteriaceae* with a microbiota dominated by *Clostridium* spp. and *Bacteroides* spp. [2]. Currently, it is commonly believed that the key players of human intestinal microbiota development are assigned to five phyla, including Actinobacteria (with the genera *Bifidobacterium* and *Colinsella*), Bacteroidetes (genera *Bacteroides* and *Prevotella*), Firmicutes (including, but not restricted to the genera *Lactobacillus*, *Clostridium*, *Eubacterium* and *Ruminococcus*), Proteobacteria (e.g. *Enterobacter*), and Verrucomicrobia (consisting of one major species, the mucin-degrading *Akkermansia muciniphila*) [3].

The microbiota plays a critical role in host immune system priming and gut maturation at early age, and can further influence the gut function through modifying gene expression of the host epithelium [20-23]. The host transcriptional pathways regulated in response to the colonizing microbiota involve nutrient uptake and metabolism, mucosal barrier function, xenobiotic metabolism, enteric nervous system and motility, hormonal and maturational responses, angiogenesis, cytoskeleton and extracellular matrix, signal transduction, and general cellular functions [24]. Interestingly, many host genes seem to be specifically altered in response to different members of the microbiota [25, 26]. Therefore, colonization of different microbes at early age may induce specific immune and metabolism system development, which consequently differentially influence the host intestinal and systemic health throughout life.

Factors influencing the development of the intestinal microbiota

There are many factors that influence the development of intestinal microbiota. Before delivery, a number of maternal and external factors during pregnancy are related to microbiota establishment in the gut, for instance, the mother's body weight and microbiota, disturbance through stress, and administration of antibiotics and probiotics. During birth, the mode of delivery (vaginally or by caesarean section) is the main factor that affects the microbiota establishment in the gut. After delivery, additional factors can influence the process of gut microbiota development at early age, such as feeding (breast feeding vs. formula feeding), antibiotic treatment, probiotics, and microbial exposure. Although gestation time is also a factor that strongly influences establishment of the infant intestinal microbiota, here we focus on the development of intestinal microbiota in term offspring since this is encountered in the majority of animal deliveries.

Prenatal and maternal factors

Prenatal factors influencing the development of intestinal microbiota remain a largely unexplored area. Recent studies have suggested that the microbial contact of the offspring with their mothers may begin already prior to birth [5, 7, 27]. Therefore, maternal

influences on the fetal gut colonization should be considered as the first stage of intestinal microbiota development. Up to now, little is known about the maternal influences on fetal gut colonization. Studies so far have only shown that the maternal status during pregnancy is related to the intestinal microbiota development after birth. Mothers' BMI, weight, and weight gain during pregnancy can influence the composition and development of infant gut microbiota after birth. Faecal *Bacteroides* and *Staphylococcus* concentrations were significantly higher in infants of overweight mothers during the first six months, whereas the abundance of bacteria related to *Akkermansia muciniphila*, *Staphylococcus*, and *Clostridium difficile* were lower in infants of normal-weight mothers and of mothers with normal weight gain during pregnancy [28]. Another study showed that overweight pregnant women had more *Bacteroides* and *Staphylococcus* than normal-weight pregnant women [29]. In addition, it was found that similarities between infant-mother microbiota increased with children's age [30]. These results suggest that the mothers' microbiota is associated with the development of gut microbiota of offspring after birth.

The mother's faecal, vaginal and possibly breast microbiota probably represent the most influential inoculum source for the development of the offspring microbiota, due to intimate contacts during and after birth. During delivery, mothers' vaginal bacteria, such as lactobacilli, can be transferred to their offspring [31]. Vaginally delivered infants acquired bacterial communities resembling their own mother's vaginal microbiota, dominated by *Lactobacillus*, *Prevotella*, or *Sneathia* spp., whereas infants born by Caesarean section harboured bacterial communities similar to those found on the skin surface, dominated by *Staphylococcus*, *Corynebacterium*, and *Propionibacterium* spp. [32]. Moreover, vaginally delivered term infants generally have higher abundance of *Bifidobacterium* at early age compared with infants born by Caesarean section [33]. It was recently found that Caesarean section is associated with a lower total microbial diversity and delayed colonisation of the Bacteroidetes phylum in the infant gut [34].

For breastfed infants, the microbiota of breast milk could also be transferred from mothers to infants after birth. The diversity and function of microbiota in breast milk has been reviewed recently [35, 36]. Proposed theories for the microbiota composition of breast milk include retrograde flow from the infant's oral cavity, transfer of organisms from maternal skin, and movement of microbiota from the maternal enteric tract to the mammary gland. Breast-fed infants were found to have significantly higher counts of bifidobacteria, whereas formula-fed babies had significantly higher proportions of *Bacteroides* and clostridia [37, 38]. Moreover, breast-fed infants were reported to be less colonized by *Clostridium difficile* and *Escherichia coli* than formula-fed infants [38].

As described above, the mother's microbiota plays an important role in the development of gut microbiota of offspring. Thus, factors that affect the maternal gut microbiota during

delivery and lactation should also be considered.

Antibiotic treatment, as a common factor disturbing the gut microbiota, has been commonly used to control infections of pregnant women in hospitals and in pre- and post-partum animals on a farm. Antibiotic treatment of mothers can impact fetus development, and associates with low birth weight of neonates and high risk of diseases such as oral clefts at early age [39]. Recently, researchers also found that antibiotic use during pregnancy altered the commensal vaginal microbiota of women [40]. Intrapartum antibiotic treatment of mothers was found associated with a decreased transmission of vaginal *Lactobacillus* to the neonate during birth [41]. Moreover, newborns delivered by mothers treated with antibiotics perinatally had lower proportions of *Bacteroides* and members of the *Atopobium* cluster [37, 38]. Most antibiotics are either injected or administered orally, thereby circulating throughout the mother's system and potentially affecting the entire mother's microbiota [42]. Thus, more studies are needed to address the antibiotic effect on milk and skin microbiota during pregnancy and lactation, as well as its effect on the gut microbiota development in the offspring.

Other external factors during pregnancy such as stress and probiotics can also impact the microbial composition in the gut of the offspring. In animal trials, prenatal stress reduced the overall numbers of bifidobacteria and lactobacilli in the gut of infant monkeys [43]. In other animal models, it was found that *L. acidophilus* and *B. lactis* administered as probiotics to mothers during late gestation were transferred to young animals born vaginally [44]. In addition, a human study showed that maternal consumption of *L. rhamnosus* strain GG affected transfer and establishment of faecal bifidobacteria in neonates [45].

Microbial exposure at early age

Apart from the mother's microbiota, the early exposure to microbes in the surrounding environment of newborns could also be a strong influencing factor in the development of the intestinal microbiota. The infants can be subjected to different microbes in different indoor environments such as home, hospital, and daycare. Multiple studies have reported links between bacteria in the home and the microbial composition of the infant gut. Recently, a study based on high-throughput sequence analysis of bacterial 16S ribosomal RNA (rRNA) genes demonstrated an association between bacterial communities of house dust and the gut of infants at three months of age [46]. Fourteen bacterial OTUs co-occurred at a significantly higher frequency in matched dust and infant stool pairs than in random pairs, despite significant differences between the dust and infant faecal microbiota. These OTUs represented the classes *Actinobacteria* (genus *Bifidobacterium*), *Bacilli* (genera *Planomicrobium, Streptococcus,* and *Lactococcus*), *Clostridia* (genera

Veillonella and Faecalibacterium, and unknown bacteria clustering within families *Lachnospiraceae* and *Peptostreptococcaceae*) and *Gammaproteobacteria* (genus *Escherichia*). These bacteria probably originate from normal household activities such as diaper changing and possibly aerosolization of faecal bacteria [46], and some gut-borne lactic acid bacteria (especially members of the genus *Lactococcus*) proliferate naturally on some organic materials under suitable conditions (e.g., dairy products, sauerkraut, dough) and subsequently deposit and accumulate in house dust [46]. Interestingly, a study on pacifier cleaning showed that the salivary microbiota differed between children whose parents cleaned their pacifier by sucking it and children whose parents did not use this practice [47]. Taken all together, normal household items and activities, family members and pets may all serve as reservoirs for infant gut bacterial inoculation. Exposure to pets or siblings of infants has been suggested to afford protection against allergic disease. In order to determine the mechanism responsible for this effect, murine models have been used to examine the interaction between the gut microbiome and house dust microbiome in two homes, one with a dog and the other without pets as control [48]. This study revealed that exposure to dog-associated household dust results in a distinct caecal microbiome composition with a significant enrichment of, amongst others, *Lactobacillus*, and *L. johnsonii* (as represented by OTUs in the clade containing *L. gasseri*) was primarily responsible for the anti-inflammatory effect. With respect to human studies, healthy full term infants living with pets exhibited under-representation of bifidobacteria and over-representation of *Peptostreptococcaceae*, whereas infants with older siblings showed the opposite effect [49, 50].

With respect to piglets, studies have shown that the surrounding environment during postnatal development can have long-term impact on gut community structure [51]. Different early-life environments were shown to be associated with major differences in mucosa-adherent microbial diversity in the ileum of adult pigs [52]. The raising environment attributes mostly to microbial association for piglets at early age. Pigs housed in a natural outdoor environment showed a dominance of Firmicutes, in particular *Lactobacillus*, whereas pigs housed in a hygienic indoor environment had reduced *Lactobacillus* and higher numbers of potentially pathogenic phylotypes [52]. Overall, environmentally-acquired bacteria influence the microbiota composition in the GI tract of piglets, as well as the microbiota of the adult pigs throughout life.

Antibiotic treatment at early age

As previously described, antibiotic treatment is a common disturbance that impacts the intestinal microbiota. Correspondingly, antibiotic administration at early age also influences the colonization process of microbiota in the gut of infants and young animals. A variety of studies have addressed the effects of antibiotics on the intestinal microbiota of infants and

piglets based on culture independent studies (summarized in **Table 1**). Generally, antibiotic treatment at early age has been shown to delay the colonization of bifidobacteria [10, 50, 53-55]. However, some researchers reported that instead of a significant alteration of the abundance of bifidobacteria, antibiotic treatment decreased the diversity of *Bifidobacterium* species at early age [54, 56, 57]. Other publications have reported antibiotic effects on the composition of faecal microbiota, but only at genus level [56]. In a murine animal model, it was found that neonatal amoxicillin treatment significantly altered the levels of lactobacilli, and led to a significant impact on the diversity of the intestinal *Lactobacillus* community [58]. In newborn piglets, a single parenteral dose of long-lasting amoxicillin at the first day of life caused a significant decrease of faecal microbiota diversity for at least five weeks after administration [59]. Considering the various targets, doses, and durations of antibiotic treatment, it is not surprising that gut microbial communities respond to different antibiotics in different ways or even in opposite ways. For example, faecal *Enterobacteriaceae* of infants younger than 6-months were reported to be significantly reduced after a 5-day ceftriaxone treatment, whereas the relative abundance of *Enterobacteriaceae* was markedly higher in the faecal samples of 1-month old infants that were treated with cefalexin in the first four days of life [53, 60]. These results also indicated that, at early age, the microbiota present before the antibiotic treatment can also influence how microbial communities respond to the antibiotic treatment. Because the intestinal microbiota at early age is more dynamic and less resilient than that of adults [37, 61], common patterns of antibiotic-induced changes of intestinal microbiota may not be easily predicted at early age, and more studies should be directed at assessing the effect of different antibiotics over time during early age, and the impact of these effects later in life.

Dietary components: Major regulators of the intestinal microbiota

Since the first introduction of solid foods to infants at 4-6 months of age, and to piglets at weaning after 1-2 months, the diet becomes a major force that shapes the composition and activity of the gut microbiota [62-64]. This is evident from the fact that the introduction of solid foods drives the infant gut microbiome into an adult type as previous discussed [13, 18], and that an alteration in gut microbiota has been shown after weaning or dietary changes [65-67]. The microbiota of healthy adult humans and other mammals is generally considered stable [68]. Especially elderly people have been shown to have a remarkably stable microbiota, although their core microbiota differs from that of younger people with greater proportion of *Bacteroides* spp. and distinct abundance patterns of *Clostridium* groups [69]. In addition, identical twin studies indicated that host genetic factors may be less important as extensive dissimilarity in gut bacterial communities was discovered [70]. Nevertheless, the faecal microbiota of monozygotic twins is more similar than that of unrelated subjects [71, 72]. Taken together, the diet may be the most important factor that impacts the intestinal microbiota in healthy humans and adult animals.

Table 1. Effects of antibiotic treatment on infant and piglet intestinal microbiota based on culture-independent studies.

Antibiotic treatment	Duration	Age	Sampling time	Effect	Reference
Infant study					
Clavulanic acid & Amoxicillin, Trimethoprim & Sulfamethoxazol	A mixture of clavulanic acid and amoxicillin for 13 days, then a mixture of trimethoprim and sulfamethoxazol		Daily for the first 2 weeks, then once a week until at least 160 days after birth	A small stable community consisting of *Ruminococcus* species. No *Bifidobacterium*	[10]
Mainly amoxicillin	During first 1 month of life		1 month	Numbers of bifidobacteria and *B. fragilis*-group species decreased	[50]
Cefalexin	In the first 4 days of life		Day 3	*Bifidobacterium* decreased Overgrowth of enterococci	[53]
			Day 5	Overgrowth of enterococci	
			1 month	*Enterobacteriaceae* markedly higher	
Amoxicillin	7 days	18 month	End of therapy	No significant alteration of bifidobacteria concentration; Numbers of *Bifidobacterium* species per microbiota significantly decreased	[56]
Ampicillin & Gentamicin	2-9 days		4 weeks, 8 weeks	Bifidobacteria detected not for all treated infants; *Bifidobacterium* spp. diversity decreased	[57]
Ceftriaxone	5 days	<6 months	Last day of antibiotic therapy (d5)	Significant reduction in total bacterial count, *Enterobacteriaceae* and enterococci; lactobacilli no longer detected	[60]
			15 days after the end of antibiotic therapy	Total bacterial count, *Enterobacteriaceae*, enterococci and lactobacilli significantly increased compared to d5, similar to the day before starting antibiotic therapy	
Ampicillin & Gentamicin	Not described	Treatment start within 48 hours of birth	4 weeks after the cessation of treatment	Significantly higher *Proteobacteria*; Lower *Actinobacteria* (genus *Bifidobacterium*) and genus *Lactobacillus*	[54]
			8 weeks after the cessation of treatment	Significantly higher *Proteobacteria*; Number of different *Bifidobacterium* species reduced	
Not described	During first 4 months		4 months	Lower *Bifidobacterium longum*; Higher counts of *Bacteroides fragilis* group	[55]
Piglet study					
Amoxicillin	1 day after birth		39 days	*Lactobacillus amylovorus* presented only in the control group; *Roseburia faecalis*-related population strongly reduced; *Shigella* spp., *Escherichia coli*, *Salmonella enterica* serovar *Typhi* enriched	[59]

The digestion of food in the intestine is a complex process (see **Figure 1** for an overview**)**. The ingested food is first rendered more digestible by processing, chewing in the mouth, and wetting in the stomach; then, the food enters the small intestine where further enzymatic digestion occurs [73]. Unabsorbed food components enter the large intestine, which is the main site of microbial metabolism as it is colonized by the highest number of microorganisms [74, 75], and become available as substrates for bacterial fermentation and transformation. Dietary components that escape absorption in the upper gastrointestinal tract are comprised to a large proportion of nondigestible dietary carbohydrates. Approximately 40 g of dietary carbohydrates reaches the colon each day having escaped digestion by host enzymes; the main categories are resistant starches (RS), non-starch polysaccharides (NSP) and oligosaccharides, although some di- and mono-saccharides (e.g. sugar alcohols) may also reach the colon [76]. Nondigestible carbohydrates provide a major source of energy for bacteria in the large intestine, and influence microbial fermentation and composition of the microbiota both in short-term dietary interventions and in response to habitual long-term dietary intake [76, 77]. In the following this introduction will mainly focus on the impact of RS on microbiota in the large intestine.

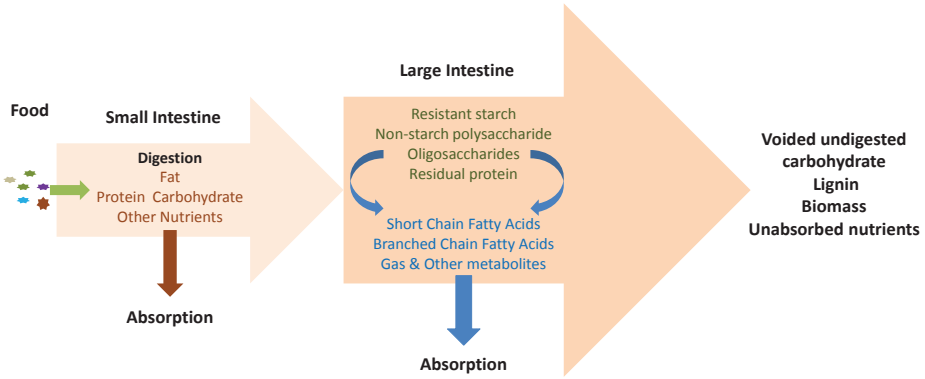

Figure 1. Fate of ingested dietary food components in the small and large intestine.

RS have been extensively reviewed in general as well as from the standpoint of their function and impact on gut health [78-82], and are defined as a portion of starch that cannot be digested by amylases in the small intestine and pass to the colon where they are fermented by the microbiota [83, 84]. The main fermentation products of RS by gut bacteria include gases (methane, hydrogen, carbon dioxide) and SCFAs (acetate, propionate, butyrate, and valerate), while much lower amounts of other organic acids (lactate, succinate, and formate), branched SCFAs (isobutyrate and isovalerate), and alcohols (methanol and ethanol) are also produced [85]. Culture studies have shown that RS have a major effect on the microbial composition in the large intestine [86-90]. Currently, culture-independent

methods allow the detection of the effects of RS on the intestinal microbiota *in vivo* [91, 92]. It has been shown that phylotypes related to *Ruminococcus bromii*, which are abundant in the large intestinal of humans, increase in response to a diet high in RS [91]. Similarly, populations closely related to *R. bromii* and *Eubacterium rectale* were shown to be the key players in degradation of RS in a dynamic *in vitro* model of the proximal colon, the TIM-2 model, using RNA stable isotope probing of models fed with ^{13}C labeled RS [93].

Different forms of RS differ in their ability to modulate the intestinal microbiota [94]. There are five types of RS (see **Table 2** for a summary). To date, studies have shown different effects of RSII, RSIII and RSIV on the human and animal intestinal microbiota. It is still unclear why different RS differ in changing the composition of the microbiota, and more studies should be conducted to identify the mechanisms by which specific bacterial taxa interact with the different forms of RS.

Table 2. Effects of resistant starches (RS) on the intestinal microbiota of human and animals based on culture-independent studies (Adapted from Birt *et at.* [85]).

Type of RS	Description	Example	Effect	Human or Animal study	Reference
RSI	Physically inaccessible starch	Coarsely ground or whole-kernel grains	-[1]	-	-
RSII	Granular starch with the B- or C-polymorph	High-amylose maize starch, raw potato, raw banana starch	Species level: *Ruminococcus bromii, Eubacterium rectale* increased	Human	[94]
			Bacteroidetes , Actinobacteria increased	rats	[95]
RSIII	Retrograded starch	Cooked and cooled starchy foods	*Ruminococcus bromii* , uncultured *Oscillibacter*, *Eubacterium rectale* increased	Human	[91], [96]
RSIV	Chemically modified starches	Cross-linked starch and octenyl succinate starch	Actinobacteria , Bacteroidetes increased Firmicutes decreased *Bifidobacterium adolescentis, Parabacteroides distasonis* increased	Human	[94]
RSV	Amylose-lipid complex	Stearic acid-complexed high-amylose starch	-	-	-

[1] "-" means that no related information has been found.

Molecular approaches for the study of the microbial communities residing in the mammalian gut

The gut microbiota has been studied via cultivation-based techniques traditionally. However, these techniques are laborious and with limitations, for instance, they require selective media or anoxic conditions in most cases, and have difficulties with assessing complex microbial communities such as those found in the gut. To circumvent some of the limitations of cultivation-based techniques, researchers can use culture-independent methods, such as fingerprinting and sequencing techniques based on small subunit (16S/18S) rRNA and the encoding genes, according to their research aim. If the research goal is only to estimate the relative abundance of certain bacteria in a community, quantitative real time PCR (qPCR) is a better analysis method considering time and cost; while for monitoring variation of the microbiota of different samples, fingerprinting techniques such as denaturing gradient gel electrophoresis (DGGE) are the most rapid methods to show overall differences of the microbial profile. Comparing to fingerprinting techniques, phylogenetic microarrays are more powerful for gaining insights into the structure and population dynamics of a complex microbial ecosystem, as well as to provide a comprehensive overview of the microbial profile of the whole sampling set. However, due to the fact that microarrays are designed based on the publicly available sequence space, next-generation technology sequencing such as 454 pyrosequencing, and more recently also Illumina MiSeq and HiSeq sequencing, can provide novel insights on composition of gut microbial communities. In the present thesis, for pig intestinal microbiota analysis we mainly used phylogenetic microarrays targeting microorganisms reported to occur in the porcine gut, as well as a supplemental analysis with fingerprinting techniques.

Fingerprinting techniques have been extensively used to monitoring community shifts among samples, e.g. in case of GI tract microbiota to assess the effect of dietary treatments, health state and age of a mammalian host. Fingerprinting techniques include DGGE, temperature gradient gel electrophoresis (TGGE), temporal temperature gradient gel electrophoresis (TTGE), single strand conformation polymorphism (SSCP) and terminal-restriction fragment length polymorphism (TRFLP) analyses. Although the principles and technical procedures vary, all these fingerprinting techniques are PCR-based and generate profiles representing the sequence diversity within the selected ecosystem. Among these fingerprinting techniques, DGGE has been most extensively used for human and animal GI tract microbiota analysis. One of the advantages of DGGE is that it enables rapid comparative analysis of microbial variation between samples, especially the microbial community response to treatment over time. In addition, the band intensities can be used as a semi quantitative measure for the relative abundance of a certain sequence in the community. Furthermore, DNA fragments can be cut from the gel and re-amplified for sequencing and thus identification of the corresponding microorganisms [97]. In general,

however, DGGE and other fingerprinting methods mentioned above, only allow to detect the most dominant bacteria that constitute at least 1% of the total bacterial community [98]. Therefore, these methods are now only being used for preliminary scanning of the gut microbiota in mammalian studies.

More recently, phylogenetic microarrays constitute a high-throughput tool targeting in most cases the SSU rRNA gene, and are a superior alternative to the more traditional fingerprinting techniques to detect the variation of a microbial community at much improved spatio-temporal resolution. Phylogenetic microarrays are in most cases glass surfaces spotted with thousands of covalently linked DNA or RNA probes. These have been successfully applied in the diversity analysis of the microbiota in the mammalian gut, such as that from human [13, 99], mouse [100] as well as pig [97, 101, 102]. The Pig Intestinal Ttract Chip (PITChip) is the main method applied for microbiota analysis in this thesis. Currently, there are two versions of the PITChip. The PITChip version 1.0 is a phylogenetic microarray, with more than 2,980 oligonucleotides based on 16S rRNA gene sequences of 627 porcine intestinal microbial species-level phylotypes [97, 101]. The PITChip version 2.0 is an update of the previous version with more than 3200 oligonucleotides targeting the 16S rRNA gene sequences of 781 porcine intestinal microbial phylotypes [102]. The PITChip as well as the corresponding Human Intestinal Tract Chip (HITChip) [103], provide a very deep and reproducible phylogenetic analysis that has been compared with deep pyrosequencing of 16S rRNA gene fragments [97, 104, 105] and next generation parallel sequencing of intestinal metagenomes [106], indicating comparable resolution and a higher sensitivity of the chip based analysis at commonly used sequencing depth.

Thesis outline and aims

The work presented in this thesis was embedded in two larger programs: 1) the EU-FP7 funded project entitled "Interplay of microbiota and gut function in the developing pig – Innovative avenues towards sustainable animal production", and 2) Wageningen University Strategic Research Agenda IP/OP funded program entitled "Satiety & Satisfaction". The main objective of this thesis is to estimate the effects of antibiotic treatment, microbial exposure and diet on the development of intestinal microbiota, focusing on the pig as an important production animal as well as a model for humans.

Chapter 1 gives an introduction of our current understanding of the development of intestinal microbiota early in life, including factors that potentially affect this development, such as perinatal and maternal factors, microbial exposure and antibiotic treatment at an early age after birth, as well as the influence of diet. Furthermore, an up to date account is given with respect to molecular approaches towards microbial community analysis, specifically focusing on the mammalian gut.

Chapter 2 provides an overview of studying the mammalian intestinal microbiome using animal models. Animal models provide an alternative way to study the in *vivo* responses to beneficial, commensal and pathogenic microorganisms in the GI tract. The main animals used to study the mammalian gastrointestinal microbiota are rodents (mainly mice and rats) and pigs. Rodent and pig models, including gnotobiotic and humanized rodents and pigs and minipigs, have been extensively employed in gut microbiota studies, and especially the piglet has been suggested as an appropriate model for studying the human infant microbiota.

The establishment and development of intestinal microbiota can be influenced by many external factors. Antibiotic treatment is one of those factors and a common disturbance of intestinal microbiota. This is also true for infants and young animals, with an additional factor that mothers can affect the intestinal microbiota of their offspring, and antibiotic treatment of mothers may indirectly affect the intestinal microbiota of their offspring. The influence of maternal amoxicillin treatment on microbiota development in piglets was estimated in **Chapter 3**. In this model, the sows received amoxicillin orally around parturition, and their offspring was serially sacrificed up to 42 days of age. Amoxicillin treatment drastically impacted the sows' faecal microbiota, and furthermore influenced specific microbial groups in the ileum and colon of the piglets before and after weaning. Maternal amoxicillin treatment may indirectly affect the gut microbiota of offspring through disturbing the maternal microbiota and the transfer of maternal microbiota to offspring.

Direct administration of antibiotics at early age can also influence the intestinal microbiota, and furthermore immune competence development of infants and young animals. Experiments described in **Chapter 4** evaluated the effect of early antibiotic treatment on the intestinal microbial colonization and immune development of piglets. Furthermore, the additional effect of stress factors associated with routine farm practice was investigated. This chapter provides direct evidence that different early-life conditions, specifically focusing on antibiotic treatment and exposure to stress, affect gut microbial colonization and intestinal immune development. This reinforces the notion that the early phase of life is critical for intestinal immune development, also under regular production circumstances.

Apart from antibiotic disturbances, early microbial association with environmental microorganisms also plays an important role in the development of intestinal microbiota and host immune system. To study this effect, a model with caesarean derived piglets is applied in **Chapter 5.** In this model, one group of piglets was inoculated with a mixture of three microbial species (*Lactobacillus amylovorus*, *Clostridium glycolicum*, and *Parabacteroides* sp. ASF519) at day 1 and 2 after birth as the simple microbial association group, whereas the other group of piglets was inoculated with the above mixture at day 1 and 2 after birth as well as a faecal inoculant of an adult sow at day 3 and 4 after birth as the complex microbial association group. The complex microbial association caused an increase of faecal microbial diversity and accelerated the faecal microbiota to develop into a stable and diverse microbiota. Complex microbial association significantly affected, althought in different ways, the microbial composition and host gene expression in the jejunum and ileum.

After the first introduction of solid foods to infants and young animals, the diet becomes a major factor that shapes the composition and activity of the intestinal microbiota. Dietary components can have a large influence on microbiota especially in the large intestine, due to the fact that they escape absorption in the small intestine, entering the large intestine as substrates for microbial fermentation and transformation. Resistant starch is an example of such feed components that can be used by bacteria in the large intestine to produce mainly short chain fatty acids (SCFA). The effects of RS on intestinal microbiota of pigs are described in **Chapter 6.** Pigs were either assigned to an RS diet or a digestible starch (DS) diet for two weeks. Intestinal samples from along the intestine were collected for measuring luminal microbiota composition, luminal SCFA concentrations and the expression of host genes involved in SCFA uptake, SCFA signalling, and satiety regulation in mucosal tissue. In both the caecum and colon, differences in microbiota composition and SCFA concentrations were observed between DS- and RS-fed pigs. Caecal tissue expression of genes encoding monocarboxylate transporter 1 (SLC16A1) and glucagon (GCG) was induced by RS. Based on these results, an additional experiment was performed as described in **Chapter 7** to identify genes the expression of which is affected by RS in the

proximal colon to infer which biological pathways were modulated. Ten pigs, fitted with a cannula in the proximal colon for repeated collection of tissue biopsies and luminal content, were fed a digestible starch (DS) diet, or a diet high in RS (34%) for two consecutive periods of 14 days in a crossover design. This study revealed that upon RS feeding, oxidative metabolic pathways, such as TCA cycle and beta-oxidation, were induced whereas many immune response pathways, including adaptive and innate immune system, as well as cell division were suppressed. The nuclear receptor PPARG was identified as a potential key upstream regulator.

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 emphasis on the different directions towards which the gut microbiota evolves under the effect of different external factors. Furthermore, this chapter provides an outlook and future perspectives with respect to the relevance and implementation of microbial function-related approaches, notably functional (meta) genomics, and their integration with host-derived data to arrive at innovative avenues towards description and prediction of development and functioning of the host microbe holo-organism using holistic systems biology approaches.

Chapter 2

Studying the mammalian intestinal microbiome using animal models

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Abstract

The gastrointestinal (GI) tract of humans and animals is colonized by microorganisms immediately after birth. The composition of the GI tract microbiota undergoes remarkable alterations during early age, reaches a relative stable status in adulthood, and is driven by external factors such as habitual diet, location along the intestine, antibiotic therapy and maternal microbiota, and intrinsic factors such as host species and genotype. Whereas usually faecal samples are used for assessing the impact on the microbiota in human intervention studies, *in vitro* and animal models provide an easier way to collect many (invasive) samples, have multiple comparisons and regulating the genotype background. Animal models, and in particular mammalian models, provide an alternative way to study the *in vivo* responses to beneficial, commensal and pathogenic microorganisms in the GI tract, including studies that aim to see the impact of the host system as well. The main animals used to study the mammalian GI tract are rodents (mainly mice and rats) and pigs. Rodent and pig models, including gnotobiotic and humanized rodents and pigs and minipigs, have been extensively employed in gut microbiota studies, and especially the piglet model has been suggested as an appropriate model for human infant studies. With pig models, several intestinal sampling techniques can be applied in kinetic microbiota studies, including small intestinal segment perfusion and cannulation. In many cases, to test a certain treatment, a tiered approach consisting of complementary methods is employed, comprising *in vitro*, *in vivo* animal models, eventually leading towards human intervention studies.

Key words: Microbiota, Pig, Mice, Rats, Gastrointestinal tract, Model

Introduction

From birth onwards the gastrointestinal (GI) tract of humans and animals is colonized by microorganisms that constitute a community or ecosystem known as the microbiota. These microorganisms, predominantly from the bacterial kingdom, but also including archaea and eukaryotes such as fungi and protozoa, can reach a diversity of at least 160 species per individual, and over 1150 different species were detected in the human gut [99, 106, 107]. This complex ecosystem increases in numbers throughout the length of the GI tract, from 10 to 1000 cells per ml in the stomach, reaching a density of 10^{11} cells per gram of intestinal content [108, 109] in the large intestine. All three domains of life are present in the large intestine where the bacterial community is dominant as well as the most phylogenetically diverse. At least nine different bacterial phyla have been detected in the large intestine, among which the phyla *Bacteroidetes* and *Firmicutes* dominate [12, 99, 110]. So far, only a minority of the bacteria in the gut have been cultured. Nonetheless molecular techniques that have emerged over the last two decades provided the opportunity to understand this complex GI tract ecosystem much better [99, 105, 111, 112]. The microbial ecosystem differs in the anatomically distinct regions in the intestinal tract, which has been reviewed elsewhere [109].

The composition of the intestinal microbiota is driven by external factors such as habitual diet, antibiotic therapy and maternal microbiota, and intrinsic factors such as host species and genotype [113-118]. Since the intestinal tract is the main point of contact of the host immune system and microorganisms, the role of microbiota in both local and systemic immune function plays an important role in immunity and health [119].

Locations and their conditions along the GI tract

pH, transit time and microbial density are just a few of the many factors that are changing along the GI-tract. These differences need to be considered during experiments.

Oral cavity

When food is chewed in the mouth, it will be broken into pieces, moisturized and mixed with digestive enzymes – amylases and lipases – from the salivary glands of the host [108]. The most common bacteria found in the mouth are species of the genera *Gemella, Granalucatella, Streptococcus* and *Veillonella* [108, 120]. Furthermore, additional niches exist in the oral cavity, such as supra- and subgingival plaque, which are densely populated by a large number of different microorganisms, the diversity of which can be similar to that of the intestinal tract [121, 122].

Stomach

The low pH of 1-2 in the stomach is too acidic for most microorganisms to survive. Until 30 years ago, it was considered to be a barrier for microorganisms, especially pathogens, to enter the body and survive [123, 124]. However, in 1984, Barry Marshall and Robin Warren isolated a gastric bacterium, which was thought to be linked to gastritis. Later on this bacterium was named *Helicobacter pylori* and is now known to be present in 50% of human beings, whereas only a minority shows gastritis [24]. Besides the understanding of *H. pylori*'s survival and maintenance in the stomach, not much is known about other species that dwell in the stomach. Bik *et al.* [123] found 128 phylotypes from 8 bacterial phyla present in the stomach. This diversity was much higher than expected thus far. Moreover, 50% of the phylotypes were assigned to uncultivated bacteria, and of these 67% were described earlier as bacteria from the mouth. Nevertheless, it remains a question whether these bacteria dwell in the stomach and whether they have adapted to different environments – mouth and stomach. To collect gastric fluid or mucosal tissue, a nasogastric or orogastric catheter can be used, which enters via the nose or mouth, respectively, and passes the esophagus to enter the stomach.

Small Intestine

The small intestine is considered the first region of the gastrointestinal tract where food meets the microbiota. It can be subdivided into the duodenum, jejunum and ileum. This region of the GI tract is hard to access compared to the mouth and large intestine and therefore less well studied. The small intestine can be sampled (like the stomach) by using an intraluminal nasogastric or orogastric catheter, that passes the stomach and part of the small intestine depending on the region where the sampling will occur by peristalsis [105, 125, 126]. The location can be determined using short-interval fluoroscopic control and calculating the distance from the pylorus to the tip [112, 125]. This method provides an indication of in which region the catheter is situated. However, due to considerable differences in the length of the small intestine in different individuals, the precise location cannot be determined. As an alternative, ileostomy subjects provide easier access to small intestinal content, and recently allowed detailed insight into structure and function of small intestinal bacterial communities [105, 125, 127]. These individuals have their colon removed, and the end of the ileum is surgically attached to an abdominal stoma. Despite not having a colon, ileostomized individuals can have a healthy life, and it could be shown by above-mentioned studies that they provide a suitable *in vivo* model system that enables analysis of the proximal small intestinal microbiota, rather than the ileum.

The microbial diversity in the small intestine is higher than in the stomach, but smaller than that found in the large intestine. The proximal small intestine is enriched with *Clostridium*

spp., *Streptococcus* spp. and *Veillonella* spp. [126]. In turn, the ileum shows a community dominated by *Bacteroidetes* and *Clostridium* cluster XIVa and is more similar to the ecosystem of the large intestine [105, 125, 127].

In addition to the possibilities outlined above, autonomous, ingestible intestinal sampling devices are being developed (e.g. http://www.micropharma.net), which would allow direct and programmed sampling of luminal as well as mucosal samples from predefined locations along the entire GI tract.

Large intestine

The large intestine can be subdivided into subparts: cecum, proximal, transversal and distal colon [109]. This region is densely populated by microbiota, the number of which can exceed 10^{11} cells per gram content. The richness is large and reaches up to 160 bacterial species per individual [106], of which 90% belong to the *Bacteroidetes* and the *Firmicutes* [128].

The transit time of the intestinal content through the large intestine is much longer than in the other regions of the intestinal tract. Here the more complex food ingredients remain at the end of the GI tract as the sole energy source for the microbiota. Undigested carbohydrates and some fraction of proteins are converted into a broad range of metabolites, of which short chain fatty acids (SCFA), including acetate, propionate and butyrate, are the most abundant. In turn, these metabolites are used by the host as an energy source. This area of the GI tract is almost entirely anaerobic, and many bacteria that inhabit this part of the intestine are (obligate) anaerobic bacteria.

To study the large intestine, usually fresh faeces are collected and analysed. However, the microbial community of faeces is quite different from that residing in the proximal large intestine [129]. This part still contains a lot of substrate for microbial growth, concentrations of which decrease towards the distal colon. Additionally, the obligate anaerobic species are much less prevalent in faeces than in the proximal large intestine [129]. To obtain samples from the large intestine colonoscopy can be used. However, to actually enter with a colonscope into the colon, patients need to take sedatives and be sober in the last hours. More importantly, in case colonoscopy is performed with prior bowel cleansing, the obtained picture on the remaining microbiota will be affected, although it should be noted that it has recently been shown that colonoscopy doesn't have a lasting effect on fecal microbiota composition [130]. Another option is to perform surgery on the patients in the large intestine; a more in depth review on sampling the large intestine is provided by Ouwehand & Vaughan [131]. Ingestible sampling devices such as those mentioned above might provide new possibilities also for undisturbed assessment of the

proximal colon.

Models of the gut

As described in the previous section, studying the different locations along the GI tract currently requires rather invasive sampling methods, however, invasive sampling from large numbers of healthy individuals is not feasible for practical and ethical reasons. *In vitro* and animal models provide an easier way to collect many (invasive) samples, have multiple comparisons and regulating the genotype background.

In vitro models

Solutions to the challenges explained above for *in vivo* studies can be the use of *in vitro* models, where a broad range of parameters can be measured during microbial fermentation. The *in vitro* models used to study the gut microbiota can be classified in batch fermentation models, continuous culture models and the TNO Intestinal Models (TIMs). The set-up and application of these different types of models has been extensively reviewed [132, 133]. *In vitro* models are usually inoculated with fecal samples. The fecal sample of an individual can be used in multiple comparisons at the same time, taking care that the different comparisons in the model are all originating from the same individual with the same genotype. By replicating particular conditions found in localized regions of the intestine, the gut microbiota in these models usually shifts to a microbial community more comparable to the corresponding intestinal region, validating to an extent the biological representativeness and value of the model.

In vitro fermentation models are mainly used to study the adaptation of the microbiota and the degradation of food or food ingredients [93, 134]. In the continuous culture and TIM models, probiotics are also tested by researchers to study their effects on the community and the washout time for these bacteria [135, 136]. Moreover by using membranes and filters, metabolites that are usually taken up by the host can be monitored during the fermentation process. However, mucus associated bacteria will not be present in these models. For this reason the M-SHIME was developed, where mucin-covered microcosms are introduced in the original SHIME model [137]. Probiotics can now be better studied in the M-SHIME model with respect to their adhesion to the mucosal layer and their colonization.

Animal models

In general, *in vitro* models do not allow researchers to study interactions between the host and the microbiota. Animal models and in particular mammalian models provide an

alternative way to study the *in vivo* responses to beneficial, commensal and pathogenic microorganisms in the GI tract. The main animals used to study the mammalian GI tract are rodents and pigs. Below we will discuss how they are used. However, to translate the knowledge gained from animal studies to the human situation, differences in physiology (see **Table 1** and **Figure 1**) and behavior between animals and humans concerning their GI tract need to be considered [109, 138, 139].

Table 1. pH of the GI tract of humans, pigs and rodents [109, 138, 139].

pH	Human	Rat	Pig
Stomach	1.0-4.4	3.3-5.1	2.2-4.3
Small intestine	5.5-7.5	6.5-7.1	6.0-7.5
Large intestine	5.9-7.0	6.6-7.4	6.3-7.1

Rodents

Conventional microbiota rodents

Rodents are often used to study the GI tract and relate this back to the human situation. These animals are relatively small, easy to keep and well known models to study drugs for humans. Like humans, the two main bacterial phyla of the rodent GI tract microbiota are the Bacteroidetes and the Firmicutes. Nevertheless, there are some differences in microbiota composition. For example, in mice *bifidobacteria* are present, but in lower abundance than in humans. Additionally, in mice, the phylum Fusobacteria is lacking, while presence of Deferribacteres and Gemmatimonadetes has been reported [117]. The mouse forestomach is colonized by a biofilm of *Lactobacillus reuteri*, and murine strains of this species are different from those found in humans that produce a vitamin B12 metabolosome [140]. Furthermore mice harbor segmented filamentous bacteria, related to clostridia, that have a profound effect on the maturation of the innate immune system but have been thought to be lacking in humans [141-143]. It should be noted that distinct populations of SFB have now also been shown in human infants during the first three years of life, even though no functional studies have yet been performed that would support a similar role in immune maturation as for their murine counterparts [144].

The microbiota composition in rodents is usually analyzed when diet-microbiota-host interactions are studied. To this end, rodents are usually on or shifted to a specific diet. After a certain time, animals are sacrificed in order to collect a range of different samples. For the purpose of microbiota analysis, in general two types of samples are collected from different locations along the GI tract, namely intestinal content as well as mucosal scrapings. The latter method allows recovery of the epithelial cell layer from the intestinal

tissue, and samples can be used to extract RNA for the analysis of host responses or to extract RNA or DNA to analyze the mucus associated microbiota.

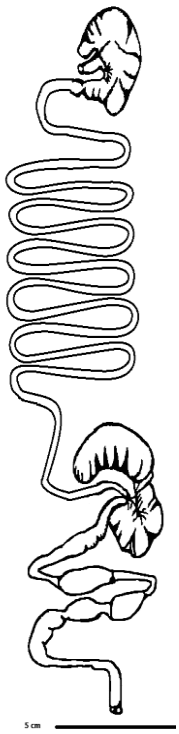

Figure 1A. Gross anatomy of the human GI tract.

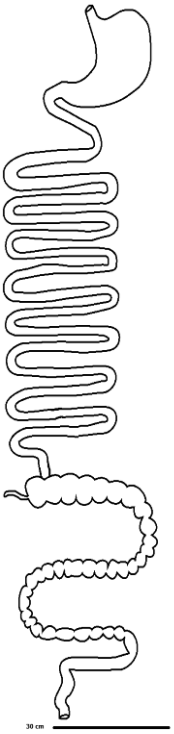

Figure 1B. Gross anatomy of the rat GI tract.

Figure 1C. Gross anatomy of the pig GI tract.

There are a large number of different strains of mice and rats available. For example, C57Bl/6 mice are generally used in studies related to diet-induced obesity, type 2 diabetes and atherosclerosis [100, 128]. Turnbaugh and co-authors [145], for example, showed that gut microbiota of obese mice have a more efficient fermentation then lean mice. This fermentation resulted in a higher energy yield for the obese mice then lean mice with the same food. Moreover the obese gut microbiota, with the corresponding phenotypes, could be transferred to germ-free mice.

The choice of an animal model with a certain phenotype, is based on strains that have or are sensitive to this phenotype. For less obvious phenotypes, like response to a change of food ingredients, the choice is more likely to be made for practical reasons, including e.g. the

animals are already bred in the facility, they are commercially available, or there is in-house experience with a given strain. However, recent studies have shown that environment and genetic background of mice have a significant impact on the microbial composition [146], and this must be taken into account when experiments are designed that compare to or proceed from previous data.

Next to these factors, behavioral aspects like eating patterns and coprophagy of rodents need to be considered when setting up experiments, as well as during the experiment itself. Rodents are usually eating around the clock, and have therefore always food in their system along the GI tract. Next to that they practise coprophagy – eating their own faeces, or that of their cage partners – which allows them to extract more nutrients from the food [147]. Also in terms of energy excretion coprophagy can have an impact, since the faeces that is eaten contains more water and nitrogen then normal faeces [148]. However, when studying the short-term effects of food intervention studies this can give a negative effect, unless at the start of intervention the faeces are removed.

Germ-free animals

Preferred animals for germ-free and gnotobiotic studies are mice. These animals are used for several purposes that include: to study the effect of colonization with one single bacterial species, a consortium of defined bacterial species or *de novo* colonization with a complex microbiota from animals with a specific genotypic and/or phenotypic background, or from other host species including humans. During conventionalization in mice it takes more than a week for the microbial community to stabilize, which needs to be taken into account in the experimental design [22]. Turnbaugh *et al.* [149] showed that transferring the microbiota of obese or lean mice into germ free mice resulted in a greater adiposity in those animals that received the faeces from obese mice. Another recent example of transferring a phenotype with its microbiota is the ability to transfer the production of testosterone from male mice to female mice. The female mice had higher testosterone levels when they received male microbiota via gavage [150].

Humanized rodents

Rats and mice that are born germ-free can be colonized by a slurry of human faeces, often referred to as “humanization” [151]. Although the difference in physiology can have an effect on the colonization of the human microbiota, these humanized animals are valuable models to study the human microbiota, and can provide information on the interaction between food ingredients, the human microbiota and the host. The microbial shifts occurring in these models due to a certain treatment are likely to take place in humans as well [152]. For instance, the microbiota of human twins discordant for obesity was studied

in mice, where the impact of the microbiota was linked with the phenotype of the human donor [132]. They could furthermore show that specific dietary changes could affect the original phenotypes concomitant with alterations in composition and activity of the microbial community.

Pigs

Pig is an important livestock for human because of their meat production. Therefore, these animals are studied intensively by researchers with the objective of production optimization. In recent years, many studies have focused on the GI tract of pigs, because manipulation of the gut microbiota can be used as an alternative of feeding antibiotics to improve pig health and growth. Additionally, pigs are scientifically important as a result of their high similarities to human beings in physiology, anatomy and nutrition [153-155]. It makes these animals essential as models for human GI tract studies.

Humanized pigs

The model of human flora-associated piglets (HFAP) was established by Pang *et al.* [156] through orally inoculating a whole fecal suspension of a healthy 10-year-old boy to cesarean section derived piglets that were raised in specific-pathogen-free (SPF) conditions. Culture-independent analysis showed that transplantation of human gut microbiota produced a donor-like microbial community in piglet gut with minimal individual variation, and the succession with aging of piglets was similar to that observed in humans. As in humans, the introduction of solid food during weaning altered the gut microbial community, resulting in a decrease in bifidobacteria. This change suggested the HFAP may share similarities with human in the process of microbial colonization, and implied the HFAP could be an attractive model to explore the effect of dietary factors on human gut microbiota. Subsequently, the HFAP model was successfully employed in prebiotic study. In order to assess the effects of short-chain fructo-oligosaccharides (scFOS) on gut microbiota, Shen *et al.* [157] applied this model and confirmed the bifidogenic property of scFOS. They found that the genus *Bifidobacterium* was stimulated consistently except during weaning, however, the effect of scFOS on non-bifidobacterial species varied at different developmental stages of the animals.

Gnotobiotic pigs

Gnotobiotic pigs have been used to study various human GI tract pathogens, such as *Helicobacter pylori* [158]. Recently, gnotobiotic pigs have been used as an animal model to study the microbial colonization during early life. Laycock *et al.* [159] used the Altered Schaedler Flora (ASF), a murine intestinal microbiota and a new “Bristol” microbiota

containing *Lactobacillus amylovorus* DSM 16698T, *Clostridium glycolicum* and *Parabacteroides sp* (ASF519), to colonize caesarean-derived gnotobiotic pigs prior to their gut closure. The ASF inoculation resulted in unreliable colonization with most (but not all) strains of the ASF. In contrast, the Bristol microbiota reliably colonized the length of the intestinal tract of gnotobiotic piglets. This microbiota can be used to study the consequences of early microbial colonization on development of the intestinal mucosa and immune system, on later colonization by a complex microbiota, and on subsequent susceptibility to disease.

Minipigs

Minipigs are proposed to be good animal models for studying obesity [160-162]. Pedersen *et al.* [163] investigated the composition of gut microbiota in relation to diet, obesity and metabolic syndrome in two pig models, Göttingen and Ossabaw minipigs. They found that diet seems to be the defining factor that shapes the gut microbiota as observed by changes in different bacteria divisions between lean and obese minipigs. In the cecum, the lean Göttingen minipigs' had significantly higher abundance of Firmicutes, *Akkermansia*, and *Methanobrevibacter*, while obese Göttingen had higher abundances of the phyla Spirochaetes, Tenericutes, Verrucomicrobia and the genus *Bacteroides*. With respect to the Ossabaw minipigs, the obese minipigs had a higher abundance of Firmicutes in terminal ileum and lower abundance of Bacteroidetes in colon compared with lean minipigs. Overall, the Göttingen and Ossabaw minipigs displayed different microbial communities in response to diet-induced obesity in the different sections of their intestine. This funding also reinforced the notion that the host genotype has to be taken into account when studying the links between microbiota, diet and phenotype.

Piglet model for infant nutrition and development

The piglet has been used extensively in infant nutrition researches. It has been suggested as an appropriate model for human infant because of similarities between piglet and infant in anatomy, physiology and gastrointestinal tract metabolism [153, 154, 164-167]. Moreover, piglet models have also been employed to evaluate the intestinal microbiota of neonates, and preterm and term infants. Development of the intestinal microbiota in neonates and infants is characterized by rapid and extensive changes in microbial abundance, diversity, and composition. These changes are influenced by medical, cultural, and environmental factors such as delivery mode, diet, familial environment, diseases, and therapies [9]. To study the effects of these factors, different piglet models have been developed. These piglet models allow us to generate more information of the dynamics of microbial colonization and its profound influence on intestinal and systemic health throughout life.

Piglet model for investigating effects of environmental microbial association on gut microbiota

To study the effects of environmental association with microbiota on gut health and development in the postnatal period, a model of caesarean derived piglets was designed by Jansman *et al.* [168]. In this model, piglets were obtained by caesarean delivery and were equally divided over two treatment groups that were housed in SPF conditions. All piglets received orally the above-mentioned Bristol microbiota consisting of *Lactobacillus amylovorus*, *Clostridium glycolicum*, and *Parabacteroides* sp. ASF519 on days 1, 2, and 3 after birth. On day 3 and 4 the piglets received either a complex microbiota by providing them with a fecal inoculant of an adult sow (complex association group) or a placebo inoculant (simple association group). By using 16S rRNA gene targeted microarray-based microbiota profiling method, they found fecal microbiota composition was less diverse in the simple association group than the complex association group. The differences of microbiota between treatments persisted for at least three weeks after birth.

Furthermore, other studies revealed that the immediate environment during postnatal development has long-term impact on gut community structure in pigs [51]. To investigate the extent to which early-life environment impacts on microbial diversity of the adult gut, Mulder and colleagues established a model with genetically-related piglets, which were housed in either indoor or outdoor environments or in experimental isolators [52]. Analysis of over 3,000 16S rRNA sequences revealed major differences in mucosa-adherent microbial diversity in the ileum of adult pigs attributable to differences in early-life environment. Pigs housed in a natural outdoor environment showed a dominance of Firmicutes, in particular *Lactobacillus*, whereas pigs housed in a hygienic indoor environment had reduced *Lactobacillus* abundance and higher numbers of potentially pathogenic phylotypes. This result revealed a strong negative correlation between the abundance of Firmicutes and pathogenic bacterial populations in the gut, and the microbial composition differences were exaggerated in animals housed in experimental isolators. This study demonstrated strong influences of early-life environment on gut microbiota composition in adult pigs, leading to a follow-up study on the impact of limiting microbial exposure during early life on the development of the gut microbiota [169]. In the following study, the outdoor- and indoor-reared piglets, exposed to the microbiota in their natural rearing environment for the first two days of life, were transferred to an isolator facility; and the gut microbial diversity of adult pigs was analyzed by 16S rRNA gene sequencing. Although the initial maternal and environmental microbial inoculum of isolator-reared animals was identical to that of their naturally-reared littermates, the microbial succession and stabilization events reported previously in naturally-reared outdoor animals did not occur. In contrast, the gut microbiota of isolator-reared animals remained highly diverse containing a large number of distinct phylotypes. These results indicated that establishment

and maturation of the normal gut microbiota requires continuous microbial exposure during the early stages of life, and this process is compromised under conditions of excessive hygiene.

Piglet model for studying gut microbiota in diseases

For preterm neonates, one of the most serious diseases is the GI inflammatory disorder necrotizing enterocolitis (NEC). The development of this disease includes the interplay of nutritional, microbial and immunological determinants. For independent studies of each determinant under clinically relevant conditions, the preterm piglet has been utilized in virtue of its beneficial characteristics compared to other animal models [170]. With preterm piglets, Sangild *et al.* [171] found that NEC pigs showed bacterial overgrowth and a high mucosal density of *C. perfringens* in some but not all animals, however mucosal microbial diversity of healthy pigs remained low and independent of diet. This finding was further confirmed by Cilieborg *et al.* [172]; their study showed a different microbiota with high *C. perfringens* abundance in preterm pigs with NEC compared with healthy individuals. However, the *C. perfringens* inoculation failed to induce NEC. This indicated that *C. perfringens* is more abundant in pigs with NEC but rather as a consequence than a cause of disease. In addition to the above studies, Azcarate-Peril *et al.* [173] have used a unique preterm piglet model to characterize spontaneous differences in microbiome composition of NEC-predisposed regions of the gut. Their study provided strong support for ileal mucosa as a focus for investigation of specific dysbiosis associated with NEC and suggested a significant role for *Clostridium* spp., and members of the Actinobacteria and Cyanobacteria in the pathogenesis of NEC.

The short bowel syndrome (SBS) piglet model is another application of the piglet model for studying gut microbiota in diseases. The development of a successful SBS model in neonatal piglets provides a possibility for characterizing the colonic microbiota following small bowel resection (SBR). By using 4-week old female piglets that received a 75% SBR, Lapthorne *et al.* [174] found a significant level of dysbiosis both two and six weeks post-SBR, particularly in the phylum Firmicutes, coupled with a decrease in overall bacterial diversity in the colon.

Sampling techniques with pig models in kinetic microbiota studies

Small intestinal segment perfusion technique with pig models

The Small intestinal segment perfusion (SISP) technique was developed to study the effects of bacteria on net absorption of fluid and electrolytes, as a more comprehensive and ethical alternative to the ligated loop test in pig models [175]. Recently, this technique has been

widely applied to study enterotoxigenic *Escherichia coli* infection [176, 177], *Salmonella typhimurium* invasion [178, 179], and mannose-specific interaction of *Lactobacillus plantarum* with jejunal epithelium [180]. Furthermore, SISP can be applied in future research to investigate the functional physiological response of probiotics and the crosstalk between probiotics and the host [181]. In the SISP test, pigs are sedated with azaperone, induced with inhalation anaesthesia and maintained with sevoflurane and nitrous oxide. For pig small intestine, five pairs of segments are prepared and each segment is 20 cm long with inlet tubes at the cranial side and outlet tubes at the caudal side. The segments can be used to study up to 10 perfused ingredients within one pig. All 10 segments are perfused simultaneously either by an infusion system or manually with syringes attached to the cranial tubes for up to 10 hours. Effluent fluid during perfusion and mucosal scraping can be sampled for microbial analysis from each segment.

Cannulation technique with pig models

Cannulation is one of the most frequently applied methods for repeated sampling of digesta from pig gut. Different cannulation techniques, such as simple T-cannula, post-valvular T-cecum cannulation and steered ileocecal valve cannulation, can be employed based on the specific research purpose. Among these cannulation techniques, the simple T-cannula is widely used for evaluating the microbial composition and function in the ileum. A simple T-cannula is normally inserted 10 to 20 cm anterior to the ileo-cecal valve. It does not transect the small intestine wall, and can maintain a normal physiological state of the intestine [182]. Currently, surgical procedures for inserting a T-cannula and sampling methods have been established for young pigs [183, 184], growing pigs [185, 186] and pregnant sows [187].

Conclusion

Studying the human GI tract can be done with a wide range of methods and technical approaches. Even though each of the methods that we described here have advantages and disadvantages, usually human faeces are used for assessing the impact on the microbiota in intervention studies, whereas animal models are used for more detailed mechanistic studies, including those that aim to see the impact of the host system as well. Nowadays mainly rodents are used to study the human GI tract, while pigs show promising and maybe better comparison to the human GI tract. Since the optimal system is not yet established, generally to test a certain treatment a combination of methods is used, first *in vitro*, then *in vivo* in an animal model, ending up with a human intervention study.

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Chapter 3

Influence of maternal antibiotic treatment on intestinal microbiota in piglets

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To be submitted.

Abstract

Amoxicillin is a commonly used antibiotic to control infections of pregnant women in the hospital, and in reproduction and growing animals on a farm. However, it is unclear to what extent the maternal antibiotic treatment affects intestinal microbiota development and gut health in the offspring. Using a pig model, two groups of five sows each were defined as control and antibiotic group, respectively. In the antibiotic group, sows were given amoxicillin orally at a daily dosage of 40 mg/kg body weight during 10 days before and 21 days after parturition. The sows' offspring (piglets) were sacrificed at 14, 21, 28 (weaning) and 42 days after birth. The microbial composition of sows' faeces and piglets' ileal and colonic content was analyzed by using the Pig Intestinal Tract Chip (PITChip). The results indicated that amoxicillin treatment drastically impacted the sows' faecal microbiota, and caused a decrease in relative abundance of bacteria related to the genera *Lactobacillus, Staphylococcus and Streptococcus*. Maternal amoxicillin treatment furthermore influenced the gut microbiota of the piglets. Especially in the ileum, maternal amoxicillin led to increased relative abundance of Proteobacteria during amoxicillin administration (at day 14 and 21), mainly driven by a stimulation of *Escherichia coli* and *Pseudomonas*. Interestingly, most microbial groups that were affected in faeces of amoxicillin-treated sows showed an opposite trend in the ileal content of their offspring. Nevertheless, a significantly higher similarity of microbial composition was detected between the faeces of amoxicillin-treated sows and ileal content of their offspring compared with the control sows and piglets. Our findings indicate that maternal amoxicillin treatment indirectly affects the gut microbiota of offspring through disturbing the maternal microbiota and the transfer of maternal microbiota to offspring.

Keywords: Maternal antibiotic treatment, Amoxicillin, Gut, Microbiota, Piglet, Sow, Offspring

Introduction

Microbiota colonizing the intestinal tract around birth plays an important role in human and animal health. Establishment of microbiota at early age is instrumental in regulating and fine-tuning the immune system throughout life [20], and is also suspected to be associated with certain diseases such as obesity and inflammatory bowel disease later in life [3, 188-190]. Establishment of gut microbiota of humans and animals can be influenced by a number of perinatal factors, including, most importantly, the maternal microbiota. Accumulating evidence suggests that the microbial contact of the offspring with their mothers may begin already prior to birth [5, 7, 27], and after birth, gut-associated bacteria can still be vertically transferred from mother to neonate via breastfeeding [191]. Thus, disturbances of maternal microbiota may influence the transfer of maternal microbiota from mother to her offspring, which may causally influence the gut microbiota development and the intestinal health of offspring throughout life.

Antibiotics have been commonly used to control infections of pregnant women in hospitals and in reproduction and growing animals on farms. Antibiotic treatment of mothers can impact fetal development and associates with low birth weight of neonates and high risk of diseases such as allergy, metabolic syndrome, and obesity at early age [39, 192]. Recently, researchers also found that antibiotic use during pregnancy altered the commensal vaginal microbiota of women [40]. Intrapartum antibiotic treatment of mothers was found associated with a decreased transmission of vaginal *Lactobacillus* to the neonate during birth [41]. Together, these results indicate that maternal antibiotic treatment may influence the gut microbial colonization of offspring through changing maternal microbiota composition. In addition, it is well known that maternal antibiotic residues can be transferred from mothers to their offspring via breastfeeding [193]. However, it is still unknown to which extent the maternal antibiotic residues affect the gut microbiota establishment of infants and young animals, and hence, studies should be directed at assessing the effect of maternal antibiotic treatment on the gut microbiota development of humans and animals at early age.

In the present study, we evaluated the effect of maternal amoxicillin treatment on sow's faecal microbiota and on the ileal and colonic microbiota of their offspring up to 42 days after birth. Amoxicillin is a broad-spectrum antibiotic widely used during pregnancy in humans [194], and also the most used antibiotic for treating mastitis and urinary tract infections of sows during pregnancy as well as after farrowing in livestock. Currently, most studies have only focused on the effect of maternal amoxicillin usage on foetal development and risk of disease at early age [39, 195]. Therefore, we developed a pig model in which the mothers received amoxicillin orally around parturition, and piglets were serially sacrificed for assessing gut microbiota development. The pig is regarded as an

appropriate model due to its similarities to humans with respect to GI anatomy, physiology, metabolism and immunity [153-155, 196]. Especially, piglets have been extensively used in infant nutrition research [164, 166, 167]. Moreover, the pig is an important livestock animal, and therefore researchers have studied piglets intensively in order to enhance their robustness and pork production through regulating the gut microbiota of piglets at early age [168, 197]. All together, the outcome of this study will be beneficial for both human infant research and livestock production.

Material and methods

Animals, feeding and sample collection

The animal experiment was conducted as described by Arnal *et al* [197]. Briefly, twenty four crossbred (Large White x Landrace) sows were used in two successive batches, taking into account parity and resistance of selected faecal bacteria (*Escherichia coli*, *Campylobacter* spp., and *Enterococcus* spp.) to amoxicillin. Sows with amoxicillin-sensitive bacteria were assigned to the antibiotic group in priority. The remaining sows were assigned to the control group. For the antibiotic group, sows were orally given the broad spectrum antibiotic amoxicillin (40 mg/kg body weight, Vetrimoxin PO containing 10% amoxicillin; CEVA Santé Animale, Loudéac, France) in their morning meal (2 kg/day) daily from 10 days before the estimated farrowing date till 21 days after farrowing. Sows and offspring were fed balanced diets formulated to meet nutritional requirements [197]: sows were fed the gestating diet (3.5 kg/day) or the lactating diet (ad libitum) in two meals, and offspring had ad libitum access to pre-starter diet from weaning at 28 days till day 42. Due to delays between conducting batch 1 and batch 2, only data from batch 1 was included in this manuscript. To this end, five sows and their offspring were randomly selected from both groups in the first batch of pigs for microbial analysis. Offspring were sacrificed at the age of 14, 21, 28 (age of weaning), and 42 days. Ileal and colonic content were collected from offspring for analyzing the microbial composition. Faecal samples were also collected from the sows at the start of the antibiotic treatment and 21 days after farrowing (the end of antibiotic treatment) for assessing amoxicillin effects on sows' microbiota.

Microbiota analysis

The microbial composition of intestinal content of piglets and faeces of sows was analyzed using the Pig Intestinal Tract Chip (PITChip) version 1.0. The PITChip is a phylogenetic microarray, with more than 2,980 oligonucleotides based on 16S rRNA gene sequences of 627 porcine intestinal microbial species-level phylotypes [97, 101]. The PITChip as well as the corresponding Human Intestinal Tract Chip (HITChip) [103], provide a very deep and

reproducible phylogenetic analysis that has been compared with deep pyrosequencing of 16S rRNA gene fragments [97, 104, 105] and next generation parallel sequencing of intestinal metagenomes [106], indicating comparable resolution and a higher sensitivity of the chip based analysis at commonly used sequencing depth. The protocol for hybridization and analysis of the generated data was performed essentially as described before for the HITChip [103]. Briefly, microbial DNA was extracted from 250 mg of intestinal content of piglets and faeces of sows using a faecal DNA extraction protocol adapted from Yu and Morrison [198] as described by Salonen *et al.* [199]. Bacterial 16S rRNA gene was amplified using the primers T7 prom-Bact-27-for and Uni-1492-rev. PCR products were *in vitro* transcribed into RNA and the purified resultant RNA was coupled with CyDye prior to fragmentation and hybridization to the array. Microarray images were processed using Agilent's Feature Extraction Software version 9.1 (http://www.agilent.com). Data was retrieved from the MySQL (version 5.1) database as describe by Rajilic-Stojanovic [200] and pre-processed using the R (Rx64 2.12.2) microbiome package (http://microbiome.github.com/), settings on default.

Statistical methods

Principal response curve (PRC) analysis was applied to detect the microbial variation between control and amoxicillin-treated sows over time. PRC is based on redundancy analysis (RDA) adjusted for overall changes in community response over time, as described by Van den Brink and Braak [201]. PRC focuses on the time-dependent treatment effects and allows these effects to be quantitatively interpreted at the species level. For PRC, the principal component is plotted against time, yielding a principal response curve of the community for each treatment. In the present study, treatment classes control and amoxicillin treatment were introduced as environmental explanatory variables. Sampling times (beginning and end of amoxicillin treatment) were introduced as co-variables. The responsive variables were the relative contribution of 143 level-2 (approximate genus-level, 90% 16S ribosomal RNA similarity threshold) phylogenetic groups targeted by the PITChip 1.0. Monte Carlo permutation tests were performed to evaluate statistical significance of the effect of amoxicillin on sow's and piglets' microbiota. These tests were also performed per sampling time, allowing to test the significance of the effect of the treatment for each sampling time.

Univariate testing was performed to detect which microbial groups were affected by maternal amoxicillin treatment in the ileal and colonic content of piglets at each sampling time by using a Mann-Whitney U signed rank test. P-values were corrected for multiple testing using Benjamini–Hochberg's approach [202].

Level-2 phylogenetic groups (143 microbial groups) of the PITChip 1.0 were used to

calculate Spearman's correlation coefficient of microbiota between each sow and its offspring. Spearman's correlation coefficients between treated sows and their offspring were compared with Spearman's correlation coefficients between control sows and their offspring by a Student's t-test.

Results

Amoxicillin affected sows' microbiota

The composition of the sows' faecal microbiota was analyzed using the PITChip phylogenetic microarray, and was found to be significantly affected by the amoxicillin treatment as demonstrated by Principal Response Curve (PRC) analysis (**Fig. 1**). While no significant differences in microbiota composition were found between control and antibiotic treated sows at the start of antibiotic treatment (P=0.24), a significant difference was found when comparing microbiota of control and treated sows at the end of antibiotic treatment (P=0.01). Level-2 (approximate genus level, 90% sequence similarity threshold) groups with high positive weight were decreased in relative abundance in the faeces of *Lactobacillus acidophilus, Lactobacillus delbrueckii, Lactobacillus gasseri, Lactobacillus plantarum, Staphylococcus aureus, Streptococcus bovis, Streptococcus intermedius, Streptococcus suis, Clostridium difficile, Clostridium perfringens, Pseudomonas* and *Staphylococcus aureus*. In contrast, bacterial groups with low negative weight increased

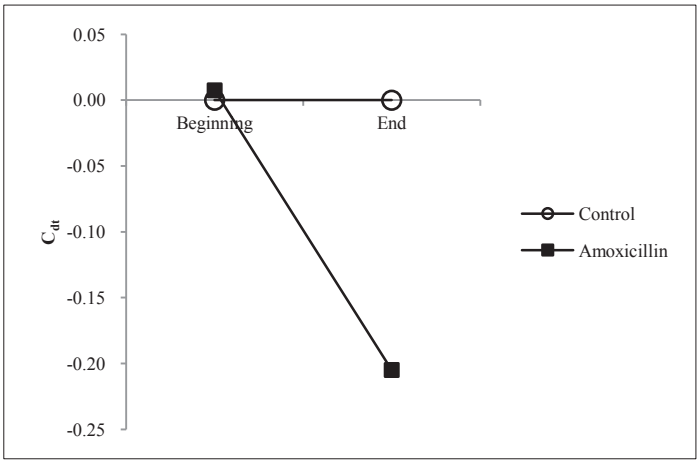

Figure 1. Principal response curves indicating the effects of amoxicillin on faecal microbiota of sows at the beginning (beginning) and the end (End) of amoxicillin treatment.

Of all variance, 10.2% is attributed to sampling date and displayed on the horizontal axis, 26.5% is attributed to treatment, and 63.3% was attributed to differences between piglets. Of the variance explained by treatment, 75.9 % is displayed on the vertical axis. The lines represent the course of the treatment levels over time.

Table 1. Amoxicillin affected microbial groups in the faeces of sows.

Class	Group	Species weight[1]	Beginning ARC[2]		End ARC[3]	
			Control	Amoxicillin	Control	Amoxicillin
Bacteroidetes	*Alistipes* et rel.	-2.56	0.28±0.29	0.38±0.25	0.16±0.17	1.10±0.98
	Bacteroides distasonis et rel.	-1.04	0.35±0.24	0.36±0.12	0.15±0.05	0.43±0.20
	Porphyromonas asaccharolytica et rel.	2.22	0.44±0.19	0.90±0.55	1.09±0.46	0.33±0.23
	Prevotella melaninogenica et rel.	-1.13	0.18±0.07	0.21±0.08	0.12±0.04	0.43±0.19
	Uncultured *Bacteroidetes*	-2.19	0.39±0.35	0.44±0.23	0.07±0.03	0.76±0.58
	Uncultured *Prevotella*	-3.47	2.13±1.75	2.50±0.71	0.84±0.35	2.97±1.45
Fibrobacteres	*Fibrobacter succinogenes* et rel.	2.51	0.57±0.13	1.03±0.40	1.26±0.47	0.34±0.15
Bacilli	*Bacillus* et rel.	1.01	2.14±0.57	1.47±0.56	2.73±0.62	2.00±0.41
	Lactobacillus acidophilus et rel.	1.37	0.45±0.30	0.27±0.10	0.46±0.15	0.10±0.04
	Lactobacillus delbrueckii et rel.	2.51	0.37±0.18	0.75±0.35	0.77±0.53	0.02±0.01
	Lactobacillus gasseri et rel.	7.00	2.41±1.22	4.63±2.83	4.27±2.82	0.04±0.04
	Lactobacillus plantarum et rel.	2.78	2.76±1.53	2.36±0.92	2.79±2.27	0.91±0.24
	Staphylococcus aureus et rel.	0.80	1.27±0.29	0.91±0.38	1.58±0.31	1.18±0.26
	Streptococcus bovis et rel.	1.15	1.12±1.46	0.77±0.66	0.64±0.35	0.27±0.11
	Streptococcus intermedius et rel.	0.83	0.54±0.81	0.27±0.19	0.30±0.18	0.09±0.10
	Streptococcus suis et rel.	1.47	1.37±2.27	0.59±0.43	0.71±0.37	0.25±0.27
Clostridium cluster I	*Clostridium perfringens* et rel.	1.18	5.10±2.21	2.91±1.40	7.59±2.37	5.66±1.94
Clostridium cluster IV	*Faecalibacterium* et rel.	-0.93	0.88±0.19	1.40±0.93	0.88±0.34	1.28±0.29
	Faecalibacterium prausnitzii et rel.	-0.85	2.14±0.22	2.89±1.07	1.95±0.32	2.52±0.19
	Ruminococcus callidus et rel	-0.93	3.83±0.86	2.67±0.95	3.13±0.62	3.91±0.47
	Sporobacter termitidis et rel.	-1.28	8.84±1.67	10.46±1.77	8.06±1.94	10.64±1.14
Clostridium cluster IX	*Megasphaera elsdenii* et rel.	0.84	0.24±0.20	0.13±0.08	0.23±0.29	0.01±0.02
Clostridium cluster XI	*Clostridium difficile* et rel.	1.31	3.06±1.53	2.07±1.25	4.25±1.06	3.02±1.07
Clostridium cluster XIVa	*Lachnospira pectinoschiza* et rel.	-0.84	2.42±0.47	2.23±0.37	2.19±0.24	2.78±0.15
Mollicutes	*Mycoplasma*	2.61	0.47±0.18	1.07±0.33	1.04±0.48	0.19±0.10
	Solobacterium moorei et rel.	-1.00	0.98±0.55	0.80±0.22	0.72±0.37	1.07±0.12
Planctomycetacia	Uncultured *planctomycetacia*	-1.40	0.03±0.02	0.05±0.05	0.02±0.01	0.39±0.28
Gammaproteobacteria	*Actinobacillus* et rel.	-0.95	0.44±0.16	0.45±0.08	0.42±0.09	0.74±0.28
	Pseudomonas et rel.	1.01	1.26±0.30	0.94±0.41	1.54±0.25	1.05±0.22

[1] Species weight: the affinity of the microbial groups with the Principal Response Curves (PRC). Microbial groups with a high negative weight are inferred to show the opposite pattern, whereas microbial groups with near zero weight either show no response or a response that is unrelated to the pattern shown by the PRC. The higher the weight, the more the actual response pattern of microbial groups is likely to follow the pattern in the PRC. Microbial groups with a species weight between -0.8 and -0.8 are not shown.

[2] Beginning ARC: Average relative contribution [%] of a microbial group at the beginning of amoxicillin treatment. Values represent means ± SDs.
[3] End ARC: Average relative contribution [%] of a microbial group at the end of amoxicillin treatment. Values represent means ± SDs.

their relative abundance in the faeces of treated sows after antibiotic treatment, including bacteria related to *Bacteroidetes* and *Clostridium* cluster IV (**Table 1)**. Despite the change in relative abundance of specific microbial groups, the antibiotic treatment did not significantly alter the microbial diversity as indicated by Shannon indices (P>0.05) (**Supplemental Fig. 1**).

Maternal amoxicillin treatment affected ileal microbiota of offspring

To identify microbial groups affected by maternal amoxicillin treatment, we used univariate testing to assess phylogenetic groups at both phylum and genus-like (level-2) level at each sampling day. At phylum level, the relative abundance of Firmicutes tended to decrease in the ileum of treated piglets at day 14 after birth, whereas the relative abundance of Proteobacteria tended to increase (**Table 2**). This increase was driven by bacteria related to level-2 groups belonging to the uncultured *Deltaproteobacteria*, *E. coli* and *Pseudomonas* (**Table 3**). Despite a decreasing trend of Firmicutes at the phylum level, bacteria related to the level-2 groups *Bacillus, Lactobacillus acidophilus*, *Staphylococcus aureus* and *Subdoligranulum* increased their relative abundance in the piglets born to amoxicillin-treated sows at day 14 after birth (**Table 3**). At day 21 after birth, the increasing trend of Proteobacteria at phylum level was still detected for the maternal amoxicillin-treated piglets. As on day 14, bacteria related to the level-2 groups *Lactobacillus acidophilus, E. coli, Pseudomonas* and *Leptospira* increased for the maternal amoxicillin-treated piglets at day 21 (**Table 3**).

Table 2. Phylum-level phylogenetic groups with different abundance in the ileum of control and maternal amoxicillin treated piglets.

	***P* value**	**Effect[1]**	**ARC[2] (%)**	
			Control	**Amoxicillin**
Day 14				
Firmicutes	0.095	-	69.876±2.328	66.667±2.527
Proteobacteria	0.095	+	16.938±1.476	20.208±2.770
Day 21				
Proteobacteria	0.095	+	18.930±2.575	22.099±3.082
Day 28				
Fusobacteria	0.032	-	0.361±0.114	0.223±0.044
Spirochaetes	0.056	-	1.439±0.270	1.088±0.271

[1] Effect: indicates whether the average relative contribution of a phylum was increased (+) or decreased (-) in the ileum of maternal

amoxicillin treated piglets in comparison with control piglets.
[2] ARC: Average relative contribution [%] of a phylum. Values represent means ± SDs. The phyla with a relative abundance lower than 0.1% in all treatments are not shown.

Table 3. Genus-level phylogenetic groups with different abundance in the ileum of control and maternal amoxicillin treated piglets.

					ARC[3]	
Class	**Group**	***p* value**	**C.*p*[1]**	**Effect[2]**	**Control**	**Amoxicillin**
Day 14						
Bacteroidetes	*Alistipes* et rel.	0.056	0.955	-	0.028±0.010	0.021±0.008
Bacilli	*Bacillus* et rel.	0.095	0.955	+	2.180±0.349	2.539±0.615
	Lactobacillus acidophilus et rel.	0.032	0.955	+	1.023±0.734	2.352±0.652
	Staphylococcus aureus et rel.	0.095	0.955	+	1.367±0.135	1.623±0.269
Clostridium cluster IV	*Subdoligranulum* et rel.	0.095	0.955	+	0.565±0.187	0.851±0.314
Clostridium cluster IX	*Megasphaera elsdenii* et rel.	0.016	0.955	-	0.026±0.024	0.006±0.001
Deltaproteobacteria	Uncultured *deltaproteobacteria*	0.095	0.955	+	0.413±0.117	0.524±0.134
Epsilonproteobacteria	*Helicobacter*	0.095	0.955	-	0.172±0.248	0.069±0.088
Gammaproteobacteria	*Escherichia coli* et rel.	0.056	0.955	+	1.595±0.534	2.634±0.719
	Pseudomonas et rel.	0.095	0.955	+	1.287±0.374	1.817±0.506
Spirochaetes	*Leptospira*	0.056	0.955	+	0.500±0.098	0.658±0.108
Day 21						
Actinobacteria	*Aeriscardovia* et rel.	0.008	0.567	-	0.284±0.133	0.105±0.034
	Bifidobacterium	0.008	0.567	-	1.435±0.615	0.641±0.192
	Olsenella et rel.	0.016	0.567	-	0.062±0.069	0.012±0.006
Bacilli	*Lactobacillus acidophilus* et rel.	0.095	0.676	+	0.937±0.596	2.485±1.206
	Tetragenococcus	0.016	0.567	-	0.121±0.080	0.049±0.010
Clostridium cluster I	*Clostridium perfringens* et rel.	0.056	0.676	+	3.272±0.581	4.405±0.890
Clostridium cluster IV	Uncultured *Clostridia* IV	0.032	0.676	+	0.764±0.111	1.001±0.109
Clostridium cluster XIII	*Peptoniphilus indolicus* et rel.	0.056	0.676	+	0.210±0.033	0.295±0.068
Clostridium cluster XIVa	*Clostridium hylemonae* et rel.	0.095	0.676	+	0.037±0.007	0.050±0.012
	Clostridium sphenoides et rel.	0.056	0.676	+	0.112±0.019	0.151±0.032
Clostridium cluster XVII	*Catenibacterium*	0.032	0.676	-	1.322±1.024	0.478±0.192
Alphaproteobacteria	*Labrys methylaminiphilus* et rel.	0.095	0.676	+	2.311±0.66	2.803±0.430
Epsilonproteobacteria	*Candidatus helicobacter*	0.095	0.676	-	0.230±0.125	0.117±0.071
Gammaproteobacteria	*Acitenobacter* et rel.	0.095	0.676	+	0.321±0.066	0.453±0.110
	Escherichia coli et rel.	0.095	0.676	+	2.072±0.910	2.988±0.702
	Pseudomonas et rel.	0.056	0.676	+	1.363±0.266	2.140±0.609
Spirochaetes	*Leptospira*	0.095	0.676	+	0.490±0.144	0.699±0.156
Day 28						
Actinobacteria	*Tonsillophilus*	0.095	0.681	-	0.401±0.209	0.222±0.079

Bacteroidetes	*Bacteroides distasonis* et rel.	0.095	0.681	-	0.089±0.029	0.055±0.008
	Bacteroides fragilis et rel.	0.056	0.681	-	0.021±0.004	0.015±0.002
	Bacteroides pyogenes et rel.	0.032	0.681	-	0.019±0.005	0.012±0.002
	Bacteroides vulgatus et rel.	0.032	0.681	-	0.011±0.002	0.007±0.001
	Paludibacter propionicigenes et rel.	0.056	0.681	-	0.011±0.002	0.008±0.001
	Porphyromonas asaccharolytica et rel.	0.095	0.681	+	0.696±0.607	1.079±0.683
	Prevotella melaninogenica et rel.	0.095	0.681	-	0.087±0.027	0.054±0.008
	Prevotella ruminicola et rel.	0.095	0.681	-	0.022±0.007	0.014±0.002
	Uncultured *Bacteroidetes*	0.056	0.681	-	0.029±0.005	0.023±0.003
	Uncultured *Prevotella*	0.095	0.681	-	0.521±0.168	0.316±0.052
Flavobacteria	*Myroides odoratus* et rel.	0.095	0.681	-	0.021±0.007	0.013±0.002
Deferribacteres	*Mucispirillum schaedleri* et rel.	0.056	0.681	-	0.010±0.002	0.007±0.001
Clostridium cluster XI	*Clostridium difficile* et rel.	0.095	0.681	-	4.796±1.780	2.370±0.707
Clostridium cluster XVII	*Catenibacterium*	0.056	0.681	-	0.487±0.151	0.330±0.040
Mollicutes	*Mycoplasma*	0.095	0.681	+	0.598±0.494	0.989±0.600
Fusobacteria	*Fusobacterium*	0.032	0.681	-	0.361±0.114	0.223±0.044
Alphaproteobacteria	*Sphingomonas* et rel.	0.016	0.681	-	0.038±0.014	0.017±0.004
Gammaproteobacteria	Uncultured *Gammaproteobacteria*	0.056	0.681	+	1.784±0.205	2.182±0.324
Spirochaetes	*Treponema* et rel.	0.032	0.681	-	0.537±0.199	0.208±0.081
Day 42						
Actinobacteria	*Eggerthella* et rel.	0.056	0.835	-	0.595±0.107	0.463±0.079
Bacteroidetes	*Bacteroides fragilis* et rel.	0.056	0.835	+	0.017±0.006	0.023±0.004
	Bacteroides vulgatus et rel.	0.056	0.835	+	0.008±0.003	0.012±0.002
	Paludibacter propionicigenes et rel.	0.095	0.835	+	0.008±0.003	0.011±0.002
Sphingobacteria	Uncultured *Sphingobacteria*	0.056	0.835	+	0.009±0.001	0.013±0.004
Deferribacteres	*Mucispirillum schaedleri* et rel.	0.095	0.835	+	0.008±0.003	0.011±0.002
Bacilli	*Tetragenococcus*	0.056	0.835	+	0.057±0.004	0.066±0.007
Mollicutes	*Mycoplasma*	0.056	0.835	-	0.407±0.276	0.183±0.072

[1] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[2]Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the ileum of maternal amoxicillin treated piglets in comparison with control piglets.
[3] ARC: Average relative contribution [%] of a microbial group. Values represent means ± SDs. The microbial groups with a relative abundance lower than 0.01% in all treatments are not shown.

On the contrary, maternal amoxicillin treatment caused a reduction in relative abundance of bacteria related to the level-2 group *Bifidobacterium* in piglets sacrificed at day 21 after birth. At day 28 after birth, we detected a significant decrease of Fusobacteria and a decreasing trend of Spirochaetes at the phylum level, and bacteria related to 10 level-2 groups within the class of *Bacteroidetes* decreased their relative abundance in the ileum of maternal amoxicillin-treated piglets. Interestingly, bacteria related to the level-2 groups of *Bacteroides fragilis*, *Bacteroides vulgatus* and *Paludibacter propionicigenes* showed an

opposite trend at day 42 in comparison with day 28 as their relative abundance increased in the ileal content of treated piglets at day 42. Similar opposing trends at day 42 compared with day 28 were observed for bacteria related to level-2 groups *Mucispirillum schaedleri* and *Mycoplasma*. Strikingly, most microbial groups that were affected both in faeces of amoxicillin-treated sows and ileal content of their offspring showed an opposite direction of change (**Supplemental Table 1**).

The similarity of the microbiota was calculated between the faecal sample of each sow and the ileal samples of its offspring by using Spearman's correlation coefficients. The amoxicillin-treated sows and their offspring showed a significantly higher similarity of their microbiota compared with the control sows and their offspring at all sampling dates ($P<0.01$, **Fig. 2**).

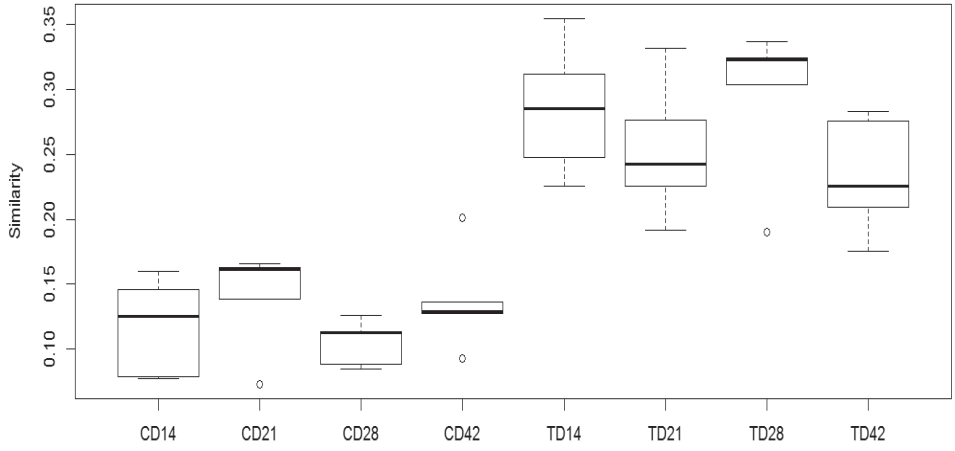

Figure 2. Box plots of total microbiota profile similarity between piglets' ileal content and sows' faeces at day 14 (D14), 21(D21), 28(D28) and 42(D42) after birth. Spearman's correlation coefficient was calculated for each sow and its offspring. The average microbiota similarity between amoxicillin treated sows and their offspring are significantly higher than between control sows and their offspring at all sampling time ($P<0.01$). Capital letters "C" and "T" represent the control piglets and maternal amoxicillin treated piglets, respectively.

Table 4. Phylum-level phylogenetic groups with different relative abundance in the colon of control and maternal amoxicillin treated piglets.

	P value	Effect[1]	ARC[2]	
			Control	Amoxicillin
Day 14				
Bacteroidetes	0.095	-	3.665±2.237	2.559±2.711
Day 21				
Fibrobacteres	0.095	-	0.865±0.211	0.535±0.279
Day 42				
Fusobacteria	0.095	+	0.218±0.076	0.279±0.099

[1] Effect: indicates whether the average relative contribution of a phylum was increased (+) or decreased (-) in the colon of maternal amoxicillin treated piglets in comparison with control piglets.
[2] ARC: Average relative contribution [%] of a phylum. Values represent means ± SDs. Phyla with a relative abundance lower than 0.1% in all treatments are not shown.

Table 5. Genus-level phylogenetic groups with different abundance in the colon of control and maternal amoxicillin treated piglets at day 14 after birth.

Class	Group	*P* value	C.*p*[1]	Effect[2]	ARC[3]	
					Control	Amoxicillin
Bacteroidetes	*Bacteroides fragilis* et rel.	0.056	0.799	-	0.123±0.108	0.046±0.062
Sphingobacteria	*Sphingobacterium thalpophilum* et rel.	0.032	0.799	-	0.118±0.062	0.034±0.021
Bacilli	*Streptococcus thoraltensis* et rel.	0.056	0.799	+	0.038±0.008	0.060±0.028
Clostridium cluster IV	*Ruminococcus bromii* et rel.	0.095	0.799	-	0.158±0.075	0.073±0.05
Clostridium cluster IX	*Veilonella*	0.095	0.799	+	0.073±0.009	0.103±0.038
Clostridium cluster XIVa	*Clostridium nexile* et rel.	0.056	0.799	+	0.089±0.048	0.300±0.203
	Clostridium symbosium et rel.	0.056	0.799	+	0.171±0.029	0.220±0.048
Deltaproteobacteria	*Desulfovibrio* et rel.	0.008	0.378	-	1.615±0.271	1.009±0.236
Epsilonproteobacteria	*Arcobacter*	0.008	0.378	-	0.321±0.038	0.214±0.035
Gammaproteobacteria	*Escherichia coli* et rel.	0.008	0.378	+	1.265±0.235	2.176±0.478
	Pseudomonas et rel.	0.095	0.799	+	1.051±0.278	1.437±0.280
	Psychrobacter et rel.	0.056	0.799	+	0.462±0.062	0.572±0.124
	Vibrio et rel.	0.095	0.799	+	0.111±0.029	0.211±0.091
Spirochaetes	Uncultured *Spirochaetes*	0.095	0.799	-	0.036±0.002	0.030±0.007

[1] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[2]Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the colon of maternal amoxicillin treated piglets in comparison with control piglets.
[3] ARC: Average relative contribution [%] of a microbial group. Values represent means ± SDs. The microbial group with a relative abundance lower than 0.01% in all treatments are not shown.

Maternal amoxicillin treatment affected colonic microbiota of offspring

At the phylum level, we detected a decreasing trend of Bacteroidetes and Fibrobacteres in the colon of maternal amoxicillin-treated piglets respectively at day 14 and 21 after birth, whereas an increasing trend of Fusobacteria was detected at day 42 after birth (**Table 4**). As in the ileum, there was an increase in relative abundance of bacteria related to level-2 groups *E. coli* and *Pseudomonas* of the maternal amoxicillin-treated piglets at day 14 (**Table 5**). At day 21, 28 and 42, there was no obvious change of any microbial groups according to their corrected *p* value (> 0.978 in all cases, data not shown).

Spearman's correlation coefficients were calculated between the microbial profiles of the faecal sample of each sow and the colonic sample of its offspring. Similar to what was observed for ileal microbiota of the offspring, treated sows and their offspring showed a significantly higher microbiota similarity compared with control sows and their offspring at day 28 after birth (P<0.05, **Fig. 3**). Although the average Spearman's correlation coefficient of the amoxicillin-treated sows and their offspring was higher than that of the control sows and their offspring at day 14, 21 and 42 after birth, the difference was not significant for these ages (P>0.05, **Fig. 3**).

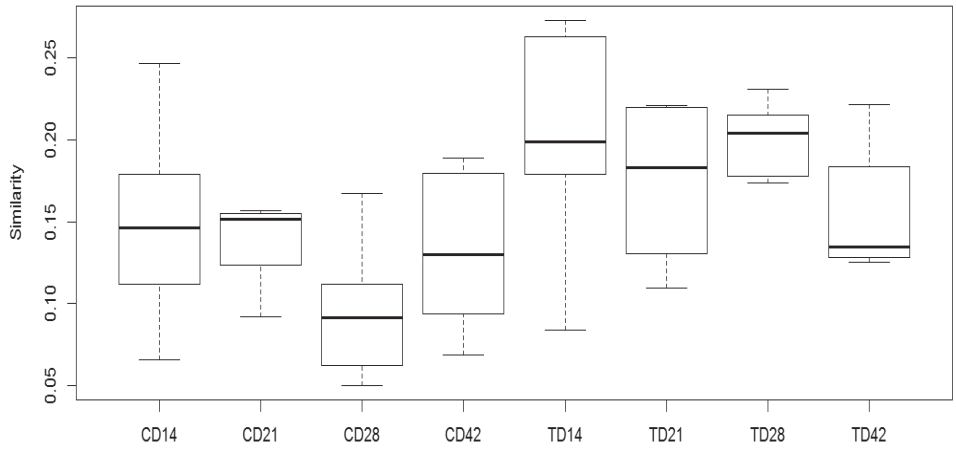

Figure 3. Box plots of total microbiota profile similarity between piglets' colonic content and sows' faeces at day 14 (D14), 21(D21), 28(D28) and 42(D42) after birth. Spearman's correlation coefficient was calculated for each sow and its offspring. Average microbiota similarity between amoxicillin treated sows and their offspring are significantly higher than between control sows and their offspring at day 28 (P<0.05). Capital letters "C" and "T" represent the control piglets and maternal amoxicillin treated piglets, respectively.

Discussion

In this study, we evaluated the effects of maternal antibiotic treatment on the microbiota development of the offspring with a pig model reared in experimental farm conditions. In this model, the sows received amoxicillin orally around parturition, and their offspring was serially sacrificed up to 42 days of age. Amoxicillin treatment drastically impacted the sows' faecal microbiota, and furthermore influenced specific microbial groups in the ileum and colon of the piglets. Overall, more microbial groups were affected by maternal amoxicillin treatment in the piglet ileum than in the colon.

Effects of maternal amoxicillin treatment on the gut microbiota of their offpring

Effect of maternal amoxicillin on ileal microbiota of offspring and its implications

Maternal amoxicillin treatment stimulated Proteobacteria in the ileum of piglets during amoxicillin administration (at day 14 and 21), which was mainly driven by higher relative abundance of *E. coli* and *Pseudomonas*. Various strains of *E.coli* are considered to be pathobionts as they carry pathogenicity-islands and have been associated with diseases such as inflammatory bowel disease and colon cancer [203, 204]. Such pathobionts can cause disease when intestinal homeostasis is disrupted. Intestinal homeostasis can be affected by antibiotic treatment, which may consequently stimulate the pathobionts. A recent study has shown that multidrug-resistant *E.coli* can expand markedly in the microbiota of antibiotic-treated mice, and induce innate immune signalling and rapid sepsis-like death [205]. In line with this observation, studies on antibiotic effects suggested that a bloom in commensal *E. coli* populations in the pig gut could be a common response of disruption in the total intestinal bacterial community [59, 206]; *E. coli* may be capitalizing on a general disruption of the microbiota, temporarily expanding its niche in part due to its short doubling time [42].

Maternal amoxicillin treatment caused age-specific responses of microbial groups in the ileum of piglets. Except bacteria of level-2 groups belonging to *Catenibacterium, E. coli, Lactobacillus acidophilus, and Leptospira,* no microbial groups were continuously affected by the maternal amoxicillin treatment at two or more consecutive sampling times. Moreover, opposing trends at day 42 (i.e. two weeks after weaning) compared with day 28 (day of weaning) were observed for some microbial groups, such as *Bacteroides fragilis* and *Bacteroides vulgatus*. It is not surprising to discover such age-specific effects of maternal amoxicillin treatment as specific temporal development patterns have been observed before. In general, the first colonizers in the human GI tract are facultative anaerobes including *E. coli* and other *Enterobacteriaceae*, followed by anaerobic bacteria such as *Bifidobacterium*, *Clostridium*, *Bacteroides*, and *Ruminococcus* [9]. Microbial

succession during the first few weeks of life in the intestinal tract of humans, chicken, pigs, and calves follows a similar pattern and both coliforms and streptococci dominate the microbiota within a few days of birth and obligate anaerobes appear some time later, even though neonatal animals are exposed to greater numbers of faecal and environmental bacteria than human neonates [11]. After this initial colonization, the microbiota undergoes consecutive changes in composition and function until a relatively stable climax community is established at around 3 years after birth [12]. On the other hand, marked changes in the gut microbiota do not only occur from birth to weaning, but also from weaning to adulthood [3]. The introduction of solid foods can significantly affect the gut microbiota and drive the microbiota into an adult-type profile [13, 18]. For instance, Bergström *et al.* reported that cessation of breastfeeding and introduction of a complementary feeding induced replacement of a microbiota characterized by *Lactobacillus, Bifidobacterium* spp. and *Enterobacteriaceae* with a microbiota dominated by *Clostridium* spp. and *Bacteroides* spp. in Danish infants [2]. Another study also showed ingestion of table foods caused a sustained increase in the abundance of Bacteroidetes [19]. Therefore, opposing trends for certain microbial groups, such as *Bacteroides fragilis* and *Bacteroides vulgatus*, may be attributed to the influence of weaning.

Maternal amoxicillin differently affects offspring ileum and colon microbiota.

Different microbial groups were affected by the maternal amoxicillin treatment in the ileum and colon of piglets in this study. Only bacteria related to genera *Escherichia* and *Pseudomonas* were affected by the maternal amoxicillin in both ileum and colon of piglets at the same age. In addition, more microbial groups were affected by the maternal amoxicillin treatment in the ileum than in the colon. These findings indicated a differential effect of maternal amoxicillin in the ileum and colon of their offspring. Due to differences in the architecture, physiological conditions and function of the gut along its length, bacterial diversity and composition also differs from the small intestine to the large intestine. Although recent studies in human adults showed that the ileum is colonized by relatives of *Streptococcus* and *Veillonella* spp. but also contains members of the *Bacteroidetes* and *Clostridium* cluster XIVa that are abundant members of large intestinal microbiota [105, 125, 127], little is known about the difference of microbiota in the ileum and colon at early age of humans. For piglets and other model animals, more detailed information is available due the possibility of taking samples from sacrificed animals [64]. In this study, we detected differences in the relative abundance of Bacteroidetes, Firmicutes, Planctomycetes and Proteobacteria in the colon compared with the ileum of the control piglets before weaning (**Supplemental Table 2**). Our study revealed that microbiota differed in the ileum and colon during microbial development at early age in piglets. Such differences could be one of the reasons why different microbial groups were affected by the maternal amoxicillin treatment in the ileum and colon.

Maternal antibiotic treatment influences the transfer of maternal microbiota to offspring

The maternal amoxicillin treatment may influence the microbiota development of the offspring through two mechanisms. One possible way is that the amoxicillin residues in milk were transferred to offspring via breastfeeding, and directly affected certain members of the intestinal microbiota of the offspring [193]. On the other hand, maternal amoxicillin may indirectly affect microbiota of the offspring through modifying the mother's microbiota of milk, skin, vagina, gut and faeces. Most antibiotics are either injected or administered orally, thereby circulating throughout the host system and potentially affecting the entire microbiota [42]. For instance, maternal antibiotic treatment during parturition can decrease transmission of vaginal *Lactobacillus* to the neonate during birth [41].

In the present study, maternal amoxicillin treatment drastically impacted sows' gut microbiota, and may consequently impact the transfer of maternal microbiota to offspring. Certain members of the maternal gut microbiota may reach breast milk via bacterial translocation and the bacterial entero-mammary pathway to subsequently colonize the gut of the breastfed neonate [191, 207]. Furthermore, the faecal-oral route of transmission is of specific importance especially for strict anaerobes. Therefore, alteration of maternal gut microbiota may result in changes of microbes colonizing in the gut of the offspring. We detected a higher similarity of microbial composition between the faeces of amoxicillin-treated sows and ileal content of their offspring compared with the control sows and their offspring. This finding seems to suggest that maternal antibiotic treatment affected gut microbiota of offspring through regulating the transfer of maternal gut microbiota to newborn piglets. Oral amoxicillin may also influence the entire sows' microbiota, including milk and skin microbiota. Further studies are needed to confirm the maternal antibiotic effect on milk and skin microbiota, as well as its effect on the gut microbiota development of offspring.

It is difficult to specify (or indicate) whether the maternal amoxicillin treatment affected the microbiota development of offspring in a direct or indirect fashion. However, some of our findings may indicate a weak direct amoxicillin effect on the microbiota of piglets. We found that amoxicillin showed an inhibition on bacteria related to genera *L.acidophilus*, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *S. aureus*, *S. bovis*, *S. intermedius* and *S. suis* in the faeces of sows. This result is in line with previous reports that amoxicillin commonly had an inhibiting effect on *Lactobacillus*, *Streptococcus* and *Staphylococcus* [59, 208-210]. However, we did not find such inhibiting effect in the ileum and colon of piglets. Moreover, most microbial groups that were affected both in faeces of amoxicillin-treated sows and ileal content of their offspring showed an opposite direction of change, such as bacteria related to *Alistipes, Bacillus, Pseudomonas* and *S. aureus.* These opposing findings suggest

breastfeeding transfer of amoxicillin may not be the main route of influencing the gut microbiota of piglets.

Conclusion

Maternal amoxicillin treatment affected the gut microbiota development of piglets. Especially in the ileum, maternal amoxicillin treatment led to a higher relative abundance of Proteobacteria during the administration, which was mainly driven by *E.coli* and *Pseudomonas*. However, most microbial groups that were affected both in faeces of amoxicillin-treated sows and ileal content of their offspring showed opposite direction of change, even though a significantly higher similarity of microbial profile was detected between the faeces of amoxicillin-treated sows and ileal content of their offspring compared with the control sows and their offspring. This finding indicates that, instead of a direct effect of amoxicillin transferred via breastfeeding, maternal amoxicillin treatment affected gut microbiota of the offspring through regulating the transfer of maternal microbiota to the offspring. Future studies are needed in order to assess the effects of antibiotic treatment on the mother's entire microbiota such as milk and skin microbiota, as well as its consequent effect on the gut microbiota development of offspring.

Acknowledgements

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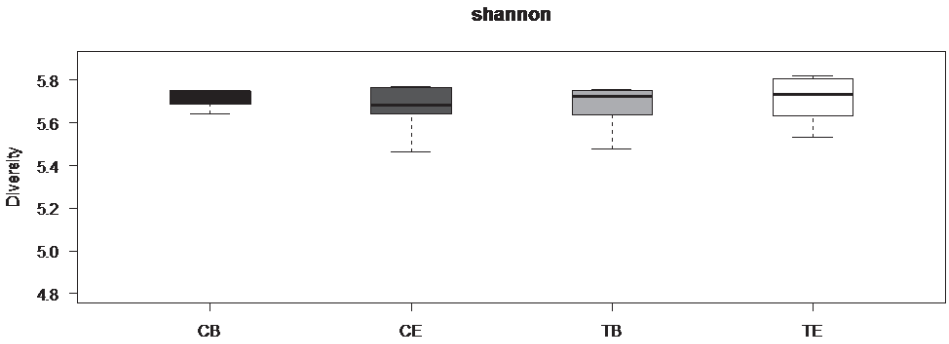
Supplemental tables and figures



Supplemental Figure 1. Microbial diversity of sows' faeces over time. The Shannon index was calculated separately for both treatments at all sampling times. CB, control group at the start of amoxicillin treatment; CE, control group at the end of amoxcillin treatment; TB, amoxicillin treating group at the start of antibiotic treatment; TE, amoxicillin treating group at the end of amoxcillin treatment.

Supplemental Table 1. Phylum-level phylogenetic groups with different relative abundance in the ileum and colon of control piglets at day 14 (D14), 21 (D21) and (D28) after birth.

	D14			D21			D28		
	Effect[1]	ARC Colon[2]	ARC ileum[3]	Effect	ARC Colon	ARC ileum	Effect	ARC Colon	ARC ileum
Actinobacteria		9.45±2.57	8.63±3.39		8.71±3.54	9.12±5.26		9.84±2.07	10.18±3.68
Bacteroidetes	0.056+	3.67±2.24	1.9±0.40		3.64±2.62	1.97±0.43	0.095+	3.91±1.89	1.77±0.44
Fibrobacteres		0.71±0.44	1.16±0.66		0.86±0.21	1.08±0.34		0.75±0.49	1.03±0.54
Firmicutes	0.095-	66.04±3.57	69.88±2.33		69.01±4.03	67.1±4.60		67.22±2.64	66.14±4.12
Planctomycetes	0.008+	0.92±1.46	0.00±0.00	0.008+	0.34±0.52	0.01±0.00	0.008+	0.13±0.13	0.01±0.00
Proteobacteria		17.58±1.29	16.94±1.48	0.016-	15.74±1.44	18.93±2.58	0.056-	16.16±1.18	19.07±2.30
Spirochaetes		1.36±0.30	1.18±0.13		1.44±0.36	1.43±0.21		1.67±0.31	1.44±0.27

[1]Effect: indicates whether the average relative contribution of a phylum was increased (+) or decreased (-) in the colon in comparison with that in the ileum of control piglets. Numbers represent the p values. P values above 0.1 are not shown.
[2] ARC Colon: Average relative contribution [%] of a phylum in the colon. Values represent means ± SDs. The phyla with a relative abundance lower than 0.1% in all treatments are not shown.
[3] ARC Ileum: Average relative contribution [%] of a phylum in the ileum. Values represent means ± SDs. The phyla with a relative abundance lower than 0.1% in all treatments are not shown.

Supplemental Table 2. Phylum-level phylogenetic groups with different abundance in the ileum and colon of maternal amoxicillin treated piglets.

	D14			D21			D28			D42		
	Effect[1]	ARC Colon[2]	ARC ileum[3]	Effect	ARC Colon	ARC ileum	Effect	ARC Colon	ARC ileum	Effect	ARC Colon	ARC ileum
Actinobacteria		8.65±3.78	8.60±6.43	0.056+	8.02±0.72	6.95±0.93		8.28±1.72	7.76±2.61	0.095+	8.69±1.89	7.13±1.24
Bacteroidetes		2.56±2.71	1.56±0.66		1.90±0.67	1.93±0.35	0.056+	2.87±0.60	1.78±0.62	0.095+	2.26±1.04	1.39±0.29
Fibrobacteres	0.056-	0.50±0.23	1.10±0.58	0.056-	0.53±0.28	0.94±0.31	0.095-	0.96±0.49	1.38±0.41		0.60±0.56	0.98±0.41
Firmicutes		67.96±1.79	66.67±2.53	0.095+	69.76±1.14	66.11±2.67		68.95±3.26	68.9±3.14		69.16±1.40	65.33±4.05
Planctomycetes	0.008+	0.30±0.48	0.00±0.00	0.008+	0.73±0.52	0.01±0.01	0.032+	0.72±1.40	0.01±0.01		0.04±0.04	0.01±0.00
Proteobacteria		18.34±1.72	20.21±2.77	0.032-	17.20±1.38	22.1±3.08		16.44±1.51	18.86±2.87	0.008-	17.29±0.59	23.18±3.83
Spirochaetes		1.41±0.60	1.55±0.39		1.57±0.04	1.62±0.35	0.056+	1.54±0.35	1.09±0.27		1.66±0.22	1.72±0.26

[1]Effect: indicates whether the average relative contribution of a phylum was increased (+) or decreased (-) in the colon in comparison with that in the ileum of maternal amoxicillin treated piglets. Numbers represent the p values. P values above 0.1 are not shown.

[2] ARC Colon: Average relative contribution [%] of a phylum in the colon. Values represent means ± SDs. The phyla with a relative abundance lower than 0.1% in all treatments are not shown.

[3] ARC Ileum: Average relative contribution [%] of a phylum in the ileum. Values represent means ± SDs. The phyla with a relative abundance lower than 0.1% in all treatments are not shown.

Chapter 4

Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets

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Abstract

Background: Early-life environmental variation affects gut microbial colonization and immune competence development, however, the timing and additional specifics of these processes are unknown. The impact of early-life environmental variations, as experienced under real life circumstances, on gut microbial colonization and immune development, has not been studied extensively so far. We designed a study to investigate environmental variation, experienced early after birth, to gut microbial colonization and intestinal immune development.

Methodology/Principal Findings: To investigate effects of early-life environmental changes, the piglets of 16 piglet litters were divided into 3 groups per litter and experimentally treated on day 4 after birth. During the course of the experiment, the piglets were kept with their mother sow. Group 1 was not treated, group 2 was treated with an antibiotic, and group 3 was treated with an antibiotic and simultaneously exposed to several routine, but stressful management procedures, including docking, clipping and weighing. Thereafter, treatment effects were measured at day 8 after birth in 16 piglets per treatment group by community-scale analysis of gut microbiota and genome-wide intestinal transcriptome profiling. We observed that the applied antibiotic treatment affected the composition and diversity of gut microbiota and reduced the expression of a large number of immune-related processes. The effect of management procedures on top of the use of an antibiotic was limited.

Conclusions/Significance: We provide direct evidence that different early-life conditions, specifically focusing on antibiotic treatment and exposure to stress, affect gut microbial colonization and intestinal immune development. This reinforces the notion that the early phase of life is critical for intestinal immune development, also under regular production circumstances.

Introduction

Maintenance of general health and prevention of infectious diseases are critically dependent on intestinal homeostasis and proper immune competence. In this regard, early colonization of the gut by microbiota as well as the concomitant development of the intestinal immune system has been proven to be important [143, 211, 212]. Immediately after birth, the intestine is colonized by bacteria derived from maternal and environmental sources [64, 213]. During the early-life period, the composition and diversity of microbiota is unstable and highly influenced by environmental conditions, including the use of antibiotics, exposure to stress, and nutrition, as observed in several recent studies using a variety of experimental conditions and models [13, 132, 169, 214-218]. A significant difference in the diversity of microbiota has, for example, been identified between naturally-reared piglets and isolator-reared piglets that were separated from each other 24 hours after birth during which period natural colonization occurred [169]. The gut microbiota in the isolator-reared piglets remained very divers and contained a large number of phylotypes, whereas in the naturally-reared piglets the microbial diversity decreased as the piglets developed from neonate (day 5) to adult-like stage (day 56: near maturity) [52]. Naturally rearing is supposed to be superior over growing-up in isolators in terms of health with improved immune development and immune homeostasis [52].

The structural and functional development of the mucosal immune system takes place concomitantly with the early-life microbial colonization of new-borns. There is now significant evidence that the process of immune maturation is influenced by the microbiota that colonize the gut at the early stages of life [119, 219-222]. A link has been discovered between the functionality of the host immune system and the early-life gut microbiota composition [21, 223, 224]. In addition, it has been observed that components of the early-life environment of piglets, most probably including the gut microbial diversity, affect the number of $CD4^+$, $CD4^+CD25^+$ effector T-cells, and $CD4^+CD25^+Foxp3^+$ regulatory T-cells, as well as the serum IgG antibody response [220]. Furthermore, it has been shown that host species specific microbiota is required for the development of the immune system [211] and that different epithelial cell lineages (e.g. paneth cells) are essential for colonization of commensal microbiota and homeostasis of the intestine [225]. Many of the studies that so far addressed the interaction between microbiota and the host immune system used (extreme) experimental conditions and do not account for the timing and specifics of events during early development encountered by the new born animals under normal production circumstances.

To investigate the impact of early-life variations, as experienced under regular production circumstances, on gut microbial colonization and immune development, we used piglets and environmental conditions common in swine husbandry systems and with similarities to

environmental conditions experienced by young infants. In intensive swine husbandry systems, piglets are frequently exposed to antibiotics at young age, mainly to prevent outbreaks of respiratory and intestinal diseases; however, the impact on intestinal health has not yet been described on microbiota and/or gene expression level. On the other hand, new born piglets are also frequently exposed to a number of stressful handlings, including ear-tagging, tail docking, and nail clipping. These treatments are known to cause stress, which is in turn expected to have a negative influence on intestinal health of animals. As shown in rats, acute stress can lead to dysfunction of the intestinal barrier [226].

The objective of this study was to investigate the effects of early-life exposure to antibiotics, and antibiotics in combination with stress at day 4 after birth on gut microbial colonization and immune development under regular production circumstances. The specific objective of this study was to identify and define changes in the composition and diversity of the microbiota in the gut and the concomitant immunological effects in intestinal tissue at day 8 after birth. We studied the effect of an antibiotic exposure on the interaction between host immune development and microbiota, in the presence and absence of stressful management procedures, using community-scale analysis of gut microbiota and genome-wide transcriptome profiling of jejunum and ileum tissue.

Material and methods

Design

The experiment was conducted with 16 sows (TOPIGS20, GY x NL) and their suckling piglets. The piglets of each sow were divided into 3 treatment groups (T1, T2, and T3) by colour-marks on their back, but stayed during the course of the experiment in the litter with their mother sow (**Figure S1**). Treatment group 1 (T1) piglets experienced no disturbance and were only handled at the time of drawing blood at day 8. Treatment group 2 (T2) piglets received an injection (subcutaneously in the neck) with 0,1 ml Tulathromycin (dosage was 2.5 mg/kg [1mL/40 kg] body weight) at day 4 after birth. Treatment group 3 (T3) piglets received an identical injection with 0,1 ml Tulathromycin at day 4 after birth and at the same time the standard management procedures (i.e. docking, clipping and weighing) used at that particular farm (VIC Sterksel, The Netherlands). At day 8 after birth from each litter (n=16) and from each treatment group (n=3) 48 piglets were sacrificed by intravenous injection of 0,5-1 ml Euthasol (20% sodium pentobarbital; 200 mg/ml). Intestinal tissues (jejunum and ileum) were taken and immediately frozen in liquid nitrogen. From the same or adjacent locations luminal contents and mucosal scrapings were taken, rinsed (with PBS) and frozen in liquid nitrogen as well.

We performed a power calculation (balanced one-way analysis of variance) in R (version

2.14.0), where the number of treatments was 3, number in each group was 4, significance level was 0.05, and power was 0.8. This resulted in an effect size of 1.07. This means that we can pick-up large effects in gene expression $\log_2$(Fold Change) ≥ 1.07 between treatments. For each treatment (T1, T2, and T3) the samples of 4 pools of 4 animals were analysed. This design reduces the effect of maternal genetics. The grouping of piglets in each pool were the same for microbiota, blood and tissue (scrapings) transcriptomic analyses, by pooling 4 animals (Supplementary Table A in File S1).

Ethics statement

This animal experiment was approved by the institutional animal experiment committee "Dier Experimenten Commissie (DEC) Lelystad" (2011077.b), in accordance with the Dutch regulations on animal experiments.

Microbiota analysis

Ileal content was not present in all piglets at day 8; therefore, only jejunal content was analysed. Jejunal contents were grouped into 4 pools as described in the 'Design' part. Microbial DNA was extracted from 250 mg of the mixture using a faecal DNA extraction protocol adapted from Yu and Morrison [198], as previously described by Salonen *et al*. [199]. After extraction of microbial DNA, the microbial composition was detected by the Pig Intestinal Tract Chip (PITChip) version 2.0. The PITChip is a phylogenetic microarray with 3,299 oligonucleotides based on 16S rRNA gene sequences of 781 porcine intestinal microbial phylotypes, it was designed according to the same principles previously described for the Human Intestinal Tract Chip (HITChip) [103] and the PITChip version 1.0 [97, 101, 227]. The PITCip and the comparable tools targeting the human (HITChip) and mouse (MITChip) intestinal microbiota provide a highly reproducible profile of microbiota composition that has been compared with deep pyrosequencing of 16S rRNA gene fragments [97, 104, 105] and metagenome sequencing of intestinal microbiota [106], indicating comparable phylogenetic resolution and a higher sensitivity of the chip-based analysis. Furthermore, microarray-based analysis is not affected by differences in read-depth per sample as is frequently observed for next generation technology sequencing-based approaches. The protocol for hybridization and analysis of the generated data was performed essentially as described before for the HITChip [103]. Briefly, the bacterial 16S rRNA gene was amplified using the primers *T7prom*-Bact-27-for (5´-TGAATTGTAATACGACTCACTATAGGGGTTTGATCCTGGCTCAG-3´) and Uni-1492-rev (5´-CGGCTACCTTGTTACGAC-3´) [103, 200]. The PCR products were transcribed into RNA and the purified resultant RNA was coupled with CyDye prior to fragmentation and hybridization to the array. Microarray images were processed using Agilent's Feature Extraction Software version 9.5 (http://www.agilent.com). Data was

retrieved from the MySQL (version 5.1) database as describe by Rajilic-Stojanovic [103] and pre-processed using the R (Rx64 2.12.2) microbiome package (http://microbiome.github.com/), settings on default. Although this data is part of a larger dataset, we only provide the data related to day 8 in this study. Diversity of microbial profiles was assessed by calculating the Shannon index of diversity using normalized signal intensities of all probes on the array (http://microbiome.github.com/). Multivariate analysis was applied for PITChip data interpretation. In order to relate changes in total microbial composition to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands) [228]. Treatment classes were introduced as environmental (explanatory) variables. The signal intensities for 151 genus-level phylogenetic groups targeted by the PITChip were used as response variables. RDA was performed focusing on inter-sample correlation, and the Monte Carlo Permutation test was applied [229] to decide whether treatment had statistically significant influence on the microbial composition. The unrestricted permutation option (since the experiment had a randomized design) that yields completely random permutations was employed [230]. Treatment was considered to significantly affect microbial composition with p-values < 0.05. Triplot diagrams were generated using CanoDraw for Windows. To test the variation of an individual microbial group between treatments we performed a Mann-Whitney-Wilcoxon signed rank test in R (version 2.14.0) with multiple testing corrections (Benjamini Hochberg).

Microarray analysis

RNA extraction blood

Blood was sampled using the PAXgene Blood RNA tube from PreAnalytiX (a Qiagen/BD company). Tubes were thawed for 2 hours at room temperature (RT). Subsequently tubes were centrifuged at 3,200x g for 10 minutes at RT and the supernatant was discarded. The pellet was suspended in RNase-free water and tubes were centrifuged as described above. The pellet was washed again with RNAse-free water by repeating the last two steps. After discarding the supernatant the pellet was dissolved in 1 ml TRIzol® reagent (Invitrogen). After 5 min at room temperature 0.2 ml of chloroform was added, the tubes were mixed vigorously for 15 seconds. After 2 min at RT, tubes were centrifuged for 15 min at 7,000x g. The watery phase containing the RNA was isolated and mixed with 0.5 ml of isopropanol. After centrifugation at 12,000x g for 10 minutes at RT, RNA pellets were washed with 75% (v/v) ethanol and dissolved in RNase-free water. Quality control was performed with the Bioanalyzer from Agilent.

RNA extraction tissue

Total RNA was extracted from 50 to 100 mg tissue samples of jejunum and ileum. The jejunum and ileum samples were homogenized using the TisuPrep Homogenizer Omni TP TH220P) in TRizol reagent (Life Technologies) as recommended by the manufacturer with minor modifications. The homogenized tissue samples were dissolved in 5ml of TRizol reagent. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated and dissolved, and quantified by absorbance measurements at 260 nm.

RNA labelling, hybridization, scanning and feature extraction

Labelling was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labeling. The input was 200 ng of total RNA and 600 ng of labelled cRNA was used on the 8 pack array.

Hybridization was performed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labeling protocol from Agilent in the hybridization oven (G2545A hybridization Oven Agilent Technologies). The hybridization temperature was 65°C with rotation speed 10 rpm for 17 hours. After 17 hours the arrays were washed as described in the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol from Agilent.

The porcine Agilent microarray slides, G2519F *Sus scrofa* (035953; V2: 026440), harbouring 43,803 probes, were used and scanned using the DNA microarray scanner with Surescan high resolution Technology (Agilent Technologies). Agilent Scan Control with resolution of 5µ, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

Data loading and statistical analysis

The files generated by the feature extraction software were loaded in GeneSpring GX 12 (also available in GEO, accession number; GSE53170, platform; GPL18045), in which a log2-transformation and quantile normalization were performed. After quantile normalization, quality control was performed and 2 samples were taken out, one sample of jejunum and one of ileum. The remaining 22 samples were analysed by principle component analysis, and a similar approach was followed for the 12 blood samples.

Subsequently, the data was filtered based on the level of expression in which only the

(20-100)th percentile was included and control probes if present were removed, resulting in 28,953 probes for the tissue samples, and 20,832 probes for the blood samples. Thereafter (multiple) probes were mapped to genes if possible, resulting in 21,660 probes/genes for tissue samples, and 15,296 probes for the blood samples. To calculate whether the difference between treatments was significant a 2-way ANOVA with multiple testing correction (Benjamini-Hochberg) was performed within GeneSpring, where we compared the following groups for blood, jejunum and ileum: T3 vs. T1, T2 vs. T1, and T3 vs. T2.

Functional annotation clustering

Functional annotation clustering analyses were performed with Database for Annotation, Visualization and Integrated Discovery (DAVID, version 6.7 [231, 232]). For each comparison, T3 vs. T1, T2 vs. T1, and T3 vs. T2, analyses were performed with lists of significantly up- and down-regulated genes (after filtering and mapping of probes). An overview of the number of up- and down-regulated genes is available in Supplementary Table B in File S1.

Gene set enrichment analysis (GSEA)

GSEA [233, 234] was performed separately for ileum and jejunum. The following settings were different from the default settings: permutations were performed on the gene set, chip platform was set to gene symbol. Six gene set databases (v3.0) were loaded for analysis, including the three Gene Ontology related gene sets biological processes, molecular function and cellular component, and three pathway related gene sets, BioCarta, Reactome and KEGG.

InnateDB interactions

Interactions were extracted from innateDB [235] and loaded into CytoScape (v2.8.1) [236, 237]. To represent the cell location for all genes in the network, the Cerebral plugin was used [238].

Quantative RT-PCR

For the quantification of expression levels based on array data, cDNA was made using random hexamer primers and reverse transcriptase, and qRT-PCR was performed targeting genes encoding IL-1βb [239], IL-6 [239], IL-8 [239] and TIMP1 [240] with on-line detection using Syber Green PCR Master Mix (Applied Biosystems, Foster City, CA, USA) in an ABI 7500 Real-Time PCR system (PE Applied Biosystems, Foster City, CA, USA). Quantitative results were determined and normalized with GAPDH gene expression

(**Figure S2**). For the quantification, a standard curve of a plasmid containing the cytokine gene of interest or GAPDH in pGEM-T easy (Promega Benelux b.v. Leiden, The Netherlands) was used.

Results

Microbiota analyses

The PITChip was used to evaluate the impact of antibiotic treatment with or without routinely stressful management on jejunal microbiota. The most dominant phylum in all samples was the Firmicutes, followed by Proteobacteria, Bacteriodetes, Spirochaetes, and Actinobacteria (**Supplementary Table C in File S1**), whereas, the relative abundances of other phyla were not above 1%. Multivariate redundancy analysis of PITChip2 profiles at the approximate genus-level showed that samples of T3 separated from most samples of T1 along the first canonical axis except for sample 12, and samples of T2 separated from those of T1 along the second canonical axis (**Fig. 1**). Both axes together explained 18.5 % of the variance in microbiota composition. Subsequently, univariate analysis was used to identify

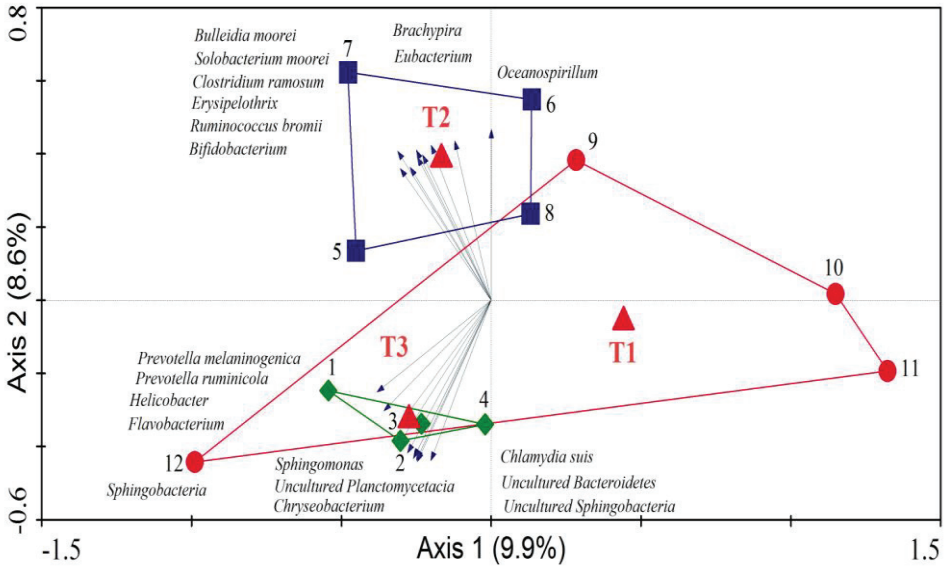

Figure 1. Triplot for RDA analysis of jejunal microbiota composition. Nominal environmental variables T1, T2 and T3 are represented by red triangles (▲). Samples are grouped by treatment: T1 (red; ○), T2 (blue; □) and T3 (green; ◇), each symbol represents a pool of four pigs, and numbers represent pool identity number. Microbial groups contributing at least 60% to the explanatory axes are represented as vectors. Both axes together explain 18.5% of the total variance in the dataset.

Table 1. Genus-level phylogenetic groups changed in T2 and/or T3.

Microbial groups	T2 vs. T1		T3 vs. T1		T3 vs. T2		ARC[2]		
	***P* value**	**C.P[1]**	***P* value**	**C.P**	***P* value**	**C.P**	**T1**	**T2**	**T3**
Actinobacteria									
Actinobacteria									
Bifidobacterium et rel.	**0.03**↑[3]	0.48	0.34	0.51	**0.03**↓	0.1	0.27±0.08	0.48±0.08	0.34±0.04
Collinsella	0.49	0.71	0.11	0.51	**0.03**↓	0.1	0.19±0.04	0.24±0.09	0.14±0.01
Olsenella et rel.	0.34	0.67	0.2	0.51	**0.03**↓	0.1	0.18±0.04	0.24±0.09	0.14±0.02
Bacteroidetes									
Bacteroidetes									
Uncultured *Prevotella*	0.2	0.64	**0.03**↑	0.33	0.89	0.96	0.20±0.03	0.29±0.08	0.32±0.05
Fibrobacteres									
Fibrobacteres									
Fibrobacter succinogenes et rel.	0.34	0.67	0.34	0.51	**0.03**↓	0.1	0.58±0.23	0.70±0.11	0.43±0.02
Firmicutes									
Bacilli									
Allofustis et rel.	0.49	0.71	0.34	0.51	**0.03**↑	0.1	0.26±0.04	0.25±0.01	0.28±0
Bacillus et rel.	**0.03**↓	0.48	0.06	0.51	0.34	0.52	0.39±0.01	0.34±0.02	0.36±0.02
Carnobacterium et rel.	1	1	0.34	0.51	**0.03**↑	0.1	0.64±0.16	0.57±0.06	0.68±0.06
Staphylococcus aureus et rel.	**0.03**↓	0.48	0.69	0.85	**0.03**↑	0.1	0.22±0.03	0.19±0.01	0.22±0.01
Streptococcus suis et rel.	0.06	0.54	0.89	1	**0.03**↓	0.1	0.55±0.16	0.83±0.09	0.56±0.1
Clostridium cluster IV									
Anaerotruncus et rel.	0.89	0.94	0.34	0.51	**0.03**↑	0.1	1.27±0.35	1.16±0.14	1.44±0.07
Faecalibacterium prausnitzii et rel.	**0.03**↑	0.48	0.2	0.51	0.2	0.38	0.22±0.09	0.37±0.04	0.31±0.08
Ruminococcus bromii et rel.	0.06	0.54	0.34	0.51	**0.03**↓	0.1	0.27±0.06	0.41±0.06	0.31±0.02
Clostridium cluster XI									
Anaerovorax et rel.	0.49	0.71	0.34	0.51	**0.03**↑	0.1	3.35±0.59	3.07±0.18	3.57±0.09
Clostridium cluster XIII									
Peptoniphilus et rel.	0.34	0.67	0.89	1	**0.03**↑	0.1	1.02±0.19	0.9±0.09	1.1±0.08
Clostridium cluster XIVa									
Clostridium sphenoides et rel.	0.49	0.71	0.34	0.51	**0.03**↑	0.1	0.69±0.15	0.62±0.07	0.8±0.04
Ruminococcus obeum et rel.	0.69	0.82	0.2	0.51	**0.03**↑	0.1	2.45±0.76	2.43±0.47	3.4±0.39
Clostridium cluster XV									
Eubacterium et rel.	**0.03**↑	0.48	0.89	1	**0.03**↓	0.1	0.15±0.05	0.26±0.03	0.16±0.03
Clostridium cluster XVII									
Catenibacterium et rel.	0.11	0.54	0.49	0.7	**0.03**↓	0.1	0.22±0.11	0.4±0.16	0.23±0.01
Erysipelotrichi									
Solobacterium moorei et rel.	**0.03**↑	0.48	0.49	0.7	**0.03**↓	0.1	0.11±0.05	0.22±0.04	0.14±0.02

Proteobacteria									
Betaproteobacteria									
Bordetella et rel.	0.11	0.54	0.89	1	**0.03**↓	0.1	0.43±0.17	0.73±0.18	0.41±0.05
Oxalobacter et rel.	0.11	0.54	1	1	**0.03**↓	0.1	0.12±0.04	0.21±0.07	0.11±0.01
Sutterella wadsorthia et rel.	0.11	0.54	0.89	1	**0.03**↓	0.1	0.12±0.06	0.24±0.09	0.11±0.01
Gammaproteobacteria									
Psychrobacter et rel.	0.2	0.64	1	1	**0.03**↓	0.1	0.31±0.08	0.41±0.07	0.33±0.01
Ruminobacter amylophilus et rel.	0.11	0.54	1	1	**0.03**↓	0.1	0.24±0.08	0.34±0.06	0.23±0.01
Spirochaetes									
Spirochaetes									
Treponema et rel.	0.49	0.71	0.89	1	**0.03**↓	0.1	1.31±0.32	1.46±0.13	1.19±0.09
Turneriella	0.2	0.64	1	1	**0.03**↓	0.1	0.2±0.13	0.32±0.07	0.15±0.01

[1] C.P: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[2] ARC: average relative contribution [%] of a microbial group. Values represent means ± SDs. The microbial groups with a relative abundance lower than 0.1% in all three treatments are not shown.
[3] "↑" or "↓" indicates whether the average relative contribution of the microbial group was increased or decreased.

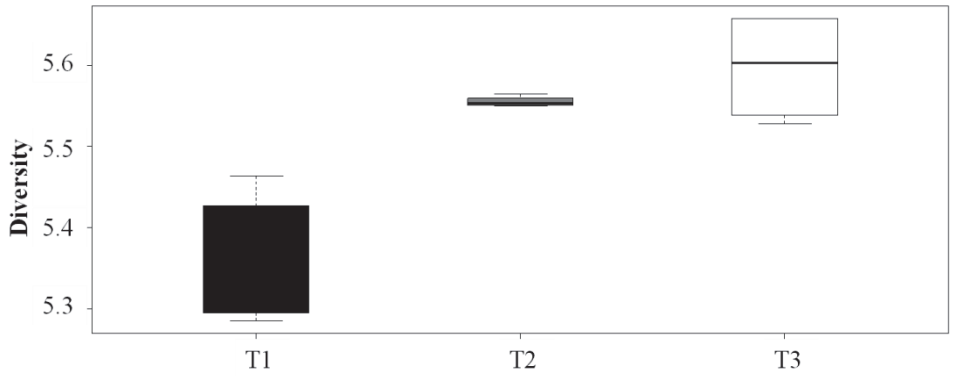

Figure 2. Diversity in microbiota in the three treatment groups. The Shannon index (y-axis) was calculated for all three treatments (T1, T2, and T3) (x-axis).

microbial groups that significantly changed between treatments (**Table 1**). In the comparison of T2 versus T1, the relative contribution of *Bifidobacterium*-like, *Erysipelotrichi*-like, *Eubacterium*-like, *Faecalibacterium prausnitzii*-like, and *Solobacterium moorei*-like bacteria increased, whereas *Bacillus*-like and *Staphylococcus aureus*-like bacteria strongly decreased. In contrast, uncultured *Prevotella* group (i.e. a genus-level phylogenetic group comprising exclusively environmental sequences and no cultured representatives) increased in its relative abundance in T3 compared to T1 piglets. When comparing T3 versus T2, multiple microbial groups in different phyla were significantly different, including groups belonging to the Actinobacteria, Fibrobacteres, Firmicutes, Proteobacteria and Spirochaetes (see **Table 1** for more detailed information).

Microbial diversity (Shannon index based on probe-level profiles) was significantly higher in T2 and T3 than in T1 (p<0.01); however, no significant difference was observed between T3 and T2. (p>0.05) (**Fig. 2**).

Transcriptomic analysis

To investigate the impact of the different treatments on the host, genome-wide gene expression was measured in intestinal tissue and blood samples. Initially, Principal Component Analysis (PCA) was performed to get more insight into the variability in blood and tissue transcriptome data and to get a visual inspection of the quality of the data. Representing transcriptome data by PCA showed clear aspects of quality control and outlier arrays and hybridization samples can be spotted and taken out based on their gene expression profile and hybridization pattern to 'control' probes. Because both intestinal tissues (jejunum, ileum) origin from the same ancestor cells, similar responses to the treatments were expected, and therefore jejunum and ileum data were analysed simultaneously (**Fig. 3**), whereas the data of blood samples were loaded separately (**Fig. 4**). For tissue-derived data, clustering occurred of similar treatments (red; T1, blue; T2, and green; T3) as well as similar tissues (squares; ileum, and triangles; jejunum). However treatment 3 samples were more dispersed compared to treatment 1 and 2, albeit grouping more closely with T1 samples (Fig. 3). Also, the different blood samples clustered, however, these clusters were dispersed when looking at principal component 1 (PC1; **Fig. 4**). This large variation within the treatment groups hinted that there were no significant differences in gene expression in blood cells between the treatments.

To investigate the effect of the three treatments in jejunum, ileum, and blood, an ANOVA was performed. All probes/genes which were characterized by $p_{cor} < 0.05$ and Fold Change > |1.5| in at least one of the six comparisons were taken for further functional and enrichment analysis (**Table B in File S1**). No differences were observed in blood, whereas in jejunum and ileum multiple probe signals (up to 80 genes) significantly differed between the treatment groups. In general more probe signals differed significantly in ileum compared to jejunum.

The significantly up- and down-regulated genes were used as input for functional analysis. First, DAVID functional annotation clustering was performed resulting in multiple groups with a significant Enrichment Score (ES). The results are presented in Tables 2-4 (ES>1), where **Table 2** shows the differences between T2 and T1, **Table 3** between T3 and T1, and **Table 4** between T3 and T2. In addition, Gene Set Enrichment Analysis (GSEA) was performed without pre-filtering of genes on e.g. p-value or fold change, so that all probes/genes were used as input. The GSEA analysis resulted in the identification of similar biological processes affected by the treatments as observed by the DAVID analysis (**Suppl.**

Table D and E in File S1). The main findings were that immune related processes appeared to be dominantly influenced by the treatments, for example cytokine/chemokine related processes differed between the treatments in both tissues. This was also verified for three genes (IL1B, IL6 and TIMP2) with qPCR, where we observed a trend between the qPCR and transcriptomics datasets (**Figure S2**).

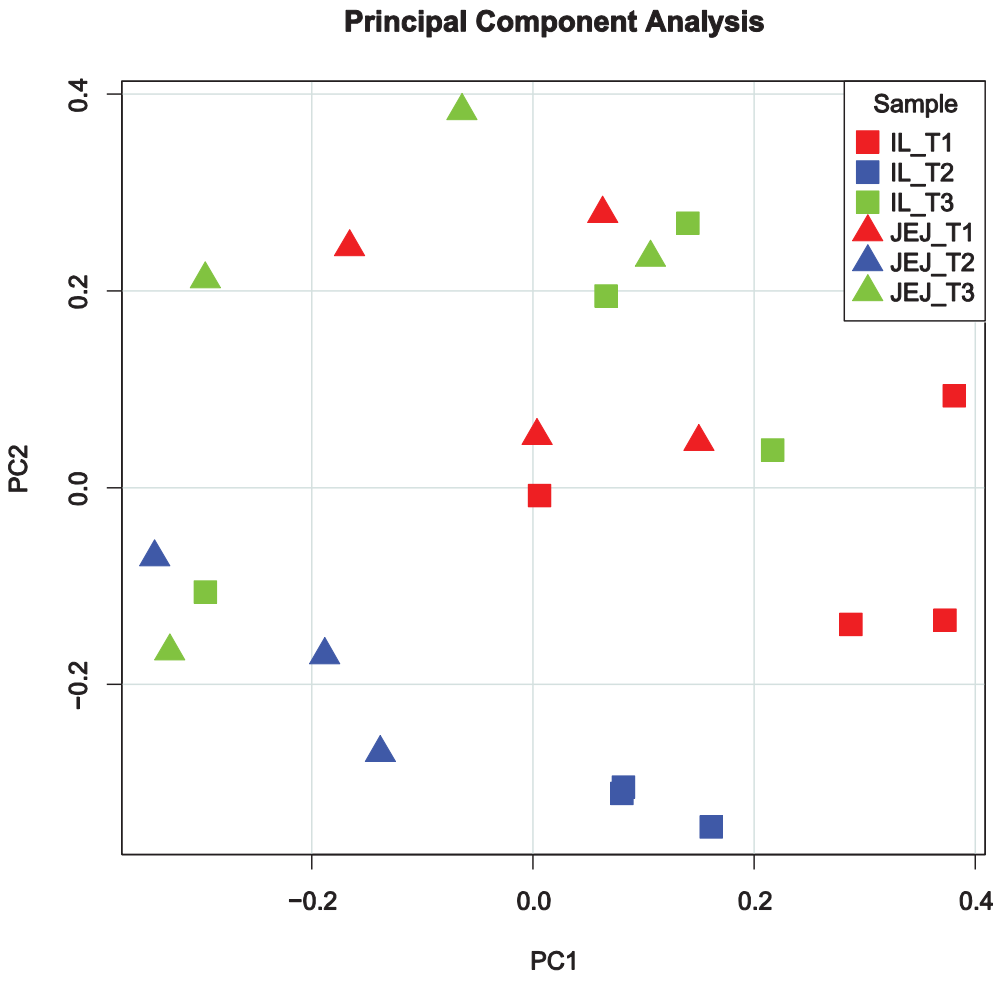

Figure 3. Principal Component Analysis on microarray data of intestinal tissue transcriptomes. A principal component analysis was performed on the 22 tissue transcriptome datasets which remained after quality control. All treatments (T1: red, T2:blue, and T3:green) are displayed for both jejunum (triangles) and ileum (squares). The x-axis depicts principal component 1 and the y-axis depicts principal component 2. Abbreviations: IL; Ileum, JEJ; Jejunum.

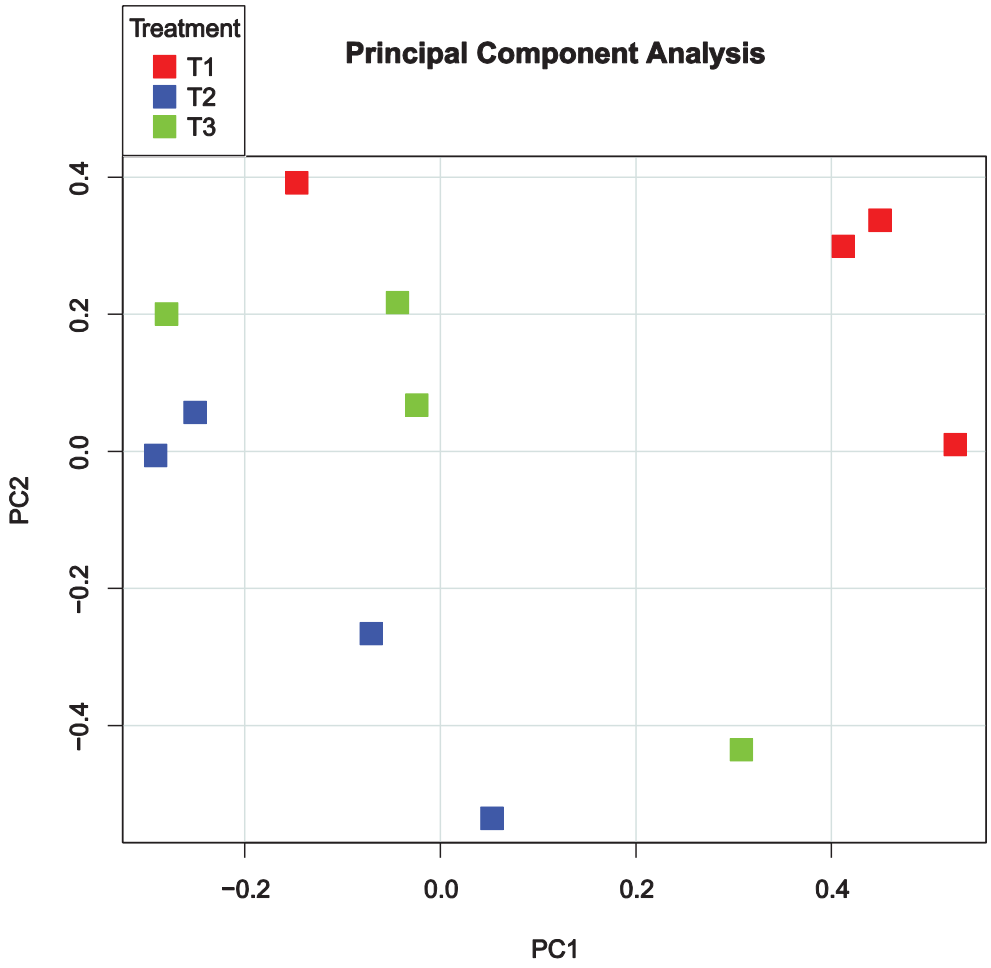

Figure 4. Principal component analysis of blood transcriptomes. A principal component analysis was performed on the 12 blood transcriptome data sets which remained after quality control. Treatment 1 (T1) is depicted in red, T2 in blue, and T3 in green. The x-axis depicts principal component 1, whereas the y-axis depicts principal component 2.

Table 2. Functional analysis of genes differentially expressed between treatment 2 versus 1.

	DOWN	
JEJUNUM	**#**	**Name**
	1	chemotaxis
	2	cytokine activity
	3	chemokine activity
	4	regulation of secretion /immune effector process
	5	cell migration/motion (leukocyte)
ILEUM	**#**	**Name**
	1	cytokine activity
	2	chemotaxis
	3	second-messenger-mediated signaling (cAMP)
	4	chemokine activity
	5	response to bacterium/regulation of systemic process

Table 3. Functional analysis of genes differentially expressed between treatment 3 versus 1.

	DOWN		UP	
JEJUNUM	**#**	**Name**	**#**	**Name**
	1	chemotaxis	**1**	nucleotide binding
	2	cytokine activity	**2**	membrane fraction
	3	extracellular region	**3**	ATP binding
	4	chemokine activity	**4**	
	5	second-messenger-mediated signaling (cAMP)	**5**	
ILEUM	**#**	**Name**	**#**	**Name**
	1	response to wounding/defense response	**1**	nucleotide binding
	2	cytokine activity	**2**	positive regulation of catalytic activity/signaling cascade
	3	chemotaxis	**3**	plasma membrane
	4	extracellular region	**4**	
	5	chemokine activity	**5**	

Table 4. Functional analysis of genes differentially expressed between treatment 3 versus 2.

	DOWN	
JEJUNUM	**#**	**Name**
	1	chemotaxis
	2	nucleotide binding
	3	
	4	
	5	
ILEUM	**#**	**Name**
	1	Immunoglobulin
	2	nucleotide binding
	3	plasma membrane
	4	nucleotide/ATP binding
	5	positive regulation of catalytic activity/signaling cascade

To visualize the differences in gene expression between the treatments, gene interactions networks associated with the dominant processes identified from the functional analysis by DAVID and GSEA were extracted from InnateDB, and the gene expression values were superimposed on these networks. Major differences were found in gene expression levels between the treatment groups for the chemokine signalling network (**Fig. 5**) and for the Toll-like Receptor (TLR) signalling network (**Fig. 6**). When focusing on the cellular location of the differentially expressed gene-encoded products of the cytokine and TLR networks, it is remarkable to see that especially the products situated extracellularly, on the cell surface, and the plasma membrane (**Fig. 7 and 8**), differed between the treatment groups. In T2 and T3 piglets lower expression was observed in these 2 networks compared to the untreated (T1) piglets. Further differentiation could be made between T2 and T3, where in T2 expression is lower compared to T3. In other words, expression of immune associated genes was high in T1, low in T2, and intermediate in T3.

Discussion

Effects of the different treatments on day 4 after birth were clearly visible at day 8 after birth, for both community-scale microbiota data and genome-wide transcriptomic data. The current study shows effects of early-life environmental variation on microbial colonization and immune development in the gut of piglets that were kept under regular-production circumstances. Here, we will focus specifically on comparison of T1 versus T2/T3 animals, for both microbiota and gene expression.

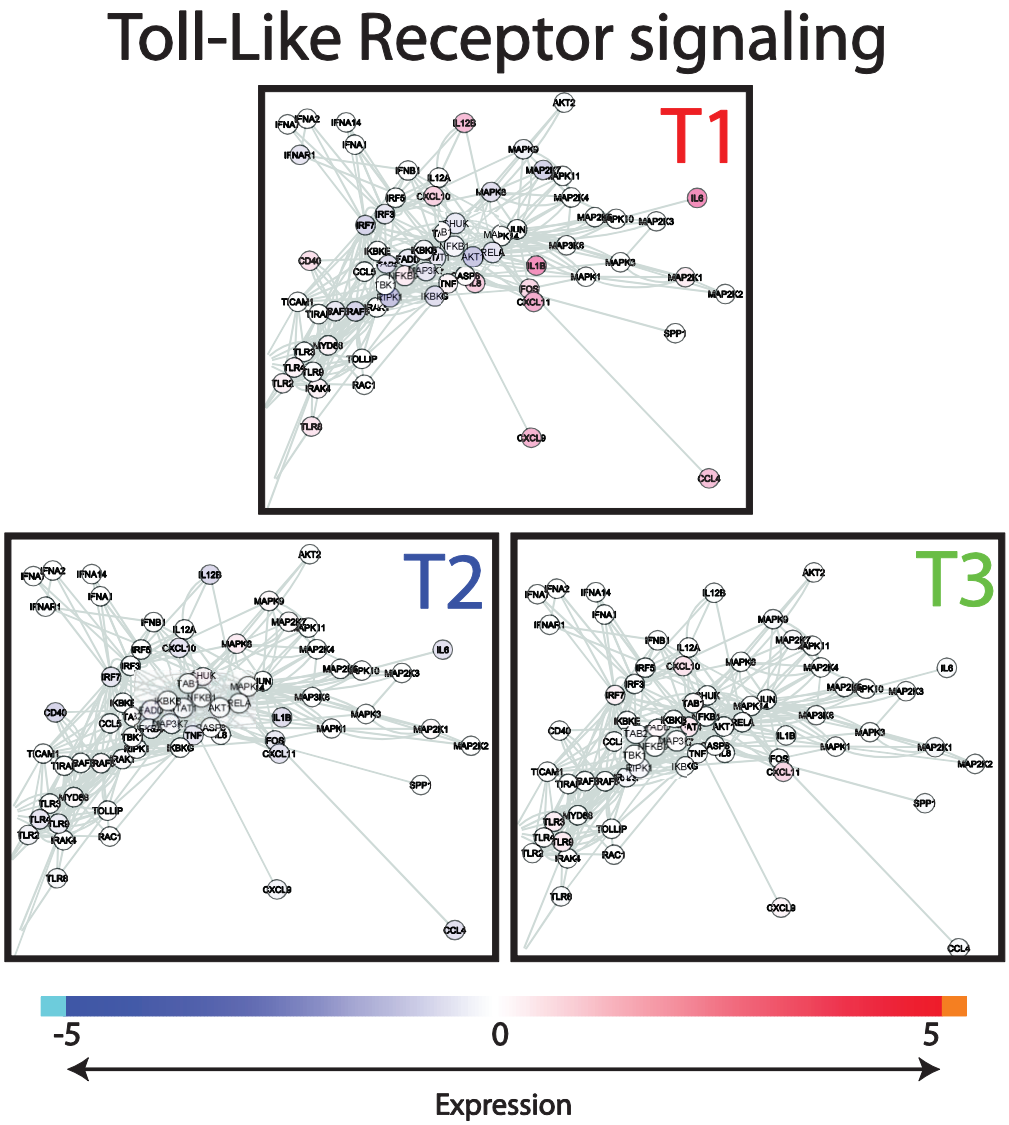

Figure 5. Snapshot of Toll-like receptor network. Interactions of genes involved in the Toll-like receptor (TLR) pathways were extracted from innateDB and visualized in CytoScape. Nodes (genes) are coloured by their expression, where blue is low expression and red is high expression (see legend on top). Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

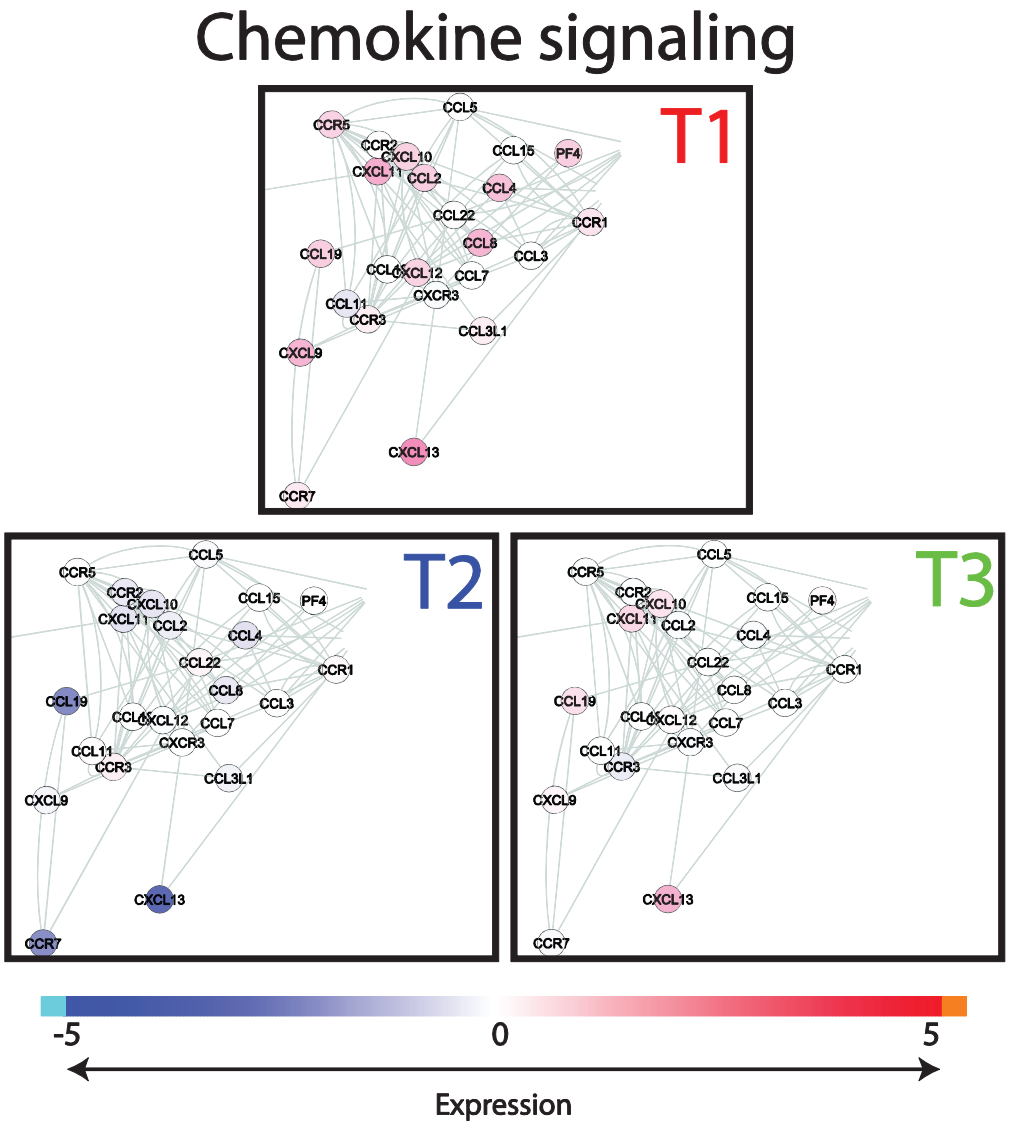

Figure 6. Snapshot of chemokine network. Interactions of genes associated with chemokine pathways were extracted from innateDB visualized in CytoScape. Nodes (genes) are coloured by their expression, where blue is low expression and red is high expression (see legend on top). Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

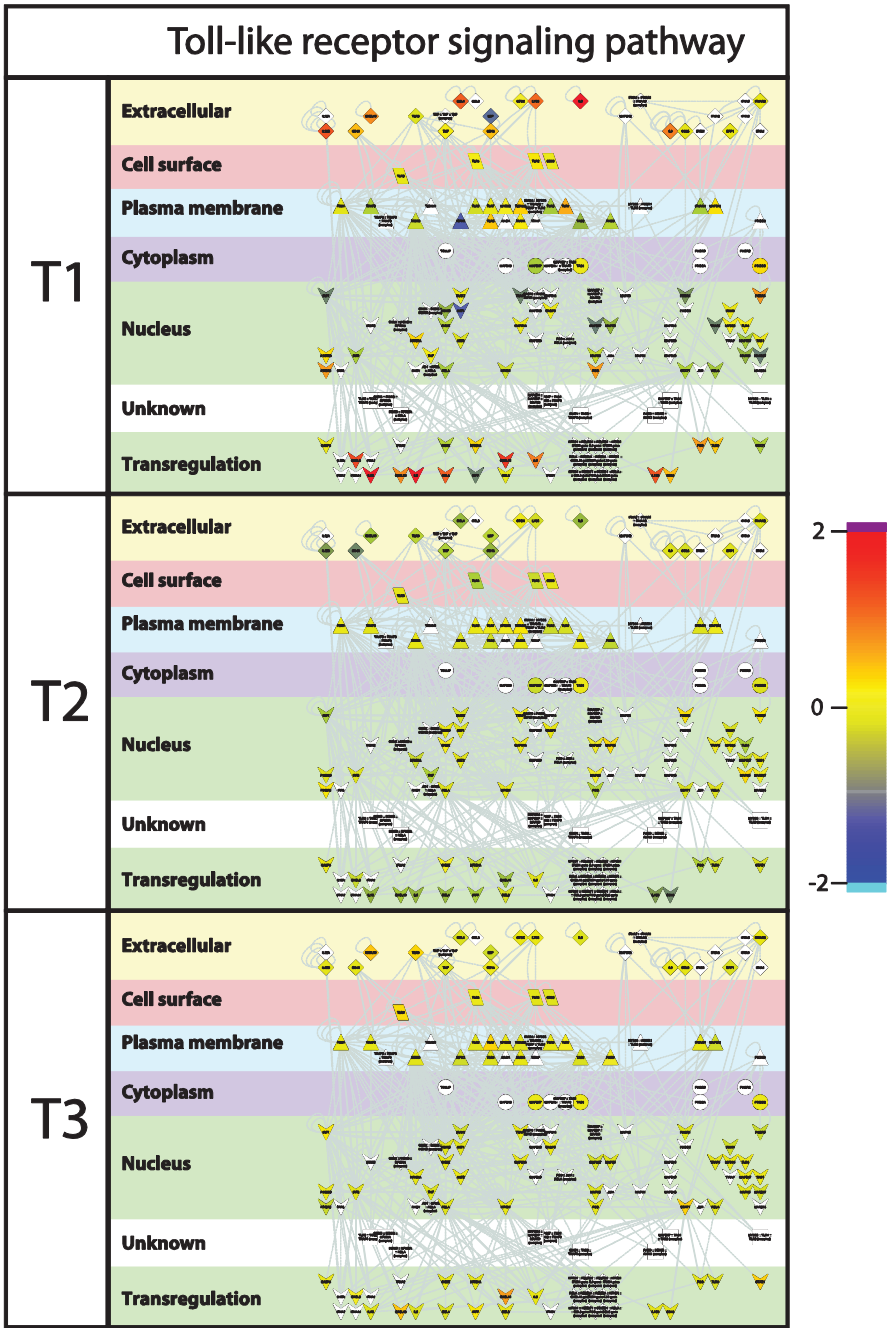

Figure 7. Toll-like receptor signalling pathway. Interactions of genes associated with Toll-like receptor pathways were extracted from innateDB [235] and visualized in CytoScape. Nodes (genes) are coloured by their expression, where blue is low expression and red is high expression. Furthermore, localization of gene products within the cell was predicted using Cerebral [238]. Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

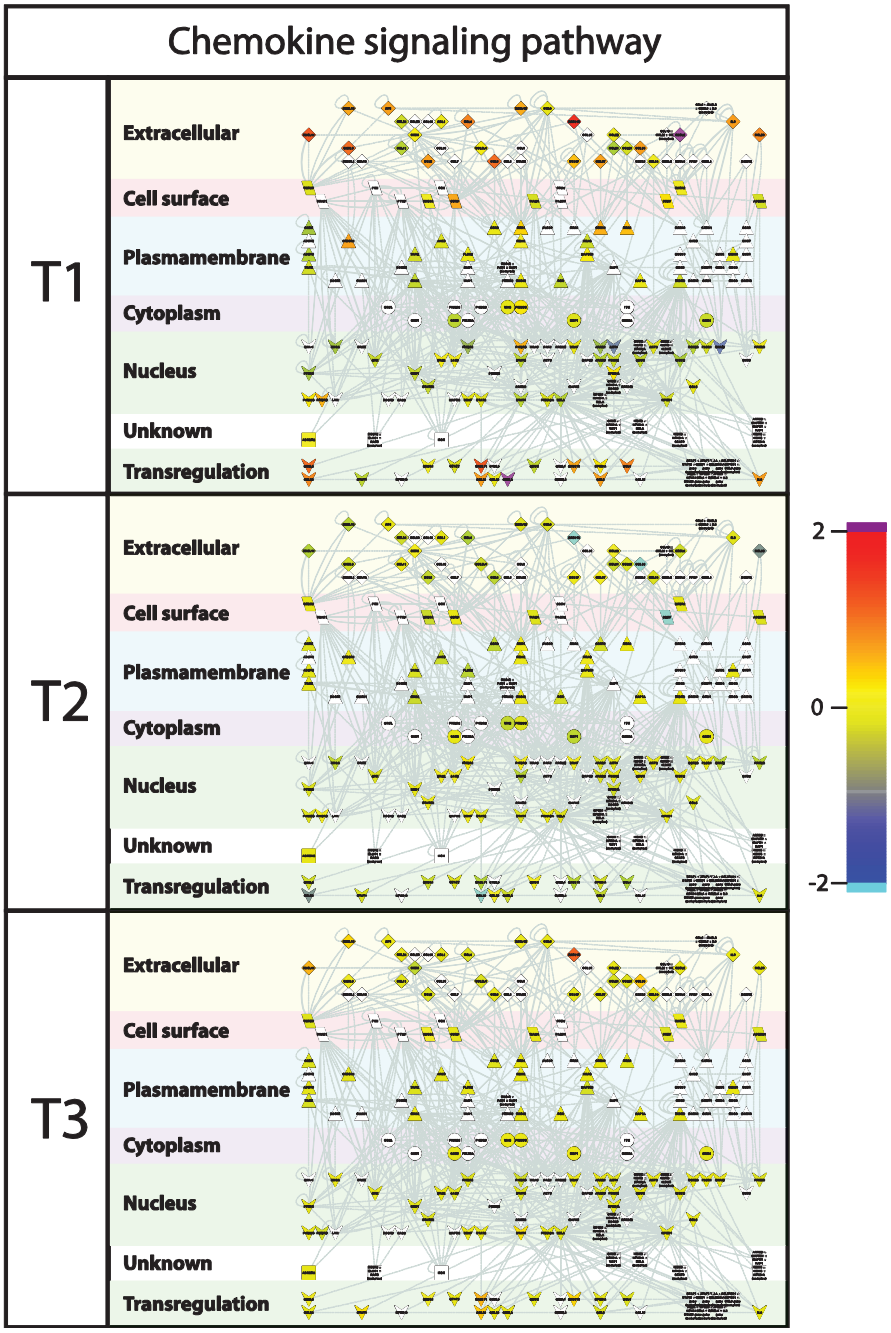

Figure 8. Chemokine signalling network. Interactions of genes associated with chemokine pathways were extracted from innateDB [235] and visualized in CytoScape. Nodes (genes) are coloured by their expression, where blue is low expression and red is high expression. Furthermore, localization of gene products within the cell was predicted using Cerebral [238]. Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

Comparison of microbiota composition and diversity between treatment groups

The diversity of microbiota as measured by the PITChip in jejunum digesta of T1 animals was significantly lower compared to that in the T2 and T3 animals. This study shows that a single antibiotic dose, administered at day 4 after birth, is able to modulate the microbial community for an extensive time. The antibiotic used in this study is regularly applied at pig farms with a one-time administration because of the long half-life (70h) of the product and elimination half-life was approximately 6-8 days (details on website European Medicines Agency). This is in line with previous observations, which showed that microbiota composition and diversity was affected for at least five weeks in new-born piglets after a single dose of parenteral amoxicillin treatment [59]. Different methodologies were used in our study as compared to Janczyk *et al.* to assess microbial diversity, i.e. microarray- and denaturing gradient gel electrophoresis (DGGE)-based profiling of PCR-amplified 16S rRNA gene fragments. Nevertheless, we observed that the trends in diversity are comparable, also with those obtained by next generation technology sequencing, albeit at different absolute values [97, 104-106]. Also the bacterial composition is not likely to return to its initial state, because the gastrointestinal tract undergoes a rapid dynamic development.

In this experiment the antibiotic treatment (T2) caused a detectable change in relative abundance of most early gut colonizers. Among these colonizers, the abundance of all anaerobic bacteria including *Bifidobacterium, Eubacterium, F. prausnitzii,* and *S. moorei* increased, whereas facultative bacteria such as *S. aureus* decreased in quantitative terms. These results may contradict some previous reports, it has been reported that a range of different antibiotics (including Amoxicillin, Ciprofloxacin, Augmentin and Trimethoprim) can reduce *Bifidobacterium* in adult faecal microbiota [241, 242], however, a strong increase of *Bifidobacterium* relative abundance was observed in this study in response to the antibiotic tulathromycin. One possible reason could be that certain antibiotic resistant *Bifidobacterium* strains, such as *B. longum* and *B. catenulatum* [56], can survive under the tulathromycin antibiotic selection. Another explanation could be a difference in the activity of antibiotics in the intestinal lumen compared to the colon. Nevertheless, a higher microbial diversity in T2 detected in this study may indicate a relatively complex microbiota of T2 animals compared to T1 animals. In the complex gut microbial system, facultative bacteria, such as *S. aureus* can not withstand the competition and environmental changes brought by the anaerobes, as shown in young infants [190]. In addition, *S. aureus* colonization has been reported negatively correlated to certain antibiotic treatments at 6 months of age in infants [243-245]. Thus, the reduction of *S. aureus* in T2 could be attributed to the acceleration of complex anaerobic microbiota establishment by the antibiotic and/or its sensibility to the antibiotic. On the contrary, we did not found a notable change of *S. aureus* between T3 and T1 animals. It appeared that the antibiotic effect was

counteracted by stress management, because the microbial groups observed in T2 animals were not observed in T3 animals. Interestingly, this was also observed for *Bifidobacterium, Eubacterium,* and *S. moorei*.

In addition to the microbial groups discussed above, there are 20 microbial groups that only showed difference between T2 and T3 in this study. This result may be attributed to a yet unknown combined effect of antibiotic and management treatment (causing stress), which may involve complex host-microbial interactions in the gut.

The results from these microbiota analyses show that microbial diversity and composition are affected by the different treatments. Earlier findings of Schmidt *et al.* also showed that continuous microbial exposure during early life stages is required for the development of a stable gut microbiota. However, it should be noted that, contrary to our study, Schmidt *et al.* measured the mucosa-adherent microbiota [169]. In the present study, piglets were housed under field conditions of commercial intensive farming systems that correspond to 'natural' and continuous exposure to microbial species. Compared to the control T1 group, the administration of tulathromycin caused a clear increase in microbial diversity, leading to a situation which is described by others as "more chaotic" [169]. The microbiota of 'natural' colonized piglets is characterized by a high abundance of lactobacilli and low abundance of microbial species related to pathogens. Such a 'natural' microbiota composition and diversity has been proposed to be important for immune homeostasis [52].

At the phylum level similar relative abundance levels of microbial groups were observed between the different treatment groups (Supplementary Table C in File S1), which is in contrast to the observation of Mulder *et al.* (2009) where antibiotic treatment resulted in a severe reduction in abundance of Firmicutes and increase of Bacteriodetes and Proteobacteria compared to the outdoor situation [52]. However, in the study of Mulder a cocktail of antibiotics was administered daily from day 1 to 28 of age, as opposed to a single dose of one antibiotic at day 4 in this study. Furthermore the phylum level data in Mulder *et al*. represents an overall abundance of (mucosa-adherent) microbial groups in the whole experiment, thus including different time-points, which makes it more difficult to compare with our data. Despite of the differences in experimental setup, it is known that both mucosal and luminal microbiota are able to interact with the host [246], however, luminal microbiota might do this more indirectly. Both studies show that the microbial diversity is affected by the antibiotic treatment, and concomitantly gene expression data showed shifts in communicative/immune processes at day 5 and/or our day 8 data.

Comparison of gene expression in host blood and intestinal tissues

Simultaneous to the microbiota sampling, gene expression measurements were performed

of intestinal tissue (scrapings) at the same location (in case of jejunum). In addition, blood transcriptome analysis was performed in order to investigate whether cross-talk between host and microbiota during this neonatal period induced systemic changes. However, the lack of significant differences between the blood samples of the treatment groups suggests that affected immunological processes are still local 4 days after treatment. When comparing the two different tissues analysed here, more genes were differentially expressed, either up or down, in ileum in contrast to jejunum. The dominant processes that were affected by the treatments (T2 or T3) are mostly involved in immunological processes and the genes involved were down-regulated in comparison to T1 animals. In connection with this, we observed that most of the biological processes affected by the treatments (T2 or T3) are involved in various immune functions. The transcriptomic data suggest that the immunological development after birth can be influenced by external factors (e.g. antibiotics and/or stress), possibly by modulating the microbial colonization of the gut [247]. Surprisingly, the top 5 processes in the functional analyses were all of communicative nature for both jejunum and ileum, including "Chemotaxis" (cell or bacterial movement towards a chemical or protein); "chemokine activity"; and "cytokine activity", the latter of which are associated with immune-modulating activities and involved in (intra)cellular communication. The term "cyclic adenosine monophosphate (cAMP) signaling" is also associated with cell communication (intracellular signal transduction, including suppression of regulatory T cells [248]), whereas "extracellular region" is associated with the space external to the outermost structure of a cell (e.g. plasma membrane). To investigate the above described immunological differences in more detail, a systems approach was applied, by extracting gene-gene interaction networks (InnateDB) for two immunological processes, namely chemokine and Toll-Like Receptor (TLR) signalling. These two networks were chosen because expression of many of the genes that were higher in T1 compared to T2 or T3 piglets belonged to these networks. Furthermore, chemokines play a major role into guiding the migration of cells, and TLRs play a key role in innate immunity and recognition of conserved microbial structures [249]. The data presented here suggests that in the intestine of T1 piglets more intensive immunological communication occurs compared to T2 and T3 piglets, which is reflected by high expression of chemokine and TLR receptors as well as their respective ligands. We hypothesize that the global down-regulation in the antibiotic treated animal (T2/T3) compared to the control (T1) piglets could be that due to the high diversity and more "chaotic" microbial population in the gut of piglets treated with the tulathromycin antibiotic. Similar observations (down regulation vs chaotic/high diversity) were observed by the Kelly group [52, 169, 216] .

Interactions between host and microbiota

The genes associated with the immunological processes that differ between the treatment groups are more strongly expressed in T1 animals compared to T2 and T3 animals, which

might be due to the observed lower diversity in microbiota and the abundance of specific (non-pathogenic) bacterial species (e.g. lactobacilli). We observed a trend towards higher abundance of lactobacilli in T1 piglets compared to the treatment groups (T1; 18%, T2; 14%, and T3; 12%). A higher abundance of lactobacilli could lead to more cross-talk between these abundant microbiota and host (immune) cells, the more so because it has previously been shown that different lactobacilli strains can modulate the expression of immune pathways [250, 251]. It is known that communication between and within intestinal cells as well as between microbiota and intestinal cells is crucial to set up a proper development (shaping) and functioning of the immune system [20]. In case of early antibiotic treatment and a concomitant increase of microbial groups related to (intestinal) pathogens, it could be hypothesized that due to the antibiotic treatment (too much) immunological tolerance is build-up during early life stages against species related to pathogens, resulting in an inefficient immune response later in life upon pathogenic challenge. This hypothesis regarding the presumed interaction between microbiota and immune development is in line with previous conclusions [215, 217, 223, 224, 252]. We intend to study this specifically in a further project.

General conclusion

Different early-life exposure under real-life circumstances, no antibiotic treatment, antibiotic treatment, or antibiotic and stressful treatment, affect the microbial colonization and immune development of piglets. The results shown here suggest a different immune development due to the early life treatments in these piglets. The next step will be to investigate which effect the early-life environment variation has on the immune competence later in life.

Acknowledgements

We want to thank the whole team of VIC Sterksel for their expertise and involvement in this research, and special thanks to Rita Hoving-Bolink for the overall organization. We also want to thank Jan Cornelissen for additional laboratory analyses (qPCR).

Supplemental tables and figures

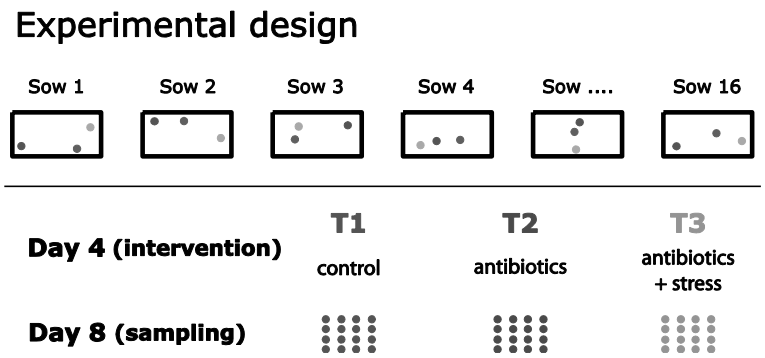

Figure S1. Depicted is the experimental design, for each sow (n=16) piglets are divided over the three treatments (T1, T2, and T3). At the day of sampling 16 piglets, 1 of each sow are taken for analysis, for analysis, for each treatment.

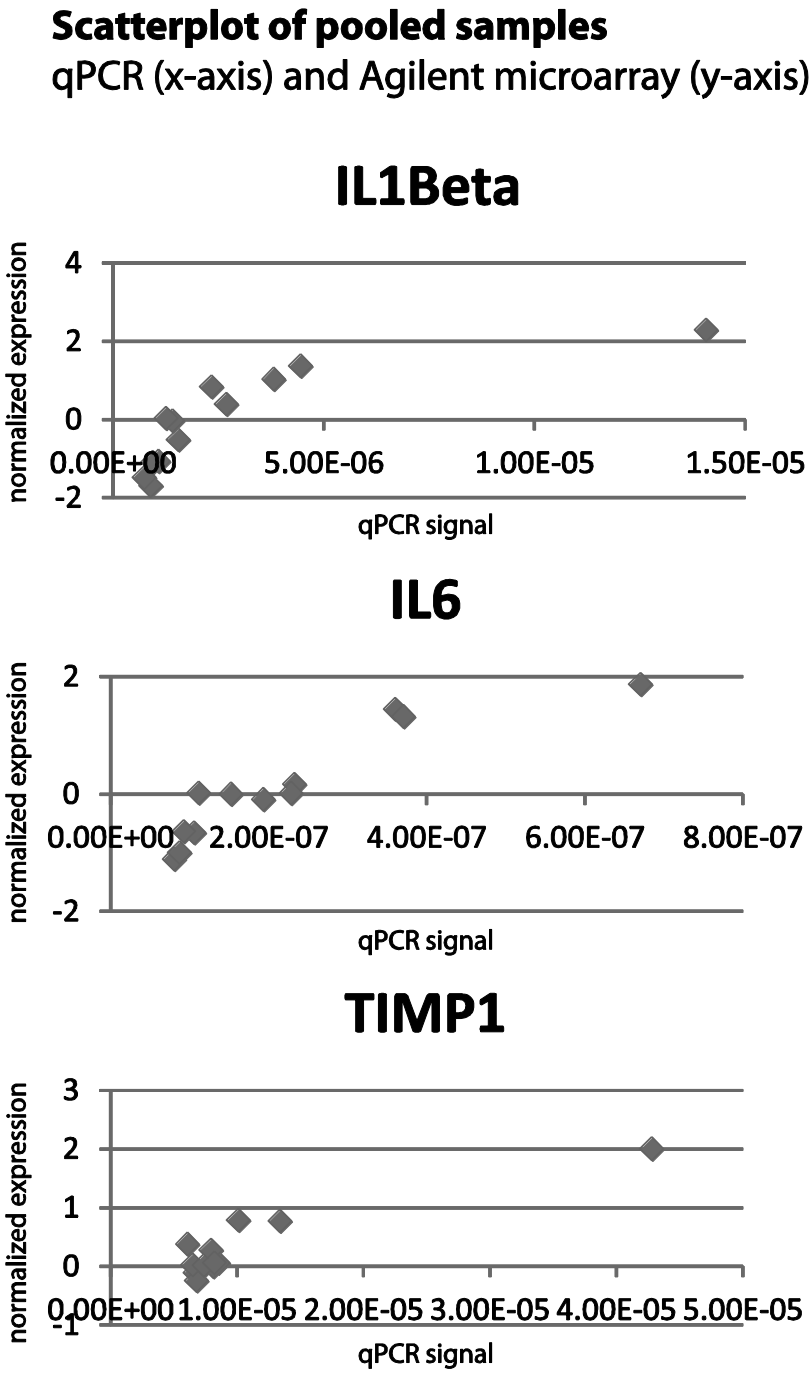

Figure S2. In the x-axis the qPCR signal is depicted and in the y-axis the normalized expression value is depicted. Three genes are depicted IL1B (upper panel), IL6 (middle panel), and TIMP1 (lower panel).

Supplementary Table A in File S1. Pools for microbiota and transcriptomic analyses.

T3*	T2	T1
Pool 1	**Pool 5**	**Pool 9**
R2679	R2679	R2679
R2705	R2705	R2705
R2592	R2592	R2592
R2704	R2704	R2704
Pool 2	**Pool 6**	**Pool 10**
R2687	R2687	R2687
R2423	R2423	R2423
R2369	R2369	R2369
R2419	R2419	R2419
Pool 3	**Pool 7**	**Pool 11**
R2336	R2336	R2336
R2504	R2504	R2504
R2692	R2692	R2692
R2514	R2514	R2514
Pool 4	**Pool 8**	**Pool 12**
R2591	R2591	R2591
R2701	R2701	R2701
R2596	R2596	R2596
R2669	R2669	R2669

* For each treatment 4 pools are created based on the same 4 piglets from 4 sows

Supplementary Table B in File S1. Number of up- or down-regulated probes and genes at day 8 after birth when comparing the different treatments (T1, T2, and T3). Numbers of annotated genes are given in parentheses.

Tissue	T2 vs. T1		T3 vs. T1		T3 vs. T2	
	Down	**Up**	**Down**	**Up**	**Down**	**Up**
Jejunum	63 (27)	24 (5)	29 (15)	38 (16)	6 (0)	55 (23)
Ileum	80 (34)	49 (16)	80 (24)	76 (29)	45 (6)	76 (32)
Blood	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)

Supplementary Table C in File S1. Phylum level bacteria changed in T2 and T3.

	T2 vs. T1	**T3 vs. T1**	**T3 vs. T2**	**ARC[1]**		
	***p* value**	***p* value**	***p* value**	**T1**	**T2**	**T3**
Firmicutes	0.34	0.89	0.11	80.15±3.04	76.86±2.01	79.22±0.88
Proteobacteria	0.34	1	0.11	11.40±2.28	13.45±1.41	12.13±0.52
Bacteroidetes	0.49	0.69	0.69	2.55±0.40	2.74±0.60	2.74±0.23
Spirochaetes	0.49	1	0.11	2.50±0.59	2.88±0.29	2.52±0.20
Actinobacteria	0.34	0.89	0.2	2.29±0.48	2.72±0.32	2.42±0.10
Fibrobacteres	0.34	0.34	**0.03**	0.58±0.23	0.70±0.11	0.43±0.02
Deferribacteres	0.11	1	0.34	0.23±0.04	0.29±0.06	0.22±0.01
Verrucomicrobia	0.11	1	0.11	0.19±0.03	0.25±0.05	0.18±0.01
Fusobacteria	1	0.34	0.49	0.10±0.01	0.10±0.01	0.11±0.01
Chlamydiae	0.49	**0.03**	**0.03**	<0.01	<0.01	<0.01
Planctomycetes	0.49	**0.03**	**0.03**	<0.01	<0.01	<0.01

[1] ARC: average relative contribution [%] of a microbial group. Values represent means ± SDs.
Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

Supplementary Table D in File S1. Summary of most prominent terms of GSEA analysis in jejunum.

T3vsT1		**T2vsT1**		**T3vsT2**	
T3	**T1**	**T2**	**T1**	**T3**	**T2**
DEGRADATION (VALINE, LEUCINE, ISOLEUCINE)	Ribosome/translation	x	response immune/defense/wounding	x	x
	LOCOMOTORY_BEHAVIOR		LOCOMOTORY_BEHAVIOR		
	beta cell development		immune cell activation		
			Ribosome/translation		
			cell cycle (arrest/apoptosis)		

Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

Supplementary Table E in File S1. Summary of most prominent terms of GSEA analysis in jejunum.

T3vsT1		**T2vsT1**		**T3vsT2**	
T3	**T1**	**T2**	**T1**	**T3**	**T2**
membrane/ receptor	Ribosome/ translation	endosome/lysosome/membrane	chemokine/cytokine	**x**	**x**
metabole	Immune/ chemokine	metabole (lipids/insulin)	intestinal immunity/inflammation		
apoptosis	behavior (locomotory)	Biocarta_Integrin_Pathway	behavior (locomotory)		
		golgi	Wounding		
		cell junction	Ribosome / translation		

Abbreviations used: T1; Treatment 1,T2; Treatment 2, T3; Treatment 3.

Chapter 5

Post-natal microbial association has impact on gut microbial colonization and intestinal gene expression in caesarean derived piglets

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To be submitted

* These authors contributed equally to this work

Abstract

To evaluate the effects of early microbial association on microbial colonization of the gastro-intestinal (GI) tract and host gene expression in the intestinal mucosa, we established an animal model with caesarean derived piglets. All piglets received orally a mixture of three microbial species (*Lactobacillus amylovorus*, *Clostridium glycolicum*, and *Parabacteroides* sp. ASF519 on day 1, 2, and 3 after birth. Moreover, on day 3 and 4, the piglets received either a faecal inoculant of an adult sow as a complex microbial association, or a placebo inoculant as simple microbial association. Microbiota composition was analysed using the Pig Intestinal Tract Chip, a 16S ribosomal RNA targeted oligonucleotide microarray, showing that complex microbial association caused an increase of faecal microbial diversity over the whole experiment period and accelerated the faecal microbiota to develop into a stable and diverse microbiota. Complex microbial association significantly affected the microbial composition and gene expression in the jejunal and ileal mucosa, albeit differently in jejunum and ileum. In the ileum, complex microbial association led to increased relative abundance of microbial groups that are known to have beneficial effects such as bacteria related to the genus *Lactobacillus* and butyrate producing members of *Clostridium* clusters, whereas it reduced the relative contribution of potential pathobionts. Immune-related gene sets were enriched by the complex microbial association in the ileal mucosa, and immuneomodulatory genes were negatively correlated with the abundance of potential pathobionts in ileal digesta. In conclusion, complex microbial association at early age has drastic effects on the development of the intestinal microbiota and the immune system. Hence it is proposed that manipulation of the microbial association at early age may be a way of supporting functional gut development.

Key words: piglets, microbiota, microbial association, transcriptome, gastrointestinal tract

Introduction

The intestinal tract of humans and animals is colonized by trillions of microorganisms that constitute a community or ecosystem known as the gut microbiota. This complex gut microbiota develops after birth as newborns are considered to be virtually sterile. During and after birth, microorganisms originating from surrounding environments, including faecal samples, rapidly colonize the gut of neonates and young animals [1-3]. However, recent studies reported the presence of microbes in the human intra-uterine environment, suggesting that some microbial colonization prior to the delivery can not be excluded [4-8].

Considerable knowledge has been assembled on the development of the intestinal microbiota in human. Generally, the first colonizers that dominate the human intistinal tract are facultative anaerobes including *Escherichia coli* and other *Enterobacteriaceae*, followed by anaerobic bacteria such as *Bifidobacterium*, *Clostridium*, *Bacteroides*, and *Ruminococcus* [9, 10]. The microbial succession during the first few weeks of life in the intestinal tract of chicken, pigs, and calves follows a similar pattern. Both coliforms and streptococci dominate the microbiota within a few days of birth and obligate anaerobes appear some time later, even though neonatal animals are exposed to greater numbers of faecal and environmental bacteria than the human neonates [11]. For human infants, after this initial colonization, the microbiota undergoes consecutive changes in composition and function until a relatively stable climax community is established at around 3 years after birth [12].

Many factors influence the microbial colonization at early age, including delivery mode (vaginally or via caesarean section), feeding (breast feeding or formula feeding), probiotic and prebiotic treatment, administration of antibiotics and exposure to environmental microbial sources. Most of these factors have been widely studied and reported to have either positive or negative impacts on infant and neonate animal development [1, 9, 50, 253]. However, the outcome of microbial association at early age cannot be easily predicted, due to the complexity and variability in especially environmental microbial exposure that newborns encounter. Infants may be subjected to different microbes in the hospital, at day care and in their home. Nevertheless, studies addressing these different sources of microbial exposure are still limited, especially those regarding exposure in hospital and day care . Regarding to the influence of the indoor house environment, studies have only shown that healthy full term infants living with pets exhibited under-representation of bifidobacteria and over-representation of *Peptostreptococcaceae*, whereas infants with older siblings exhibited under-representation of *Peptostreptococcaceae* and higher numbers of bifidobacteria [49, 50].

With respect to animals, especially piglets, studies have shown that the surrounding

environment in the postnatal period can have long-term impact on gut community structure [51]. Early-life environment was shown to cause major differences in mucosa-adherent microbial diversity in the ileum of adult pigs [52]. The raising environment attributes mostly to microbial association of piglets at early age. Pigs housed in a natural outdoor environment showed a dominance of Firmicutes, in particular *Lactobacillus*, whereas pigs housed in a hygienic indoor environment had reduced *Lactobacillus* and higher numbers of potentially pathogenic phylotypes [52]. Overall, environmentally-acquired bacteria influence the microbiota composition in the gut of piglets, as well as the microbiota of the adult pigs throughout life.

The microbiota plays a critical role in host immune system priming and gut maturation at early age, and can further influence the gut function through modifying gene expression of the host epithelium [20-23]. The host transcriptional pathways regulated in response to the colonizing microbiota involve nutrient uptake and metabolism, mucosal barrier function, xenobiotic metabolism, enteric nervous system and motility, hormonal and maturational responses, angiogenesis, cytoskeleton and extracellular matrix, signal transduction, and general cellular functions [24]. Interestingly, many host genes seem to be specifically altered in response to different members of the microbiota [25, 26]. Therefore, colonization of different microbes at early age may induce specific immune and metabolism system development, which consequently differentially influence the host intestinal and systemic health throughout life.

The pig is an important production animal as well as an appropriate model for humans, because of its similarities to humans in terms of anatomy, physiology and GI tract metabolism [153, 154, 164-167]. Moreover, piglets have been extensively used as model for infant nutrition and for studies on GI tract microbiota thus providing valuable information to understand the dynamics of microbial colonization of the intestinal tract. Furthermore, what we provide here is also important for developing new strategies for improving animal development and performance in animal production. In the present study, we used a piglet model designed by Jansman *et al*. [168] to investigate the impact of postnatal association with a simple or a complex microbiota on microbiota development and gene expression of the intestinal mucosa at early age. This study includes two animal experiments. Firstly, we compared the faecal microbiota of piglets colonized with a simple microbial community to that of animals associated with a complex microbiota (i.e. faecal inoculum) over a time course of 28 days starting immediately after birth. Secondly, we compared the microbiota and host gene expression in the jejunal and ileal mucosa of piglets at age of 16 days using the same association model. The results of this study confimed that early microbial exposure can impact the gut microbiota and host gut gene expression. This study provides evidence for the interaction between environmental factor, microbiota and

host. The gained insight is expected to be instrumental in improving sustainable pig management, as well as to provide important insight for the study of human gut microbiota.

Material and methods

Animals, feeding and sample collection

Experiment 1 was conducted as previously described by Jansman *et al.* [168]. Thirty piglets from two sows [(Great York×Pie) ×'Dalland' cross] were obtained by caesarean delivery (day 0). The piglets were divided over two treatment groups, housed in separate clean, non-sterile rooms, balanced for body weight and litter of origin. Each piglet received 30 ml of blood serum of an adult sow on day 0 and 20 ml on each of days 1, 2, and 3 and 5 mL of serum on day 4, as a source of immunoglobulins. All piglets received daily an oral inoculation of starter microbiota consisting of *Lactobacillus amylovorus* (3.6×10^7 cfu), *Clostridium glycolicum* (5.7×10^7 cfu) and *Parabacteroides* sp. ASF519 (4.8×10^7 cfu) from day 1 to 3 after birth. These three bacterial species have been reported to colonize throughout the length of the intestinal tract when administered to gnotobiotic piglets and induced a systemic increase in serum immunoglobulins. They were shown to be of value for highly controlled, reproducible experiments addressing the consequences of early microbial colonization [159]. On day 3 and 4, the piglets received either a complex microbiota by providing 2 mL of an inoculant consisting of 10% saline diluted faeces (DF) of an adult sow (complex association, CA), or a placebo (10% saline) inoculant (simple association, SA). Piglets were fed ad libitum a milk replacer diet during a period of 5 days (days 0-4) and a commercial moist diet during the remainder of the study as described previously [168]. Piglets were studied during a period of 28 days, and faeces of four representative piglets were repeatedly obtained for microbiota composition analysis on day 3, 5, 7, 14 and 28. On day 28, faecal samples were only collected from three piglets due to a loss of one animal of the SA group on day 26.

A second experiment was executed for a further evaluation of microbiota composition and mucosal gene expression in the jejunum and ileum using the same experimental model as used in experiment 1 [168]. Briefly, the piglets were assigned to one of the two treatments (SA or CA) taking into account litter origin, body weight and gender. Each piglet received a total of 50, 20, 20 and 20 ml of serum on day 0, 1, 2, and 3 after birth, respectively. For the first two days of the experiment, serum was used originating from experiment 1, whereas on day 3, serum was used originating from sows who delivered the piglets of the present experiment. As described before, all piglets received orally a mixture of *Lactobacillus amylovorus*, *Clostridium glycolicum* and *Parabacteroides* sp. ASF519 on day 1, 2 and 3 after birth. On day 3 and 4 the piglets received either treatment SA or CA. Piglets were fed milk for SPF piglets (Sloten bv, Deventer Nederland), consisting of 35% skimmed milk

powder, 35% whey powder, 22% vegetable oil, 0.5% hydrolysed wheat protein, 3.3% wheat starch, 2.7% sugar and a premix containing 23% crude protein for the first 5 days. For the remaining period, a slurry diet for SPF piglets (Nutreco, Trouw Nutrition, Belgium) was fed to the piglets, which consisted of 33.1% whey powder, 20.2% maize,13.5% wheat, 9% coconut oil, 5% wheat gluten, 2.5% sugar, 5.1% soybean meal, 2.5% potato starch, 2.5% potato protein, 1.25% rice protein, 0.6% extruded wheat and a vitamin/mineral premix. At day 16, six piglets of each treatment were euthanized based on balance of litter, body weight and sex. Intestinal digesta and mucosal tissue samples were collected from the jejunum and ileum. Jejunal and ileal mucosal tissue were quickly rinsed in PBS and immediately frozen in liquid nitrogen and stored at -80°C for analysis of gene expression.

Microbiota analysis

Microbial DNA extraction

Microbial DNA was extracted from 250 mg of DF, faeces and intestinal digesta of piglets using a faecal DNA extraction protocol adapted from Yu and Morrison [198], as described by Salonen *et al.*[199]. Briefly, cell lysis was achieved by bead beating in a Precellys®24 (Bertin, Montigny-le-Bretonneux, FR) instrument, in the presence of 4% (w/v) sodium dodecyl sulphate (SDS), 500 mM NaCl, 50 mM Tris-HCL (pH 8), and 50 mM EDTA. Following bead beating, samples were incubated at 95℃ for 15 min, centrifuged and precipitated with 260 µL of 10 M ammonium acetate. Nucleic acids were subsequently recovered by precipitation with isopropanol. Nucleic acid pellets were washed with 70% ethanol, dried and re-suspended in 100 µl of TE buffer. Purification was attained by adding 2 µl (10 mg/ml) RNase and incubating the samples for 15 min at 37°C. After incubation, 15µl proteinase K and 200 µl Buffer AL was added, followed by the use of QIAamp columns to complete purification.

Microbiota analysis

The microbial composition of DF, faeces and intestinal digesta of piglets was analyzed by using the Pig Intestinal Tract Chip (PITChip), version 1.0 and 2.0, respectively, in experiment 1 and experiment 2. The PITChip is a phylogenetic microarray, with more than 2,980 oligonucleotides based on 16S rRNA gene sequences of 627 porcine intestinal microbial species-level phylotypes for version 1.0, and with more than 3290 oligonucleotides based on 16S rRNA gene sequences of 781 porcine intestinal microbial species-level phylotypes respectively for version 2.0 [97, 101]. Like its human microbiota counterpart, the Human Intestinal Tract Chip (HITChip) [103], it provides a very deep and reproducible phylogenetic analysis that has been compared with deep pyrosequencing of 16S rRNA gene fragments [97, 104, 105] and next generation parallel sequencing of the

intestinal metagenome [106], indicating comparable resolution and a higher sensitivity of the chip based analysis. The protocol for hybridization and analysis of the generated data was performed essentially as described before for the HITChip [103]. The bacterial 16S rRNA gene pool was amplified using the primers T7 prom-Bact-27-for and Uni-1492-rev. The PCR products were *in vitro* transcribed into RNA that was subsequently purified and coupled with CyDye prior to fragmentation and hybridization to the array. Microarray images were processed using Agilent's Feature Extraction Software version 9.1 for experiment 1 and version 9.5 for experiment 2 (http://www.agilent.com). Data were retrieved from the MySQL (version 5.1) database as describe by Rajilic-Stojanovic [200] and pre-processed using the R (Rx64 2.12.2) microbiome package (http://microbiome.github.com/), using default settings.

Denaturing Gradient Gel Electrophoresis (DGGE) analysis was applied to detect the faecal microbiota of the excluded piglets. See **Supplemental methods** for experimental details.

Gene expression of intestinal tissue

RNA extraction

Total RNA was extracted from 50 to 100 mg jejunal and ileal mucosal tissue. The jejunum and ileum samples were homogenised using the TisuPrep (Homogenizer Omni TP TH220P) in TRizol reagent (Life Technologies) as recommended by the manufacturer with minor modifications. The homogenised tissue samples were dissolved in 5 ml of TRizol reagent. After centrifugation the supernatant was transferred to a fresh tube. Subsequently a phase separation with chloroform was performed as described by the manufacturer. The RNA was precipitated, dissolved and quantified by absorbance measurements at 260 nm. Furthermore quality control was performed with the BioAnalyzer 2100 (Agilent Technologies, Santa Clara, CA).

Labelling, hybridization, scanning and feature extraction

Labelling was done as recommended by Agilent Technologies using the One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling. The input was 10 ng of total RNA and 600 ng of labelled cRNA was used on an 8-pack array, porcine Agilent microarray slides, G2519F *Sus scrofa* (035953; V2:026440).

Hybridization was performed as described in the Agilent One-Color Microarray-Based Gene Expression Analysis Low input Quick Amp Labelling protocol in a dedicated hybridization oven (G2545A hybridization Oven Agilent Technologies). The hybridization temperature was 65°C with rotation speed 10 rpm for 17 hours. After 17 hours the arrays

were washed as described in the above-mentioned labelling protocol.

The arrays were scanned using an Agilent Technologies DNA microarray scanner with Surescan high resolution Technology, using Agilent Scan Control with resolution of 5µ, 16 bits and PMT of 100%. Feature extraction was performed using protocol 10.7.3.1 (v10.7) for 1 colour gene expression.

Data loading and processing

For each sample (n=24) a technical replicate was available, thus in total 48 samples were analyzed. The 48 files generated by the feature extraction software were loaded in GeneSpring GX 12, in which a log2-transformation and quantile normalization were performed. After quantile normalization, quality control was performed and data belonging to one ileal mucosa sample were removed. For each technical replicate pair the average was calculated and subsequently analysed by principle component analysis for each tissue separately.

Statistical methods

Microbiota analysis

Multivariate analysis was applied for detecting the microbial variation between CA and SA piglets. Principal response curves (PRC) analysis was employed to detect the faecal microbial variation over time in experiment 1. As described by Van den Brink and Ter Braak [201], PRC is based on redundancy analysis (RDA) adjusted for overall changes in community response over time. PRC focuses on the time-dependent treatment effects and enables a quantitative interpretation of effects towards the microbial composition. For PRC, the principal component is plotted against time, yielding a principal response curve of the community for each treatment. For the present study, treatment classes (CA or SA) were introduced as environmental (explanatory) variables and five sampling times (day 3, 5, 7, 14 and 28) were introduced as co-variables. The responsive variables were the relative contribution of 143 level 2 (approximate genus-level, 90% 16S ribosomal RNA similarity threshold) phylogenetic groups targeted by the PITChip (version 1.0). To evaluate the statistical significance of treatment effects on microbiota, Monte Carlo permutation tests were performed. These tests were also performed per sampling date, allowing the significance of the effect of a treatment regime to be tested for each sampling time. For day 28, since only three faecal samples of SA piglets were collected, the geometric mean of the relative contribution of 143 approximate genus-level phylogenetic groups of the other 3 SA piglets was calculated and included in the dataset.

To relate the variation of microbiota in jejunal and ileal content to treatments in experiment 2, RDA was used as implemented in the CANOCO 4.5 software package (Biometris, Wageningen, The Netherlands). RDA is the canonical form of principle component analysis and is a multivariate linear regression method where several response parameters are related to the same set of environmental variables of reduced rank [254]. The response variables in experiment 2 were the relative contribution of 150 approximate genus-level phylogenetic groups detected by the PITChip (version 2.0), and treatment classes (CA or SA) were introduced as environmental (explanatory) variables. RDA was performed focusing on inter-samples correlation, and the Monte Carlo Permutation test was applied to evaluate whether treatment class significantly contributed to the observed differences in microbial composition between CA and SA groups [228, 255]. Since the experiment had a randomized design, we used the unrestricted permutation option that yields completely random permutations. Treatment classes were considered to significantly affect microbial composition at P values < 0.05. Diagrams were plotted as biplots using CanoDraw (Biometris, Wageningen, The Netherlands).

Univariate testing of differences for individual microbial groups in experiment 1 and experiment 2 was done using a Mann-Whitney U signed rank test. P-values were corrected for multiple testing using Benjamini–Hochberg's approach [202].

Gene Set Enrichment Analysis (GSEA)

GSEA [9,10] was performed separately for the ileal and jejunal mucosal tissue in experiment 2. The following settings were different from the default settings. Permutations were performed on the gene set, and chip platform was set to gene symbol. Six gene set databases (v3.0) were loaded for analysis, namely three Gene Ontology (GO) related gene sets, i.e. biological processes, molecular function and cellular component, and three pathway related gene sets, i.e. BioCarta, Reactome and KEGG.

Correlation analysis

Correlation analysis of microbiota (PITChip data) and gene expression (tissue) datasets was performed as previously described [256]. In brief, a correlation matrix between the PITChip data and intestinal tissue gene expression was computed using the $^{2}\log$ transformed expression values. To reduce the probability of type I errors (false positives) for multiple testing, the Benjamini–Yekutieli method [202] was employed and the data was corrected for multiple testing using an FDR method. FDR was set at < 0.05 % for expected proportion of false positive correlations in the multiple comparison testing. Detailed explanation of the methods used to perform correlation analysis can be obtained from the authors upon request.

Results

Faecal microbial colonization

In order to evaluate the effects of inoculation with diluted faeces (DF) on microbiota establishment over time, we calculated the similarity of microbiota between DF and faeces of CA piglets over time using Pearson's correlation, taking the similarity between DF and SA piglets as a control (**Fig. 1**). The similarity was at the lowest value at day 3 for both SA (66.7±4.7%) and CA (63.7±4.3%) piglets. After inoculation with DF, the similarity between DF and CA piglets significantly increased at day 5 ($P<0.05$). It slightly dropped at day 7 and then significantly increased at day 14 ($P<0.05$), reaching its highest value (86.6±4.1%) at day 28. As a control, the similarity between DF and SA piglets did not significantly change over time, although there was an increasing trend at day 5 and 28.

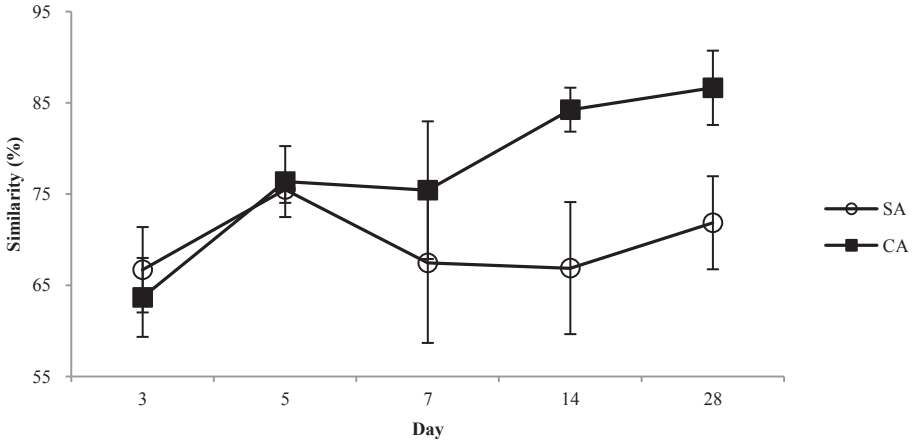

Figure 1. Similarity of microbial profiles of sow faeces used for early microbial association and faeces of piglets in the SA and CA treatment over time in experiment 1. The microbiota similarity was calculated between DF and piglets at day 3 (D3), 5(D5), 7(D7), 14(D14) and 28(D28) using Pearson's correlation. SA, simple association with a placebo inoculant; CA, complex association with inoculant consisting of diluted faeces of an adult sow.

Microbial diversity of faecal samples as based on the Shannon index appeared to be higher for all piglets with increasing age, except at day 28 (**Fig.2**). When comparing the microbial diversity in CA-piglets to that of SA-piglets, we found that the CA piglets had a significantly lower diversity than SA piglets at day 3 ($P<0.05$). After DF inoculation, however, the diversity of CA piglets was higher than that of SA piglets, with significant differences observed for day 5 and 14 ($P<0.05$).

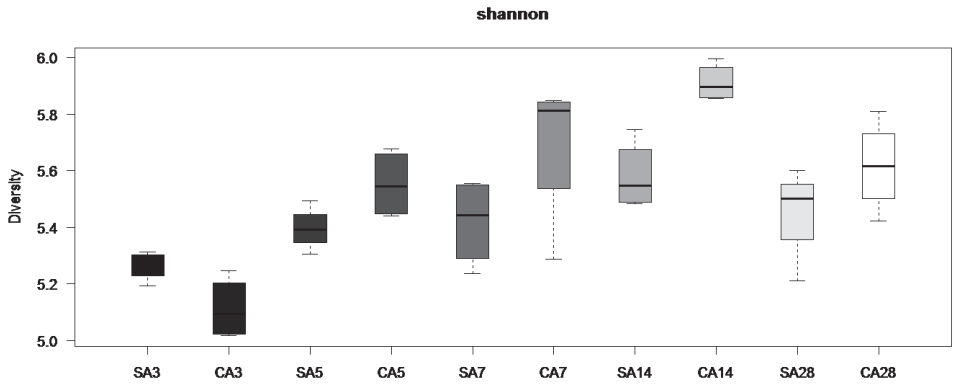

Figure 2. Microbial diversity of piglets' faeces over time in experiment 1. The Shannon index was calculated separately for both treatments at all sampling times. SA, simple association with a placebo inoculant; CA, complex association with inoculant consisting of diluted faeces of an adult sow. Numbers following SA or CA indicate sampling time: day 3, 5, 7, 14 and 28.

Principle Response Curves (PRC) analysis indicated that after DF inoculation, microbiota composition in CA piglets increasingly deviated from that of SA piglets especially after day 5 (**Fig. 3**). Mont Carlo permutation testing confirmed that association with DF contributed significantly to the microbial differences between CA- and SA-piglets (P=0.045). The composition of microbiota of CA piglets tended to be different from that of SA piglets at day 7 (P=0.060) and day 28 (P=0.065), and the difference was significant at day 14 (P=0.045).

Univariate testing was applied to detect differences in microbial groups between CA and SA piglets at each sampling day. At day 3, we detected a different relative abundance of bacteria related to the genera *Allofustis*, *Weissella*, *Sphingomonas* and *Leptospira* (**Table 1**). After DF inoculation, there was an increase of six bacterial groups of *Clostridium* cluster XIVa and *Treponema* at approximate genus level at day 5 (**Table 1**). More bacterial groups varied between the SA and CA piglets at day 7. At phylum level, the relative abundance of Fibrobacteres decreased in CA-piglets in comparison with SA-piglets (**Supplemental Table 1**). At level 2 (90% 16S ribosomal RNA similarity threshold), there was a reduction in the relative abundance of bacteria related to *Fibrobacter succinogenes, Gemella haemolysans, Lactobacillus gasseri, Turicibacter,* uncultured *Clostridium* cluster IV*, Clostridium sphenoides, Pseudomonas*, and *Brachyspira*, whereas an increase was detected for the relative abundance of a number of anaerobic bacterial groups typically found in faeces, including fermenting microorganisms and populations especially within the Firmicutes known or predicted to be involved in production of short chain fatty acids, such

Table 1. Level 2 phylogenetic groups with different relative abundance in piglets faeces over time in experiment 1.

Class	Group	Effect[1]	*P* value	C.*p*[2]	ARC[3]	
		CA vs SA			SA	CA
Day 3						
Bacilli	*Allofustis*	-	0.03	0.62	0.43±0.10	0.25±0.06
	Weissella et rel.	-	0.03	0.62	0.36±0.13	0.19±0.08
Alphaproteobacteria	*Sphingomonas* et rel.	-	0.03	0.62	0.05±0.01	0.04±0.01
Spirochaetes	*Leptospira*	+	0.03	0.62	0.52±0.08	0.65±0.03
Day 5						
Clostridium cluster XIVa	*Clostridium lactifermentans* et rel.	+	0.03	0.48	0.11±0.05	0.27±0.12
	Clostridium symbosium et rel.	+	0.03	0.48	0.14±0.03	0.36±0.14
	Coprococcus eutactus et rel.	+	0.03	0.48	0.23±0.07	0.44±0.11
	Eubacterium plexicaudatum et rel.	+	0.03	0.48	0.03±0.00	0.06±0.04
	Ruminococcus obeum et rel.	+	0.03	0.48	0.87±0.20	3.13±2.07
	Uncultured *Clostridia* XIVa	+	0.03	0.48	1.04±0.30	2.38±0.51
Spirochaetes	*Treponema* et rel.	+	0.03	0.48	0.15±0.04	0.32±0.10
Day 7						
Actinobacteria	*Aeriscardovia* et rel.	+	0.03	0.20	0.12±0.04	0.26±0.11
	Collinsella	+	0.03	0.20	0.13±0.06	0.29±0.06
	Olsenella et rel.	+	0.03	0.20	0.01±0.00	0.04±0.01
Sphingobacteria	*Sphingobacterium thalpophilum* et rel.	+	0.03	0.20	0.02±0.01	0.05±0.02
Fibrobacteres	*Fibrobacter succinogenes* et rel.	-	0.03	0.20	1.39±0.75	0.20±0.08
Bacilli	*Gemella haemolysans* et rel.	-	0.03	0.20	0.37±0.13	0.17±0.07
	Lactobacillus gasseri et rel.	-	0.03	0.20	6.18±4.82	0.04±0.05
	Turicibacter et rel.	-	0.03	0.20	1.46±0.96	0.12±0.04
Clostridium cluster IV	*Clostridium leptum* et rel.	+	0.03	0.20	0.16±0.12	0.48±0.12
	Faecalibacterium prausnitzii et rel.	+	0.03	0.20	1.73±0.83	3.49±0.73
	Ruminococcus bromii et rel.	+	0.03	0.20	0.01±0.00	0.03±0.02
	Sporobacter termitidis et rel.	+	0.03	0.20	3.66±1.48	6.49±0.63
	Subdoligranulum et rel.	+	0.03	0.20	0.32±0.07	0.58±0.22
	Uncultured *Clostridia* IV	-	0.03	0.20	1.12±0.20	0.67±0.12
Clostridium cluster IX	*Mitsuokella multiacida* et rel.	+	0.03	0.20	0.04±0.01	0.18±0.14
Clostridium cluster XIVa	*Clostridium sphenoides* et rel.	-	0.03	0.20	0.25±0.05	0.12±0.04
Clostridium cluster XIVb	Uncultured *Clostridia* XIVb	+	0.03	0.20	1.62±0.40	2.11±0.09
Gammaproteobacteria	*Pseudomonas* et rel.	-	0.03	0.20	1.32±0.51	0.56±0.22
	Psychrobacter et rel.	+	0.03	0.20	0.27±0.09	0.46±0.08
Spirochaetes	*Brachyspira*	-	0.03	0.20	0.03±0.02	0.01±0.00
Day 14						
Bacteroidetes	*Alistipes* et rel.	+	0.03	0.19	0.05±0.03	2.21±1.28
	Bacteroides pyogenes et rel.	+	0.03	0.19	0.04±0.07	0.60±0.31

	Bacteroides vulgatus et rel.	+	0.03	0.19	0.02±0.03	0.41±0.13
	Prevotella melaninogenica et rel.	+	0.03	0.19	0.07±0.07	1.68±0.36
	Prevotella ruminicola et rel.	+	0.03	0.19	0.03±0.04	0.20±0.12
	Uncultured *Bacteroidetes*	+	0.03	0.19	0.08±0.12	2.74±2.26
	Uncultured *Prevotella*	+	0.03	0.19	0.73±1.00	11.79±4.76
Flavobacteria	*Flavobacterium cucumis* et rel.	+	0.03	0.19	0.01±0.00	0.06±0.04
Sphingobacteria	Uncultured *Sphingobacteria*	+	0.03	0.19	0.02±0.02	0.17±0.06
Bacilli	*Streptococcus intermedius* et rel.	+	0.03	0.19	0.34±0.25	1.33±0.45
Clostridium cluster IV	*Clostridium cellulosi* et rel.	+	0.03	0.19	0.20±0.10	0.62±0.25
Clostridium cluster IX	*Megasphaera elsdenii* et rel.	+	0.03	0.19	0.01±0.00	0.65±0.47
	Mitsuokella multiacida et rel.	+	0.03	0.19	0.06±0.04	0.65±0.26
	Peptococcus niger et rel.	+	0.03	0.19	0.00±0.00	0.02±0.01
	Phascolarctobacterium faecium et rel.	+	0.03	0.19	0.63±0.07	1.22±0.11
Mollicutes	*Acholeplasma* et rel.	-	0.03	0.19	0.29±0.06	0.17±0.06
Planctomycetacia	Uncultured *planctomycetacia*	+	0.03	0.19	0.01±0.00	0.09±0.06
Gammaproteobacteria	*Avibacterium*	-	0.03	0.19	0.27±0.14	0.04±0.01
	Bisgaard	-	0.03	0.19	0.21±0.10	0.03±0.01
	Escherichia coli et rel.	-	0.03	0.19	1.93±0.39	1.13±0.33
	Pasteurella	-	0.03	0.19	0.43±0.18	0.10±0.04
	Ruminobacter et rel.	-	0.03	0.19	0.26±0.12	0.04±0.02

[1] Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the CA group in comparison with the SA group.
[2] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[3] ARC: Average relative contribution [%] of a microbial group. Values represented means ± SDs.

as *Clostridium leptum, Faecalibacterium prausnitzii*, *Sporobacter termitidis*, *Ruminococcus bromii* and *Mitsuokella multiacida* (**Table 1**). The observed differences also reflected the significantly higher similarity between the faecal inoculum and CA piglet faecal microbiota profiles as described above. At day 14, we found an increase in Bacteroidetes and Planctomycetes, with a decrease in Proteobacteria at phylum level in CA-piglets (**Supplemental Table 1**). At approximate genus level, there was an increase of 16 bacterial groups belonging to the classes of *Bacteroidetes*, *Flavobacteria*, *Sphingobacteria*, *Bacilli*, *Clostridium* cluster IV, *Clostridium* cluster IX and *Planctomycetacia* in CA-piglets respectively, while a lower relative abundance was detected for *Acholeplasma* and bacterial groups belonging to *Gammaproteobacteria*, including *Avibacterium*, *Bisgaard*, *Escherichia coli*, *Pasteurella* and *Ruminobacter* (**Table 1**). At day 28, no significant differences in relative abundance of bacterial groups were detected between treatments, however, observed differences were similar to those found at day 7 (**Supplemental Table 2**).

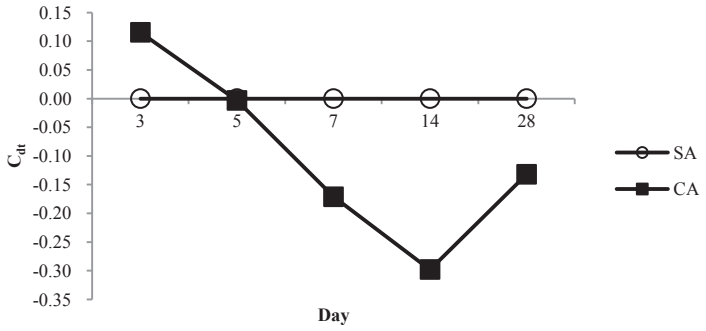

Figure 3. Principal response curves indicating the faecal microbial variation over time of CA-and SA-piglets in experiment 1. Of all variance, 28.1% is attributed to sampling date and displayed on the horizontal axis, 21.5% is attributed to treatment, and 58.8% was attributed to differences between individual piglets. Of the variance explained by treatment, 51.6 % is displayed on the vertical axis. The SA treatment was set as the baseline throughout the experiment. C_{dt}, C_{dt} is calculated based on canonical coefficients in the output of CANOCO software according to formula: C_{dt} = Regression and canonical coefficient of environmental variable*total standard deviation/standad deviation of environmental variable.

Microbial profile in jejunal and ileal digesta

RDA showed that the complex microbial association significantly contributed to the observed difference of jejunal (P=0.002) and ileal (P=0.002) microbiota between CA and SA piglets in Experiment 2 (**Fig. 4**). Univariate analysis showed differences in microbial groups in digesta of the jejunum and ileum between treatments. In the jejunum, the relative abundance of the phyla Deferribacteres, Spirochaetes and Verrucomicrobia was significantly higher in the CA group, whereas the relative abundance of Bacteroidetes, Fibrobacteres and Fusobacteria was lower compared to the SA group (**Supplemental Table 3**). At the genus level (i.e. 90% sequence similarity), CA resulted in a decrease of certain early gut colonizers, including *Bifidobacterium* and *Faecalibacterium prausnitzii,* as well as some opportunistic pathogens such as *Streptococcus suis*. A few presumed beneficial microbes such as *Akkermansia muciniphila* and *Eubacterium rectale* were increased; opportunistic pathogens such as *Clostridium perfringens* were increased in the CA piglets as well compared to SA piglets (**Table 2**).

In ileal digesta, there was a significantly higher relative abundance of Firmicutes and Verrucomicrobia at phylum level in CA group, while a lower relative abundance was detected for Proteobacteria, Bacteroidetes, Fibrobacteres and Fusobacteria (**Supplemental Table 3**). At the approximate genus level, a stimulation of CA was found for some presumed beneficial microbes, including bacteria related to *Akkermansia muciniphila*, *Lactobacillus plantarum*, *Lactobacillus salivarius* and butyrate-producing *Ruminococcus bromii* and *Eubacterium rectale*, whereas opportunistic pathogens including *Clostridium*

leptum, *Clostridium difficile*, and members of the *Gammaproteobacteria*, e.g. *Escherichia coli* and *Pseudomonas* spp. were reduced in the CA group (**Table 3**).

Despite the change in relative abundance of several microbial groups, CA did not significantly alter the microbial diversity in the jejunal and ileal digesta as indicated by Shannon indices (P>0.05) (data not shown).

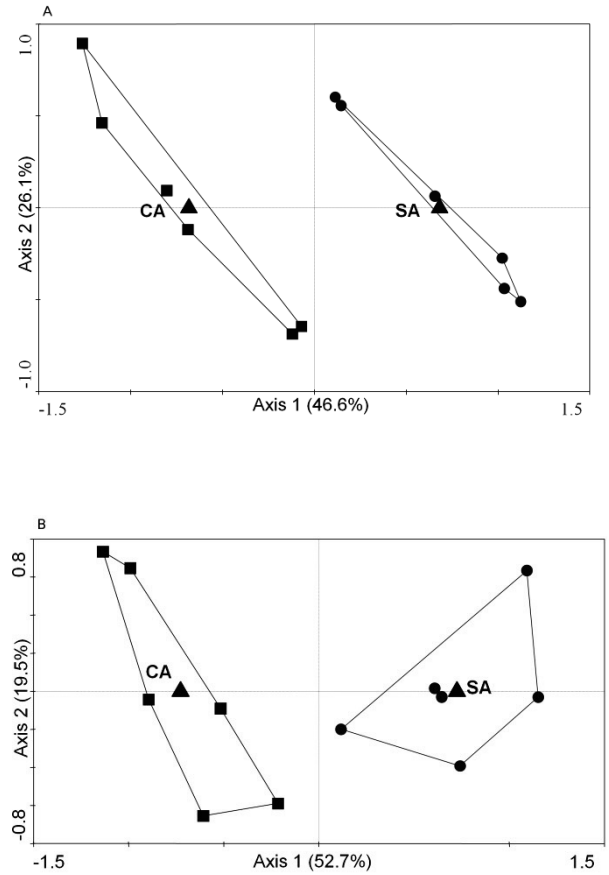

Figure 4. Biplots for RDA analysis of microbiota in jejunal (A) and ileal digesta (B) in experiment 2. Nominal environmental variables simple association (SA) and complex association (CA) are represented by triangles (▲). Samples are grouped by treatment: CA (■) and SA (●), each symbol represents a piglet. Both axes together explain 72.7% of the total variance in the data for microbial composition in digesta obtained from the jejunum (A) and ileum (B).

Table 2. Level 2 phylogenetic groups with different relative abundance in the digesta of the jejunum of piglets at day 16 of age in experiment 2.

Class	Group	Effect[1] CA vs SA	*P* value	C.*p*[2]	ARC[3] CA	ARC[3] SA
Actinobacteria	*Bifidobacterium* et rel.	-	<0.01	0.02	0.65±0.08	0.94±0.17
Bacteroidetes	*Bacteroides distasonis* et rel.	-	<0.01	0.02	0.25±0.13	0.71±0.18
	Prevotella melaninogenica et rel.	-	<0.01	0.02	0.12±0.05	0.27±0.06
	Uncultured *Porphyromonadaceae*	-	0.03	0.07	0.40±0.04	0.46±0.06
	Uncultured *Prevotella*	-	<0.01	0.02	0.67±0.27	1.52±0.35
Deferribacteres	*Mucispirillum schaedleri* et rel.	+	0.03	0.07	0.28±0.03	0.22±0.04
Fibrobacteres	*Fibrobacter succinogenes* et rel.	-	0.01	0.03	0.23±0.14	0.61±0.23
Bacilli	*Aerococcus urinaequi* et rel.	+	<0.01	0.02	0.10±0.07	0.02±0.00
	Bacillus et rel.	-	<0.01	0.02	0.40±0.05	0.58±0.13
	Carnobacterium et rel.	+	<0.01	0.02	0.65±0.05	0.44±0.09
	Enterococcus et rel.	+	0.01	0.03	4.42±0.51	3.35±0.58
	Lactobacillus salivarius et rel.	+	<0.01	0.02	3.41±0.47	1.56±0.27
	Lactococcus et rel.	-	<0.01	0.02	0.24±0.14	1.03±0.41
	Staphylococcus aureus et rel.	+	0.02	0.05	0.18±0.02	0.14±0.03
	Streptococcus bovis et rel.	-	0.03	0.07	2.25±1.30	4.35±1.19
	Streptococcus salivarius et rel.	-	<0.01	0.03	0.85±0.48	2.38±0.68
	Streptococcus suis et rel.	-	<0.01	0.02	1.04±0.58	2.60±0.68
	Uncultured *Bacilli*	+	0.03	0.07	0.14±0.02	0.10±0.02
Clostridium cluster I	*Clostridium perfringens* et rel.	+	<0.01	0.03	0.74±0.06	0.52±0.18
Clostridium cluster IV	*Anaerotruncus* et rel.	+	0.01	0.03	1.24±0.14	0.91±0.16
	Faecalibacterium et rel.	-	0.01	0.03	0.51±0.05	0.62±0.08
	Faecalibacterium prausnitzii et rel.	-	0.01	0.03	0.36±0.10	0.61±0.11
	Ruminococcus callidus et rel.	+	0.02	0.05	2.90±0.30	2.22±0.43
	Uncultured *Clostridia* IV	+	0.04	0.10	3.81±0.38	3.21±0.54
Clostridium cluster IX	*Megasphaera elsdenii* et rel.	-	<0.01	0.03	0.13±0.07	0.28±0.07
	Mitsuokella multacida et rel.	-	0.01	0.03	1.02±0.61	2.39±0.56
	Peptococcus niger et rel.	+	0.02	0.05	0.33±0.05	0.25±0.04
	Phascolarctobacterium faecium et rel.	-	0.01	0.03	0.16±0.04	0.25±0.04
	Veillonella	-	0.01	0.03	0.14±0.03	0.18±0.02
Clostridium cluster XIVa	*Butyrivibrio crossotus* et rel.	-	0.04	0.10	1.16±0.06	1.24±0.05
	Coprococcus eutactus et rel.	-	<0.01	0.02	0.48±0.10	0.82±0.12
	Eubacterium hallii et rel.	-	0.01	0.03	0.59±0.04	0.68±0.05
	Eubacterium rectale et rel.	+	0.04	0.10	2.26±0.18	1.92±0.30
	Lachnobacillus bovis et rel.	+	0.01	0.03	1.41±0.22	1.08±0.16
	Roseburia intestinalis et rel.	-	0.01	0.03	1.08±0.10	1.35±0.17
	Ruminococcus obeum et rel.	+	0.04	0.10	3.08±0.31	2.58±0.39
Clostridium cluster XIVb	Uncultured *Clostridia* XIVb	-	0.03	0.07	1.39±0.16	1.53±0.08
Mollicutes	Uncultured *Mollicutes*	+	0.01	0.03	0.68±0.07	0.49±0.11
Fusobacteria	*Fusobacterium* et rel.	-	0.01	0.03	0.46±0.17	0.90±0.26
Alphaproteobacteria	*Caulobacter* et rel.	+	0.01	0.03	1.04±0.11	0.80±0.17
	Labrys methylaminiphilus et rel.	+	<0.01	0.02	0.86±0.05	0.72±0.07
	Rhodobacter et rel.	+	0.04	0.10	0.79±0.12	0.62±0.13
Betaproteobacteria	*Neisseria* et rel.	-	<0.01	0.03	0.30±0.05	0.44±0.08
	Sutterella wadsorthia et rel.	-	0.04	0.10	0.37±0.08	0.49±0.05
Deltaproteobacteria	*Desulfovibrio* et rel.	-	0.01	0.03	0.4±0.07	0.57±0.11
	Lawsonia intracellularis et rel.	-	<0.01	0.03	0.15±0.09	0.37±0.10
	Uncultured *Deltaproteobacteria*	+	0.03	0.07	0.28±0.03	0.22±0.04

Gammaproteobacteria	*Aeromonas*	+	0.04	0.10	0.18±0.02	0.13±0.05
	Ignatzschineria et rel.	+	0.01	0.03	0.44±0.05	0.30±0.06
	Psychrobacter et rel.	-	0.03	0.07	0.42±0.04	0.51±0.07
	Vibrio et rel.	+	0.04	0.10	0.18±0.02	0.13±0.05
Spirochaetes	*Brachyspira*	-	0.04	0.10	0.20±0.06	0.25±0.05
	Treponema et rel.	+	<0.01	0.02	1.29±0.23	0.75±0.17
	Uncultured *Spirochaetes*	+	0.02	0.05	0.51±0.06	0.37±0.09
Verrucomicrobiae	*Akkermansia muciniphila* et rel.	+	0.04	0.10	0.25±0.03	0.20±0.04

[1] Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the CA group in comparison with the SA group.
[2] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[3] ARC: Average relative contribution [%] of a microbial group. Groups with a relative abundance lower than 0.1% in all treatments are not shown. Values represent means ± SDs, $n = 6$ per treatment.

Table 3. Approximate genus-level phylogenetic groups with different relative abundance in the ileum of piglets in experiment 2.

Class	Group	Effect[1] CA vs SA	*P* value	C.*p*[2]	ARC[3]	
					CA	SA
Bacteroidetes	*Prevotella melaninogenica* et rel.	-	<0.01	0.02	0.13±0.04	0.23±0.06
	Uncultured *Prevotella*	-	<0.01	0.01	0.68±0.23	1.33±0.36
Bacilli	*Aerococcus urinaequi* et rel.	+	<0.01	0.01	0.14±0.05	0.02±0.01
	Allofustis et rel.	+	0.02	0.04	0.25±0.04	0.15±0.05
	Carnobacterium et rel.	+	0.01	0.02	0.66±0.12	0.33±0.15
	Enterococcus et rel.	+	0.02	0.04	4.92±1.02	3.18±0.82
	Lactobacillus plantarum et rel.	+	0.02	0.04	3.99±1.39	1.76±1.03
	Lactobacillus salivarius et rel.	+	<0.01	0.01	3.68±0.74	1.49±0.38
	Uncultured *Bacilli*	+	0.02	0.04	0.13±0.04	0.08±0.03
Clostridium cluster IV	*Anaerotruncus* et rel.	+	<0.01	0.01	1.18±0.09	0.56±0.26
	Clostridium cellulosi et rel.	+	<0.01	0.01	1.71±0.15	0.81±0.36
	Eubacterium cellulosi et rel.	+	<0.01	0.02	0.40±0.07	0.16±0.09
	Eubacterium siraeum et rel.	+	0.02	0.04	0.59±0.09	0.43±0.12
	Faecalibacterium et rel.	+	0.02	0.04	0.52±0.11	0.40±0.06
	Papillibacter cinnamivorans et rel.	+	<0.01	0.01	1.50±0.15	0.69±0.27
	Ruminococcus bromii et rel.	+	<0.01	0.01	1.02±0.15	0.58±0.14
	Ruminococcus callidus et rel.	+	<0.01	0.01	2.70±0.14	1.44±0.50
	Sporobacter termitidis et rel.	+	<0.01	0.01	6.17±0.46	4.31±0.79
Clostridium cluster IX	*Dialister* et rel.	+	0.02	0.04	0.73±0.18	0.48±0.19
	Peptococcus niger et rel.	+	<0.01	0.02	0.28±0.04	0.10±0.08
Clostridium cluster XI	*Anaerovorax* et rel.	-	<0.01	0.01	1.08±0.67	3.67±1.06
	Clostridium difficile et rel.	-	<0.01	0.01	1.75±1.78	9.65±2.81
	Eubacterium pyruvativorans et rel.	-	<0.01	0.01	0.04±0.04	0.18±0.07
Clostridium cluster XIII	*Peptoniphilus* et rel.	-	0.01	0.02	0.41±0.27	1.16±0.39
Clostridium cluster XIVa	*Clostridium herbivorans* et rel.	+	0.01	0.02	0.68±0.10	0.42±0.13
	Clostridium hylemonae et rel.	-	<0.01	0.01	0.03±0.02	0.10±0.03
	Clostridium lactifermentans et rel.	+	0.02	0.04	0.62±0.08	0.46±0.08

	Clostridium nexile et rel.	+	0.01	0.02	0.17±0.02	0.10±0.03
	Clostridium oroticum et rel.	-	<0.01	0.02	0.42±0.33	1.39±0.35
	Clostridium sphenoides et rel.	-	<0.01	0.01	0.25±0.13	0.65±0.24
	Clostridium symbiosum et rel.	+	0.01	0.02	0.36±0.05	0.21±0.07
	Dorea et rel.	-	0.02	0.04	0.29±0.21	0.84±0.38
	Eubacterium hallii et rel.	+	<0.01	0.01	0.57±0.05	0.37±0.08
	Eubacterium plexicaudatum et rel.	+	0.01	0.02	0.35±0.05	0.20±0.07
	Eubacterium rectale et rel.	+	<0.01	0.01	2.24±0.13	1.18±0.40
	Eubacterium ventriosum et rel.	+	<0.01	0.02	0.19±0.03	0.10±0.04
	Lachnobacillus bovis et rel.	+	<0.01	0.01	1.40±0.13	0.50±0.26
	Lachnospira pectinoschiza et rel.	+	<0.01	0.01	3.41±0.33	1.77±0.61
	Roseburia intestinalis et rel.	+	<0.01	0.01	1.03±0.11	0.62±0.15
	Ruminococcus obeum et rel.	+	<0.01	0.01	2.89±0.32	1.65±0.53
	Uncultured *Clostridia* close to *Clostridium symbiosum* et rel.	-	<0.01	0.02	0.40±0.20	0.87±0.16
	Uncultured *Clostridia* XIVa	+	<0.01	0.01	2.25±0.19	1.28±0.35
	Uncultured *Clostridia* XIVa close to *Anaerostipes caccae*	+	0.01	0.02	0.11±0.01	0.06±0.02
Clostridium cluster XIVb	Uncultured *Clostridia* XIVb	-	<0.01	0.01	1.26±0.22	2.21±0.40
Mollicutes	Uncultured *Mollicutes*	+	0.01	0.02	0.60±0.06	0.43±0.13
Alphaproteobacteria	*Caulobacter* et rel.	+	<0.01	0.01	1.04±0.13	0.58±0.27
	Rhizobium et rel.	-	<0.01	0.01	0.25±0.21	1.00±0.26
Deltaproteobacteria	*Desulfovibrio* et rel.	-	0.01	0.02	0.34±0.06	0.47±0.05
	Lawsonia intracellularis et rel.	-	<0.01	0.02	0.13±0.07	0.30±0.09
Gammaproteobacteria	*Aeromonas*	-	<0.01	0.01	0.13±0.04	0.30±0.09
	Escherichia coli et rel.	-	0.01	0.02	0.89±0.25	1.51±0.25
	Halomonas et rel.	-	0.01	0.02	0.18±0.05	0.28±0.04
	Ignatzschineria et rel.	+	0.01	0.02	0.37±0.05	0.25±0.07
	Pseudomonas et rel.	-	<0.01	0.01	0.56±0.18	1.16±0.26
	Psychrobacter et rel.	-	<0.01	0.01	0.40±0.04	0.56±0.05
	Thiocapsa et rel.	-	<0.01	0.01	0.13±0.06	0.33±0.04
	Vibrio et rel.	-	<0.01	0.01	0.13±0.04	0.29±0.09
Spirochaetes	*Leptospira*	-	<0.01	0.01	0.19±0.10	0.45±0.07
	Turneriella	+	0.01	0.02	0.78±0.12	0.40±0.25
	Uncultured *Spirochaetes*	+	<0.01	0.02	0.53±0.10	0.25±0.13
Verrucomicrobiae	*Akkermansia muciniphila* et rel.	+	0.04	0.08	0.21±0.02	0.16±0.06

[1] Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the CA group in comparison with the SA group.
[2] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[3] ARC: Average relative contribution [%] of a microbial group. Groups with a relative abundance lower than 0.1% in all treatments are not shown. Values represent means ± SDs, $n = 6$ per treatment.

Gene expression in the ileal and jejunal mucosal tissue

We performed gene set enrichment analysis (GSEA) to investigate whether gene sets were differently expressed in the jejunal and ileal mucosa between the different association treatments. GSEA showed fewer significantly different gene sets (FDR < 5%) in the jejunum compared to ileum (**Table 4, 5**). In the jejunum, there were no clear differences in mucosal gene expression between SA and CA piglets. In piglets of both SA and CA treatments, gene sets involved in (generic) metabolic processes were expressed (**Table 4**). In contrast, in the ileum clear differences between treatments were observed. Many gene sets were enriched in CA pigs, especially related to immune related processes. 'T cell activation', 'cytokine activity', and 'regulation of lymphocyte activation' were processes especially observed using the GO database, whereas 'RIG I like receptor signalling pathway' and 'antigen processing and presentation' were found to be stimulated using the KEGG database (**Table 5**). Compared to the ileum, fewer gene sets involved in immune related processes were observed to be expressed in the jejunal mucosa.

Table 4. Significantly enriched gene sets in the jejunal mucosa on day 16 of piglets based on the KEGG and GO database**.**

NAME	SIZE	NES[1]	NOM p-val	FDR q-val[2]
KEGG database				
CA				
Lysosome	61	2.775	<0.001	<0.001
Vibrio cholerae infection	29	1.977	<0.001	0.019
Aldosterone regulated sodium reabsorption	22	1.865	0.002	0.030
Sphingolipid metabolism	16	1.879	0.005	0.033
SA				
Ribosome	48	-2.098	<0.001	0.002
Peroxisome	40	-1.953	0.001	0.005
Metabolism of xenobiotics by cytochrome p450	16	-1.996	0.001	0.005
Proteasome	23	-1.809	0.002	0.024
GO database				
SA				
Structural constituent of ribosome	40	-2.177	<0.001	0.005

[1] NES: Normalized Enrichment Score
[2]FDR < 5%

Table 5. Significantly enriched gene sets in ileal mucosa on day 16 of CA-piglets based on KEGG and GO database.

NAME	SIZE	NES[1]	NOM p-val	FDR q-val[2]
KEGG database				
Proteasome	23	2.675	<0.001	<0.001
RIG I like receptor signaling pathway	42	2.251	<0.001	<0.001
Cytosolic dna sensing pathway	29	2.023	0.001	0.004
Antigen processing and presentation	28	1.906	0.001	0.013
Glycine serine and threonine metabolism	16	1.903	0.005	0.013
Metabolism of xenobiotics by cytochrome p450	16	1.876	0.002	0.017
Primary immunodeficiency	24	1.786	0.006	0.032
Glycolysis gluconeogenesis	32	1.768	0.003	0.036
GO database				
Alcohol metabolic process	39	2.255	<0.001	0.009
T cell activation	29	1.961	0.001	0.035
Gtpase activity	49	1.928	0.001	0.039
Cytokine metabolic process	30	1.961	<0.001	0.039
G protein signaling coupled to camp nucleotide second messenger	39	1.985	<0.001	0.040
Cytokine activity	69	1.913	<0.001	0.042
Lymphocyte activation	38	1.929	<0.001	0.043
Cytokine biosynthetic process	30	1.962	0.001	0.044
Regulation of lymphocyte activation	23	1.990	<0.001	0.045
Guanyl nucleotide binding	26	1.894	0.003	0.047

[1] NES: Normalized Enrichment Score
[2]FDR < 5%

A criterion for selection of differentially expressed genes was introduced by setting a cut-off of fold change of expression (FC ≥2.0). This resulted in 338 differentially expressed genes in the jejunal mucosa and 412 genes in the ileal mucosa between association treatments. A total of 109 genes were found differentially expressed both in ileal and jejunal mucosa. Intriguingly, the majority of these identified genes displayed opposite direction of differential expression in the ileal versus the jejunal mucosa (**Fig.5**). In order to group the common annotated up- and down-regulated genes on shared biological function, or regulation, functional annotation clustering was performed at the online DAVID Database (version 6.7). In the ileal mucosa (and contrarily in the jejunum), CA treatment appeared to induce a set of genes involved in lipid, glutathione and amino acid metabolism, apoptosis, defence response and regulation of gastric motility (**Fig. 5**). However, genes known to be involved in inflammatory responses were repressed in the ileal mucosa of CA compared to

SA piglets (**Fig. 5**).

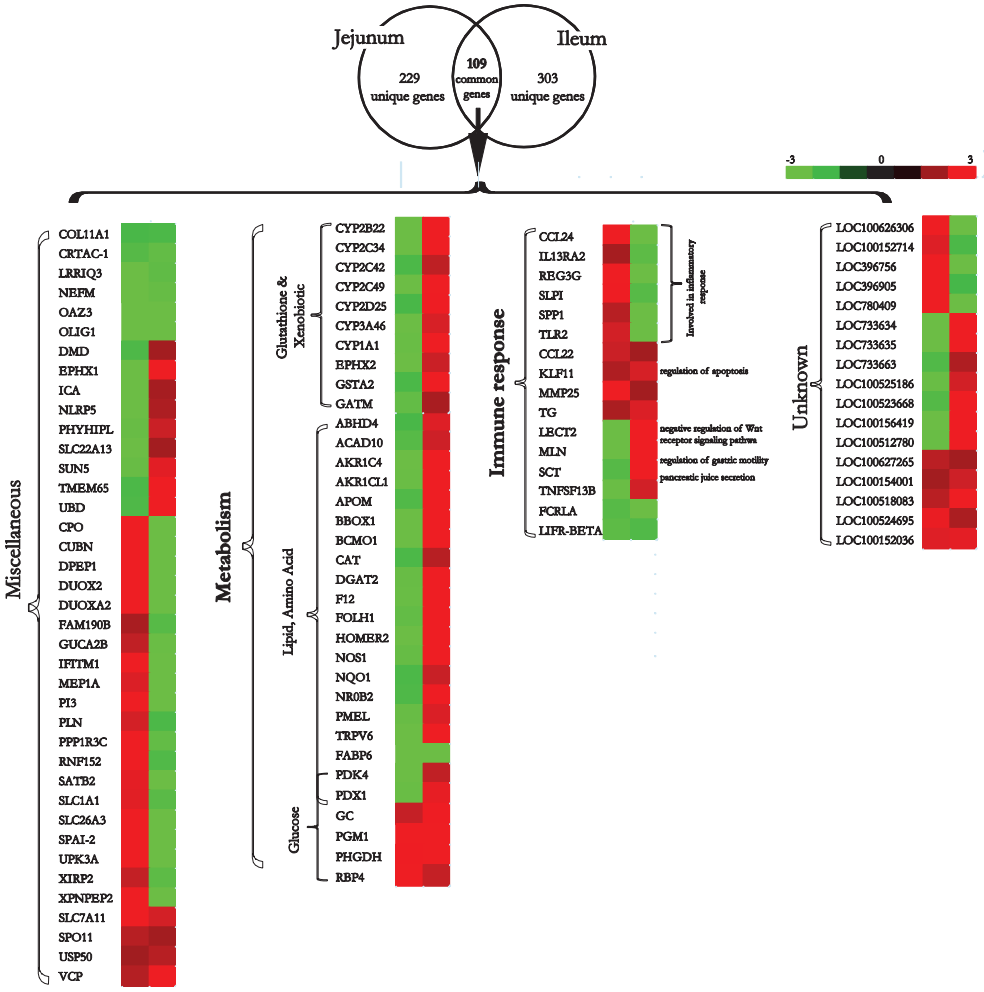

Figure 5. Gene expression analysis in jejunal and ileal mucosa on day 16 in experiment 2. Venn diagram illustrating the numbers of genes the expression of which was differentially expressed comparing CA and SA treatments (FC≥2.0) in either jejunal or ileal mucosa, or in both compartments (Top). Heat-maps showing the GO of the genes observed both in jejunum and ileum in CA treated piglets (Bottom).

In parallel, Ingenuity Pathway Analysis (IPA) showed GO annotation enrichment for genes that were differentially expressed in the jejunum and ileum in the CA piglets. The ileal mucosa comprised more modulated biological functions and signalling pathways compared to the jejunal mucosa. The majority of the modulated biological processes were involved in metabolism in the jejunal mucosa, whereas processes related to immune responses, cell trafficking and signalling were expressed at higher levels in the ileal mucosa (**Figure 6**). In

the ileum, the most significantly (P<0.05) altered signalling pathways were iNOS signalling, interferon signalling, and IL-12 signalling and production of macrophages (**Figure 6**).

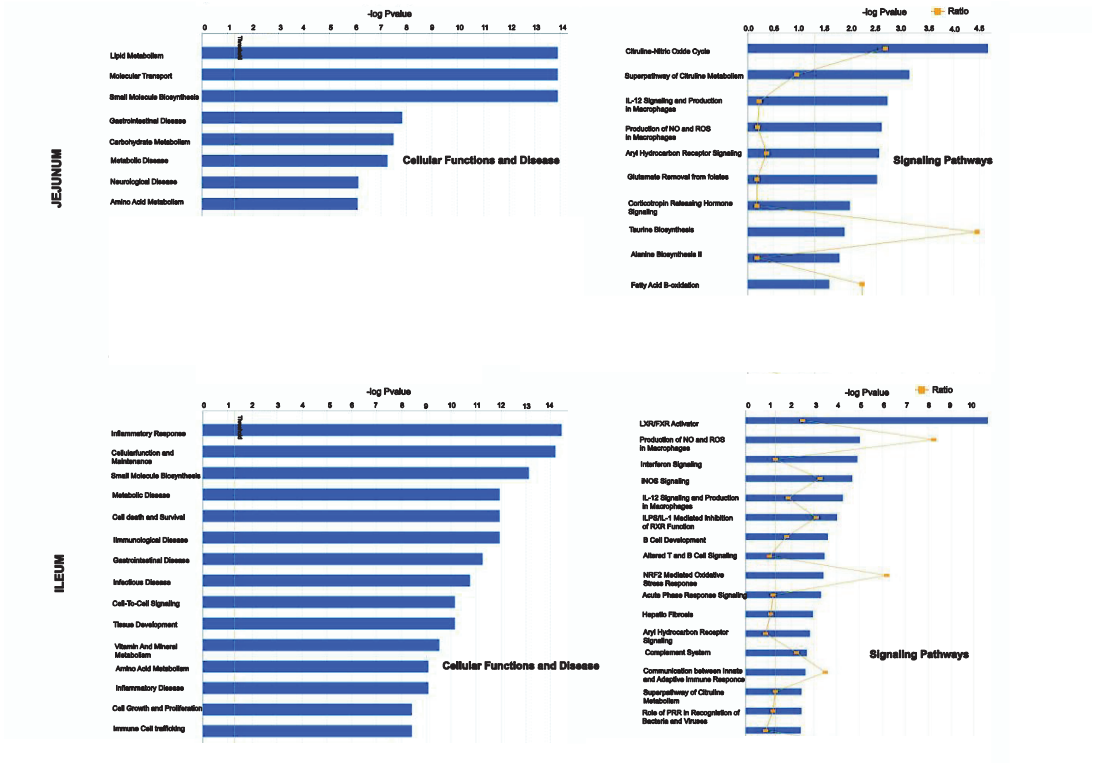

Figure 6. Biological functions and signaling pathways in the jenunal and ileal mucosa modulated in the CA treatment. Cellular functions and disease annotations calculated using Ingenuity Pathway Analysis (IPA) in (A) jejunum and (C) ileum. Signaling pathways significantly modulated in (B) jejunum and (D) ileum in CA treated piglets. Pathways were determined using IPA, and significance of differences in pathway modulation was calculated via a one-tailed Fischer's Exact test in IPA and represented as –log (P-value); -log values exceeding 1.30 were significant (P<0.05).

Correlation between the host transcriptome and microbiota composition

To further investigate the relationship between gene expression in the intestinal mucosa and composition of microbiota in digesta in the corresponding intestinal sections, correlation analyses were performed for each intestinal segment. A false discovery rate (FDR) method was employed to explore possible association of the small intestinal microbiota with gene expression patterns of CA-piglets. Genes which were differentially expressed in response to the CA treatment in jejunal and ileal mucosa were correlated to the microbial groups that varied between CA and SA piglet as identified by PITChip analysis (**Supplemental figure**

1 and 2). Significant correlations (FDR<0.01; Benjamini-Yekutieli correction) between the microbiota and specific mucosal gene expression changes were only detected in the ileum for several microbial groups previously identified as pathobionts such as *C. difficile, C. hylemonae, C. oroticum, E. coli, Pseudomonas and Leptospira.* The identified microbial groups, which decreased in abundance in the ileum of CA piglets, displayed a negative correlation to several genes involved in metabolism, immune modulation and SCFA (butyrate) transport. More specifically, negative correlations were found between the above mentioned microbial groups and acyl-CoA dehydrogenase family, member 10 (ACAD10), SMAD family member 1(BSP-1), fragments of the low-affinity receptor for IgE (FCER2/CD23), interleukin 12 receptor, beta 2 (IL12RB2), proopiomelanocortin (POMC), prostaglandin F receptor (PTGFR), solute carrier family 16, member 1 (monocarboxylic acid transporter 1, SLC16A1), member 7 (SLC16A7), transporter 1 (TAP1), and transmembrane protein 89 (TMEM89) (**Fig. S2**).

Discussion

In this study, caesarean derived piglets were associated with either a three component ("simple") microbiota consisting of *Lactobacillus amylovorus, Clostridium glycolicum* and *Parabacteroides* sp. ASF519, or with both the simple microbiota and diluted faeces of an adult sow, as a complex microbial association model. The complex microbial association had a significant effect on microbiota establishment at early age. The complex microbial association caused an increase in faecal microbial diversity, and accelerated the faecal microbiota to develop into a stable composition. Meanwhile, the complex microbial association also had drastic effects on the microbiota in the jejunum and ileum, and gene expression in the ileal mucosa at early age.

Diversified microbiota in the lower gut benefits the host at early age

In experiment 1, a higher microbial diversity was detected in the faeces of CA piglets than in SA piglets after the inoculation with diluted faeces, and an increasing similarity of microbiota was discovered between CA piglets' faeces and the faecal inoculant originating from an adult sow. The slightly lower similarity at day 7 may be caused by the change from the milk based diet towards the slurry diet with a different ingredient composition on day 5 in combination with an increase in absolute feed intake in time after day 5. A drastic shift in diet composition as occurring at weaning of piglets, can result in changes of absorptive, secretory, and barrier properties of the piglet's intestine, as well as in gut microbial composition [257, 258]. Consequently, many transient species may reduce and a subsequent complex microbial succession process may occur. In the present study, the effect of the diet change was more obvious in SA piglets compared to CA piglets. All SA piglets showed transient diarrhoea on day 7 and 8, whereas no piglets in the CA group showed diarrhoea

during the study (data not shown). Meanwhile, the microbial similarity of faeces of SA piglets and the faecal inoculant was temporally increased at day 5. This increase suggests a rapid microbial colonization in the SA piglet gut. The transient diarrhoea and gut dysbiosis of SA piglets may have resulted in the decrease of similarity between DF and SA at day 7. From day 12 onwards, all piglets in the SA group showed periods of diarrhoea. In total, nine piglets from the SA group were removed from the experiment due to severe health problems and poor physical conditions during the course of the study. Autopsy on these piglets revealed signs of an *E. coli* infection, which was confirmed by bacterial examination of the liver, pericard and abdominal fluid of several piglets, showing the presence of haemolytic *E. coli* in a number of cases. During infection, *E. coli* could become a dominant species in the intestinal tract and outcompete other species, as was confirmed by DGGE analysis of the faecal microbiota composition of piglets that were euthanized because of poor health status or died before day 28. On the DGGE gel, we found less than three bands for each of these piglets at the age of 7 and 14, and *E.coli* was the predominant band for all excluded piglets (data not shown). The above findings suggest that a higher intestinal microbial diversity and a more adult type of microbiota in the lower gut at early age may help the residing microbiota to resist external influences such as change in diet and outgrowth of pathobionts, such as *E.coli*. Furthermore, a low diversity of faecal microbiota at early age has been correlated with a higher predisposition for immune diseases in humans. It has been shown that a low microbiota diversity in early infancy preceded asthma at school age [259], and a more diversified microbiota early in life might prevent allergy development [260].

Microbial colonization at early age differed along the GI tract

Microbial groups that were found different between SA and CA differed along the GI tract. Distinct regions of the GI tract can represent unique habitats to which the resident microbiota are well adapted [108]. One of the reasons is that the function and architecture of the gut differs along its length. For instance, the first regions of the small intestine (duodenum and jejunum) are the primary site of contact between bacteria and the host, and this region makes bacteria face bile and pancreatic enzyme-mediated stress [74]. Moreover, several physiological conditions, such as pH, peristaltic movement of the tract, desquamation of epithelial cells and mucosal flow, also influence the microbiota distribution in the GI tract [261]. Consequently, bacterial diversity and composition differs from the small intestine to large intestine. For the human adult, the concentration of bacteria increases along the length of the gut, from 10^4 cells per g in the duodenum to 10^{12} cells per g in the colon [74]. Studies on mucosal biopsies showed the bacterial diversity is lowest in the jejunum, and highest in the ascending colon while the diversity in the distal ileum, ascending colon and rectum is not significantly different from each other [262]. Up to now, most human neonate and infant studies are performed with faecal samples, while

studies with newborn animals seldomly compared the difference of microbiota between the jejunum and ileum. Studies of adult humans have shown that the proximal small intestine is enriched with *Clostridium* spp., *Streptococcus* spp. and *Veillonella* spp. [126], while the ileum shows a community dominated by *Bacteroidetes* and *Clostridium* cluster XIVa and is more similar to the ecosystem of the large intestine [105, 125, 127]. However, little is known about the difference of microbial colonization and composition in the jejunum and ileum at early age of humans and animals. Our study showed that the complex microbial association significantly increased Deferribacteres and Spirochaetes in the jejunum, while in the ileum, a significant increase of Firmicutes and a decrease of Proteobacteria was found. Correspondingly, we analysed the microbial composition of feacal samples on day 14 and found that Bacteriodetes and Planctomycetes increased their relative abundance, whereas the relative abundance of Proteobacteria decreased. These results indicated that the complex microbial association promoted the development of the piglet ileal and faecal microbiota into a more adult type. Members of the Bacteroidetes are the principal degraders of complex plant polysaccharides in the adult GI tract, and a shift of a community dominated by Actinobacteria and Proteobacteria to one dominated by Firmicutes and Bacteroidetes suggests the establishment of an adult like microbiota characterized by a full suite of functions and greater stability [213]. The beneficial effect was more obvious when we evaluated the composition of genus-like level 2 microbial goups in the ileum. Most groups increased in piglets from the CA group were related to the class *Bacilli, Clostridium* cluster IV and *Clostridium* cluster XIVa, including presumed beneficial members of *Lactobacillus* and butyrate producing *Ruminococcus*, *Eubacterium* and *Roseburia*. On the contrary, potentially pathogenic Proteobacteria, such as *E.coli* and *Pseudomonas* were decreased in the CA piglets. With respect to the jejunum, the effect of complex microbial association on the microbial colonization was not as clear as in the ileum. We found a decrease of several butyrate producing *Clostridia*, bacteria related to genus *Bifidobacterium* spp., and potential pathogenic *Strepococococcus*. Taken together, the above findings indicate that complex microbial association may regionally benefit the gut microbiota, e.g. it may benefit the microbiota in the ileum while not affecting that in the jejunum. Reports have shown particular species that are beneficial to young age groups (e.g. *Lactobacillus* and *Bifidobacterium*) might actually be harmful if transplanted into elderly populations [213]. Accordingly, microbial groups that are beneficial in the ileum may not be of benefit in the jejunum. It should be noted, however, that we did not study the succession of mucosa-associated microbiota as was done previously by other studies [52, 169].

Early microbial association modified gut gene expression

The gut microbiota has been proposed to have a crucial role in the establishment and maintenance of adaptive immunity and homeostasis [263]. In this study, complex microbial association lead to more immune signalling in the ileal mucosa. This result is consistent

with studies that have been performed with germfree and conventional mice. Gaboriau – Routhiau *et al*, with the main focus on the terminal ileum, have reported nearly 50% of the genes differentially expressed in the intestine of gnotobiotic mice regulated T-cell development in the response to colonizing gut microbiota [142]. More recently, studies of Larsson *et al*. and El Aidy *et al*. confirmed "immune response" is the largest category of genes that is regulated in response to microbial colonization in the gut [21, 264]. However, unlike the ileum, fewer gene sets involved in immune related processes were observed in the jejunum, and hence, the effect of complex microbial association in the jejunum is difficult to predict in the present study. Moreover, the host gene expression pattern differed between the jejunal and ileal mucosa. The majority of the modulated biological processes were involved in metabolism in the jejunum, whereas in the ileal mucosa processes were more related to immune responses, cell trafficking and signalling such as iNOS signalling, interferon signalling, and IL-12 signalling and production of macrophages. These results can also be attributed to the differentiation of function and architecture of the GI tract. The main functions of duodenum and jejunum are related to the enzymatic degradation of dietary nutrients and constituents and subsequent absorption of nutrients, whereas the ileum is the predominant immune sampling site, because of a higher abundance of Peyer's patches in the ileal mucosa. Therefore, the ileum is generally assumed to be an important site for interaction between the intestinal microbiota and the host's immune system [74].

Correlation of pathobionts and host transcriptome

In the ileum, the decreased abundance of pathobionts in the CA group was associated with induction of immunmodulatory genes. Pathobionts are normally considered harmless symbionts that can become pathogenic under certain environmental conditions [204, 265]. The colonization of pathobionts in the gut may be a risk factor in IBD, colon cancer and perhaps for diseases outside of the intestinal compartment [204]. Hereby, identifying the molecular interactions between pathobionts and the mammalian immune system might be critical to understanding the etiology of certain diseases. Our study revealed a negative correlation between the relative abundance of a number of pathobionts and FCER2. FCER2 plays a role in regulation of IgE synthesis when expressed on B cells, and has been reported to relate to asthma [266-269]. It encodes a low-affinity IgE receptor, CD23, and the activation of CD23 results in down regulation of IgE-mediated immune responses. IgE production is a hallmark of asthma and atopic disease. A high level of IgE can increase the risk of asthma. Therefore, complex microbial association at early age resulted in a decrease in pathobionts which may decrease the IgE level and reduce the risk of asthma. Besides FCER2, genes including BSP-1, IL12RB2 and TAP1 were also negatively correlated to most pathobionts. These genes have been reported to be involved in many immune response pathways. For instance, BSP-1 is involved in a range of biological activities including cell growth, apoptosis, morphogenesis, development and immune responses

[270-272].

Conclusion

Complex microbial association at early age accelerated faecal microbiota to develop into an adult type microbiota that may resist changes of GI tract conditions caused by diet and occurrence of pathogens. Complex microbial association significantly affected luminal microbiota development, and gene expression in the jejunal and ileal mucosa. Especially in the ileum, the complex microbial association influenced genes involved in immune pathways. Expression of immune modulatory genes was negatively correlated with the abundance of pathobionts in digesta. In conclusion, complex microbial association at early age has a drastic effect on the development of intestinal microbiota and the immune system. Manipulation of microbial association of the gut at early age may be a way to support the development of the immune system.

Acknowledgements

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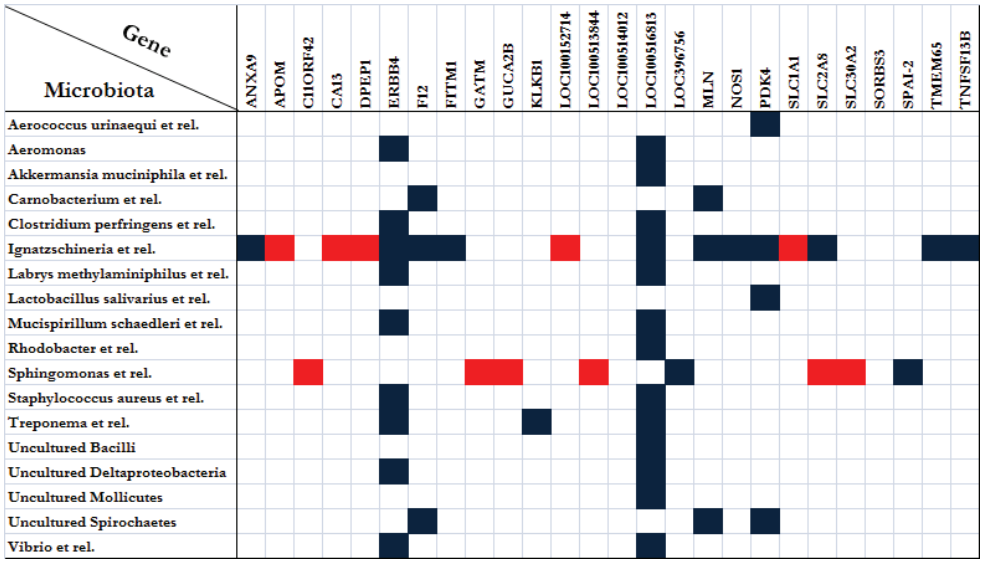
Supplemental methods

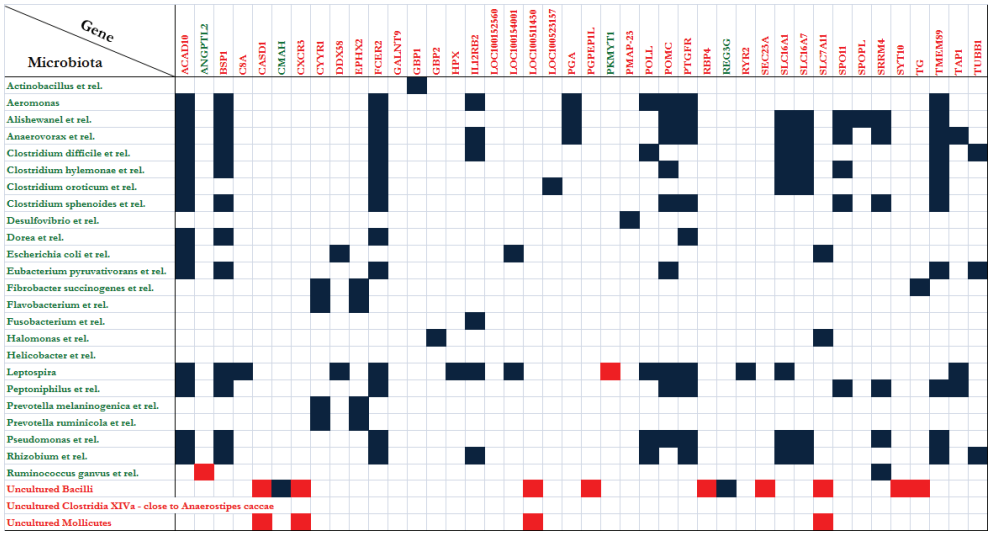
Denaturing Gradient Gel Electrophoresis (DGGE) analysis

PCR performed by using GoTaq® polymerase kit from Promega (Madison, WI, USA). Primers GC-968-f (5'-CGC CCG GGG CGC GCC CCG GGC GGG GCG GGG GCA CGG GGG GAA CGC GAA CCT TAC-3') and 1401-r (5'- CGG TGT GTA CAA GAC CC-3') were used to amplify the V6 to V8 regions of the bacterial 16S ribosomal RNA (rRNA) gene. 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. A thermocycler T1 (Whatman Biometra, Gottingen, Germany) was used for amplification. The program was set as follow: pre-denaturation at 95°C for 2 min, 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 40 sec, extension at 72°C for 60 sec and final extension at 72°C for 5 min. The size of the PCR products was determined by electrophoresis on a 1 %(W/v) agarose gel containing ethidium bromide.
The PCR amplicons were separated by denaturing gradient gel electrophoresis [273] with a Dcode TM system (Bio-Rad Laboratories, Hercules, CA, USA). Briefly, samples were loaded onto 8% polyacrylamide gels with a denaturant gradient of 30 - 60% (100% was defined as 40% formamide and 7 M urea), pre-run for 5 min at 200 V, and subsequently electrophoresed at 85 V for 16 h at 60 ℃. Gels were developed by silver staining [274], and scanned at 400 d.p.i.. Further analysis was performed with BioNumerics 4.5 software (Applied Maths).

Supplemental tables and figures



Supplemental Figure 1. Association of specific jejunal bacterial groups with differentially regulated genes in CA treatment**.** Correlation heat map shows the positive (red boxes) and negative (dark blue boxes) association between the approximate genus level of PITChip output and the genes that were differentially regulated in the CA treatment.



Supplemental Figure 2. Association of specific ileal bacterial groups with differentially regulated genes in CA treatment. Correlation heat map showing the positive (red boxes) and negative (dark blue boxes) association between the approximate genus level of PITChip output and the genes that were differentially regulated in the CA treatment. Green-labeled text represents reduced abundance in case of the microbiota and repression in case of genes, red labeled text represents increased bacterial abundance or gene induction.

Supplemental Table 1. Phylum-level phylogenetic groups with different relative abundance in faeces between CA and SA piglets over time in experiment 1.

		Effect[1]	ARC[2]	
	***P* value**	**CA *vs* SA**	**CA**	**SA**
Day 5				
Deferribacteres	0.06	-	0.02±0.01	0.05±0.02
Firmicutes	0.06	+	64.13±6.08	52.81±4.34
Day 7				
Fibrobacteres	0.03	-	0.20±0.08	1.39±0.75
Planctomycetes	0.06	-	0.00±0.00	0.01±0.00
Day 14				
Bacteroidetes	0.03	+	21.92±8.66	2.40±2.00
Deferribacteres	0.06	+	0.03±0.01	0.01±0.01
Planctomycetes	0.03	+	0.09±0.06	0.01±0.00
Proteobacteria	0.03	-	12.52±1.95	19.05±2.85
Day 28				
Bacteroidetes	0.06	+	5.41±2.96	1.64±0.17
Deferribacteres	0.06	+	0.02±0.00	0.01±0.00
Spirochaetes	0.06	+	1.69±0.32	1.19±0.09

[1] Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the CA group in comparison with the SA group
[2] ARC: Average relative contribution [%] of a microbial group. Values represent means ± SDs.

Supplemental Table 2. Approximate genus-level phylogenetic groups tending to be different in faeces between CA and SA piglets over time in experiment 1**.**

Class	**Group**	**Effect[2]**	***P* value**	**C.*p*[1]**	**ARC[3]**	
		CA vs SA			**SA**	**CA**
Day 5						
Bacteroidetes	*Paludibacter propionicigenes* et rel.	-	0.06	0.48	0.39±0.18	0.14±0.09
	Prevotella melaninogenica et rel.	-	0.06	0.48	0.28±0.11	0.13±0.06
	Uncultured *Porphyromonadaceae*	-	0.06	0.48	0.68±0.29	0.28±0.15
Flavobacteria	*Myroides odoratus* et rel.	-	0.06	0.48	0.07±0.03	0.03±0.01
Sphingobacteria	Uncultured *Sphingobacteria*	-	0.06	0.48	0.48±0.25	0.17±0.12
Deferribacteres	*Mucispirillum schaedleri* et rel.	-	0.06	0.48	0.05±0.02	0.02±0.01
Clostridium cluster XIVa	*Bryantella* et rel.	+	0.06	0.48	0.79±0.29	1.44±0.41
	Eubacterium ventriosum et rel.	+	0.06	0.48	0.02±0.00	0.03±0.00
	Lachnospira pectinoschiza et rel.	+	0.06	0.48	1.84±0.52	2.88±0.37
	Roseburia intestinalis et rel.	+	0.06	0.48	0.15±0.03	0.22±0.06
Day 7						
Actinobacteria	*Actinomyces* et rel.	-	0.06	0.26	0.66±0.37	0.22±0.12
	Bifidobacterium	+	0.06	0.26	0.68±0.23	1.43±0.58
	Microbacterium	+	0.06	0.26	0.75±0.17	1.04±0.15

Bacilli	*Bacillus* et rel.	-	0.06	0.26	2.64±1.26	0.94±0.47
	Weissella et rel.	-	0.06	0.26	0.24±0.10	0.08±0.04
Clostridium cluster IV	*Clostridium cellulosi* et rel	+	0.06	0.26	0.14±0.09	0.27±0.07
Clostridium cluster XIVa	*Dorea* et rel.	-	0.06	0.26	0.51±0.11	0.26±0.10
Planctomycetacia	Uncultured *planctomycetacia*	-	0.06	0.26	0.01±0.00	0.00±0.00
Betaproteobacteria	Uncultured *betaproteobacteria*	+	0.06	0.26	0.02±0.01	0.07±0.03
Gammaproteobacteria	*Vibrio* et rel.	-	0.06	0.26	0.25±0.11	0.09±0.08
Spirochaetes	*Leptospira*	-	0.06	0.26	0.51±0.27	0.17±0.05
Day 14						
Bacteroidetes	*Paludibacter propionicigenes* et rel.	+	0.06	0.26	0.02±0.04	0.08±0.03
	Uncultured *Porphyromonadaceae*	+	0.06	0.26	0.10±0.12	0.53±0.26
Flavobacteria	*Chryseobacterium* et rel.	+	0.06	0.26	0.04±0.02	0.12±0.06
Deferribacteres	*Mucispirillum schaedleri* et rel.	+	0.06	0.26	0.01±0.01	0.03±0.01
Bacilli	*Gemella haemolysans* et rel.	-	0.06	0.26	0.30±0.05	0.19±0.05
	Lactobacillus paracasei et rel.	-	0.06	0.26	0.04±0.03	0.01±0.00
	Streptococcus salivarius et rel.	+	0.06	0.26	0.50±0.29	1.09±0.32
	Streptococcus suis et rel.	+	0.06	0.26	0.68±0.40	1.47±0.47
Spirochaetes	*Treponema* et rel.	+	0.06	0.26	0.18±0.06	0.48±0.23
Day 28						
Bacteroidetes	*Alistipes* et rel.	+	0.06	0.23	0.02±0.00	0.61±0.4
	Bacteroides distasonis et rel.	+	0.06	0.23	0.13±0.05	0.50±0.31
	Bacteroides fragilis et rel.	+	0.06	0.23	0.02±0.01	0.07±0.04
	Bacteroides pyogenes et rel.	+	0.06	0.23	0.02±0.00	0.10±0.06
	Bacteroides vulgatus et rel.	+	0.06	0.23	0.01±0.00	0.06±0.04
	Paludibacter propionicigenes et rel.	+	0.06	0.23	0.01±0.00	0.03±0.01
	Prevotella melaninogenica et rel.	+	0.06	0.23	0.08±0.02	0.27±0.07
	Prevotella ruminicola et rel.	+	0.06	0.23	0.02±0.01	0.05±0.01
	Uncultured *Bacteroidetes*	+	0.06	0.23	0.03±0.00	0.73±0.59
	Uncultured *Prevotella*	+	0.06	0.23	0.53±0.14	2.36±1.39
Flavobacteria	*Chryseobacterium* et rel.	+	0.06	0.23	0.03±0.00	0.08±0.03
	Flavobacterium cucumis et rel.	+	0.06	0.23	0.01±0.00	0.02±0.01
Deferribacteres	*Mucispirillum schaedleri* et rel.	+	0.06	0.23	0.01±0.00	0.02±0.00
Bacilli	*Lactobacillus delbrueckii* et rel.	-	0.06	0.23	0.64±1.01	0.02±0.00
	Lactobacillus gasseri et rel.	-	0.06	0.23	1.85±2.89	0.02±0.01
	Streptococcus intermedius et rel.	+	0.06	0.23	0.16±0.14	0.87±0.30
	Streptococcus suis et rel.	+	0.06	0.23	0.27±0.13	0.81±0.29
Clostridium cluster IV	*Clostridium cellulosi* et rel	+	0.06	0.23	0.31±0.04	0.64±0.17
	Eubacterium cellulosi et rel	+	0.06	0.23	0.06±0.01	0.11±0.03
	Ruminococcus bromii et rel.	+	0.06	0.23	0.03±0.01	0.23±0.21
	Subdoligranulum et rel.	-	0.06	0.23	1.02±0.11	0.48±0.10

Clostridium cluster IX	*Dialister* et rel.	+	0.06	0.23	0.01±0.00	0.01±0.00
	Megasphaera elsdenii et rel.	+	0.06	0.23	0.01±0.00	0.05±0.04
	Mitsuokella multiacida et rel.	+	0.06	0.23	0.03±0.00	0.21±0.15
	Peptococcus niger et rel.	+	0.06	0.23	0.00±0.00	0.03±0.01
Clostridium cluster XI	*Clostridium difficile* et rel.	+	0.06	0.23	2.68±1.47	5.77±0.60
Clostridium cluster XIVa	*Butyrivibrio crossotus* et rel.	+	0.06	0.23	0.31±0.04	0.48±0.10
	Clostridium sphenoides et rel.	-	0.06	0.23	0.37±0.21	0.18±0.06
	Dorea et rel.	-	0.06	0.23	0.36±0.02	0.30±0.04
	Ruminococcus ganvus et rel.	-	0.06	0.23	0.14±0.05	0.09±0.01
Mollicutes	*Mycoplasma*	-	0.06	0.23	0.57±0.46	0.15±0.09
Gammaproteobacteria	*Avibacterium*	-	0.06	0.23	0.08±0.02	0.04±0.01
	Bisgaard	-	0.06	0.23	0.08±0.02	0.04±0.01
	Pasteurella	-	0.06	0.23	0.19±0.03	0.13±0.04
Spirochaetes	*Treponema* et rel.	+	0.06	0.23	0.30±0.09	0.82±0.16
	Turneriella	-	0.06	0.23	0.47±0.06	0.27±0.02

[1] C.*p*: P value corrected for multiple testing according to the procedure of Benjamini-Hochberg
[2] Effect: indicates whether the average relative contribution of a microbial group was increased (+) or decreased (-) in the CA group in comparison with the SA group
[3] ARC: Average relative contribution [%] of a microbial group. Values represent means ± SDs.

Supplemental Table 3. Phylum-level phylogenetic groups with different abundance in the jejunum and ileum between CA and SA piglets in experiment 2.

Phylum	**CA *vs* SA**		**Jejunum ARC[1]**		**Ileum ARC**	
	Jejunum	**ileum**	**CA**	**SA**	**CA**	**SA**
Bacteroidetes	-[2]	-	2.97±0.27	4.47±0.55	2.85±0.34	3.77±0.44
Deferribacteres	+	NSD[3]	0.28±0.03	0.22±0.04	0.23±0.03	0.19±0.06
Fibrobacteres	-	-	0.23±0.14	0.61±0.23	0.27±0.17	0.55±0.18
Firmicutes	NSD	+	76.94±1.12	76.31±1.50	78.38±0.82	74.31±1.72
Fusobacteria	-	-	0.46±0.17	0.90±0.26	0.46±0.12	0.72±0.16
Proteobacteria	NSD	-	13.37±0.88	12.22±1.69	11.99±1.19	15.30±1.43
Spirochaetes	+	NSD	2.88±0.35	2.27±0.25	2.81±0.40	2.39±0.60
Verrucomicrobia	+	+	0.25±0.03	0.20±0.04	0.21±0.02	0.16±0.06

[1] ARC: average relative contribution [%] of a phylum. Values represent means ± SDs. The microbial groups with a relative abundance lower than 0.1% in all treatments are not shown.
[2] " +" or "-" indicates whether the average relative contribution of the microbial group was increased or decreased in the CA group in comparison with SA group.
[3] NSD: no significant difference of a of a phylum observed in the CA group in comparison with SA group

Chapter 6

A diet high in resistant starch modulates microbiota composition, SCFA concentrations and gene expression in pig intestine

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Abstract

Resistant starch (RS) is highly fermentable by microbiota in the colon, resulting in the production of SCFAs. RS is thought to mediate a large proportion of its health benefits, including increased satiety, through the actions of SCFAs. The aim of this study was to investigate the effects of a diet high in RS on luminal microbiota composition, luminal SCFA concentrations and the expression of host genes involved in SCFA uptake, SCFA signalling and satiety regulation in mucosal tissue obtained from small intestine, caecum and colon. Twenty adult female pigs were either assigned to a digestible starch (DS) diet or a diet high in RS (34%) for a period of 2 wk. After the intervention, luminal content and mucosal scrapings were obtained for detailed molecular analysis. RS was completely degraded in caecum. In both the caecum and colon, differences in microbiota composition were observed between DS- and RS-fed pigs. In the colon these included the stimulation of the healthy gut-associated butyrate-producing *Faecalibacterium prausnitzii*, whereas potentially pathogenic members of the *Gammaproteobacteria*, including *Escherichia coli* and *Pseudomonas* spp., were reduced in relative abundance. Caecal and colonic SCFA concentrations were significantly greater in RS-fed pigs, and caecal gene expression of monocarboxylate transporter 1 (*SLC16A1*) and glucagon (*GCG)* was induced by RS. In conclusion, our data show that RS modulates microbiota composition, SCFA concentrations and host gene expression in pig intestine. Combined, our data provide an enhanced understanding of the interaction between diet, microbiota and host.

Introduction

Obesity and related disorders, such as cardiovascular diseases and type 2 diabetes, have become major public health issues [275, 276]. Various lifestyle factors, of which diet is a major one, play an important role in the development of these disorders [277, 278]. A large amount of research has reported an inverse relationship between fibre consumption and the risk of obesity and diabetes [279]. Moreover, consumption of dietary fibre prevents the accumulation of fat mass [280, 281], increases insulin sensitivity [282, 283] and can enhance feelings of satiety [284].

Resistant starch (RS) is a type of dietary fibre that includes all starch and starch degradation products that are not absorbed in the small intestine of healthy humans [79]. It is known to be fermented to a large extent by microbiota in the colon, resulting in the production of SCFAs [73, 90]. These SCFAs can diffuse across the epithelial cell membrane, but SCFA absorption by the enterocytes is also mediated by the monocarboxylate transporter 1 (SLC16A1, also known as MCT1) and sodium-coupled monocarboxylate transporter 1 (SLC5A8 or SMCT1) [285].

Enhanced SCFA production provides an important link between dietary fibre consumption and health benefits. First, SCFAs lower the pH in the colon, which can prevent the overgrowth of pathogenic bacteria [286]. Acetate, propionate and butyrate are the major SCFAs produced in the colon, of which butyrate is thought to be most beneficial for health. Among the health benefits observed with butyrate are the prevention and inhibition of colon carcinogenesis, protection against mucosal oxidative stress, strengthening of the colonic defence barrier and butyrate also has anti-inflammatory properties [287]. Propionate has the potential to reduce cholesterol concentrations in blood [288].

In addition to these health benefits, SCFAs are thought to be involved in the increase in satiety observed with fibre consumption [289]. Studies in rodents have provided evidence that fermentation of RS is an important mechanism for increased endogenous secretion of the gut hormones glucagon-like peptide 1 (GLP-1) and peptide YY (PYY) [280, 290-292]. GLP-1 and PYY are satiety-stimulating hormones that are released in response to nutrient intake, mainly in the ileum and colon. SCFAs are agonists for free fatty acid receptor 2 and 3 (FFAR2 and FFAR3 respectively), 2 G protein-coupled receptors present in the gastrointestinal tract. Both of these G protein-coupled receptors are expressed in enteroendocrine cells in the gut epithelium. It has been proposed that the activation of these receptors trigger the production and release of GLP-1 and PYY by enteroendocrine L-cells [293, 294].

In the experiments described in this chapter, we investigated the effects of a diet high in RS

on luminal microbiota composition, luminal SCFA concentrations and the expression of host genes involved in SCFA uptake, SCFA signalling and satiety regulation in mucosal tissue obtained from small intestine, caecum and colon. Adult pigs were used as a model for humans because the anatomy and physiology of the gastrointestinal tract of pigs and the pig genome are similar to those of humans [153, 154]. Because we recently reported that a diet high in RS decreases feeding motivation in pigs [295], special attention was given to selected genes involved in SCFA sensing and regulation of satiety.

Materials and Methods

Experimental design, pigs and housing

Two independent studies were performed. First, a pilot experiment was carried out to ascertain whether the gene expression profile along the intestine of pigs is similar to the sites of expression in humans and rodents. Three multiparous female pigs with a mean (± SEM) body weight of 273 ± 1.15 kg were included is this study.

The main study had a parallel design. Two groups of 10 female pigs (PIC Benelux B.V.), aged 22 mo, with an initial body weight of 268 ± 3.85 kg were assigned to 1 of 2 treatments. Treatments differed with regard to the type of starch in the diet: digestible starch (DS) or RS. Siblings were equally distributed between the 2 groups. Pigs were individually fed and housed in pairs that received the same diet. The area of each pen was 11 m^2 and contained 2 drinking nipples and 2 feeding troughs. Artificial lights were on from 06:30 h until 22:00 h and dimmed during the dark period. The animal protocol was approved by the Animal Care and Use Committee of Wageningen University. The same pigs were used previously for feeding motivation studies [295].

Diets and feeding

The 2 experimental diets used in the main study were identical except for type of starch. The main source of starch in the DS diet was pregelatinized potato starch (Paselli WA4; AVEBE), which was replaced on a dry matter basis in the RS diet by retrograded tapioca starch (Actistar; Cargill). According to the supplier, this starch was ≥50% resistant to digestion in the small intestine. On the basis of physical and chemical characteristics, the RS used in this study can be classified as RS type 3 (RS3) [79]. Diets were isoenergetic on a gross energy basis. The detailed composition of the experimental diets is presented in **Supplemental Table 1**. Each diet was given to 10 pigs in pelleted form at 07:00 h and 17:00 h for a period of 14 d. The daily feed allowance was 1.13 times the energy requirements for maintenance [net energy = 293 kJ/($kg^{0.75}$ · d)], and pigs were allowed 1 h to consume the meal. All pigs had free access to water throughout the entire day.

Blood collection

Blood was collected 5 h postprandially on 2 separate days. Blood sampling was performed before the start of the treatment period when all pigs had consumed the DS diet for 2 d and at the end of the dietary intervention when pigs had been fed the DS or RS diet for 12 d. Blood was drawn from the jugular vein and collected in a BD Vacutainer EDTA tube with protease (Complete, EDTA-free; Roche) and dipeptidyl peptidase-4 (Millipore) inhibitors. Tubes were centrifuged for 10 min at 1300 *g* at 4°C immediately after blood collection. Plasma was separated into aliquots and stored at -80°C.

Collection of digesta and tissue

Digesta and tissue samples were collected 5 h after the morning meal. This time point was selected on the basis of a previous study in which an increase in SCFA concentration was observed 4-5 h after feeding [296]. Pigs were stunned and exsanguinated, after which the abdominal cavity was opened. The gastrointestinal tract from stomach to anus was removed from the cavity and the length of the small intestine and colon was determined. The small intestine was divided into 10 parts of equal length, the caecum was divided into 2 parts and the colon into 4 parts. The luminal content was collected from the 4 most distal parts of the small intestine (segment 7 to 10) and from all caecal and colonic parts. For subsequent analysis of SCFA concentrations, part of the content was collected in tubes with 1 mL of H_3PO_4, after which the samples were thoroughly mixed and stored at -20°C. Luminal content was also stored in empty tubes at -20°C to determine dry matter and degradation of RS. The remaining amount of digesta was collected in 1.5 mL Eppendorf tubes to determine microbiota composition. These tubes were immediately frozen in liquid nitrogen and stored at -80°C until further analyses. In addition, a small piece from the middle of each intestinal segment was excised and rinsed in PBS. Epithelial cells were collected from these tissues by scraping the mucosal lining with a glass slide. These samples were immediately snap-frozen in liquid nitrogen and then stored at -80°C for subsequent RNA isolation.

Analysis of RS in luminal samples

RS was analysed according to methods used by Goñi *et al.* [297], adapted for intestinal samples, including extra washing steps and the total volume set at 35-40 mL. Impurities were removed by deionization of the sample by using equal amounts of Q-sepharose and S-sepharose beads equilibrated with phosphate buffer, 20 mmol/L, pH 7. An aliquot (100 µL) of the sample obtained in step 9 of the procedure was combined with an equal amount of the ion exchange mix and thoroughly mixed for 5 min at room temperature. For the glucose assay, 100 µL supernatant obtained after 5 min of centrifugation at 14,000 *g* was

used. Glucose was determined in the samples by using the Glucose Assay Kit from Sigma-Aldrich (product no. GAGO20). The amount of RS in the sample was calculated by using the method as described [297].

Microbiota analysis

Microbial DNA was extracted from 250 mg of intestinal contents by using a faecal DNA extraction protocol [199]. Denaturing gradient gel electrophoresis (DGGE) analysis was performed for a preliminary scanning of the microbial profile. Briefly, universal primers S-D-Bact-0968-a-S-GC and S-D-Bact-1401-a-A-17 [298] were used to amplify the V6 to V8 variable regions of the bacterial 16S rRNA gene. The V6-V8 PCR amplicons were separated by DGGE according to the specifications of Muyzer *et al.* [273] by using a DCode system (Bio-Rad Laboratories). Gel images were digitally normalized by comparison with an external standard pattern by using Bionumerics software package version 4.5 (Applied MathS). Observed bands were classified across all samples, and band class information, including relative band intensity, was used for multivariate analysis.

After the preliminary scanning of microbiota composition by DGGE analysis, the luminal contents from the first part of caecum (from 9 DS-treated and 7 RS-treated pigs) and colon (from 7 DS-treated and 8 RS-treated pigs) were selected for further analysis by using the Pig Intestinal Tract Chip (PITChip). The PITChip is a phylogenetic microarray with >2900 oligonucleotides based on 16S rRNA gene sequences of 627 porcine intestinal microbial species-level phylotypes [227]. The PITChip provides a very deep and reproducible phylogenetic analysis that has been compared with deep pyrosequencing of 16S rRNA gene fragments [104, 105, 227] and next-generation parallel sequencing of intestinal metagenomes [106], indicating comparable resolution and a higher sensitivity of the chip-based analysis.

The protocol for hybridization and analysis of the generated data was performed essentially as previously described for the Human Intestinal Tract Chip [103]. The bacterial 16S rRNA gene was amplified by using the primers *T7prom*-Bact-27-for and Uni-1492-rev [103]. The PCR products were transcribed into RNA and the purified resultant RNA was coupled with CyDye (GE Healthcare Life Sciences) before fragmentation and hybridization to the array. Microarray images were processed using Agilent's Feature Extraction Software, version 9.1 (Agilent Technologies). Data normalization and processing were performed as described [103, 227].

Dry matter and SCFA measurement

Dry matter was determined by drying the intestinal content to a constant weight at 103°C

(ISO standard 6496; International Organization for Standardization, 1999).

The digesta samples collected in tubes with H_3PO_4 were thawed, mixed on a vortex and centrifuged at 20,000 *g* for 5 min. The supernatant was collected and diluted 1:1 with a solution containing isocaproic acid. SCFA concentrations were determined in the effluent by gas chromatography (Fisons HRGC Mega 2; CE Instruments) at 190°C by using a glass column fitted with Chromosorb 101 (Supelco). The carrier gas was N_2 saturated with methanoic acid, and isocaproic acid was used as an internal standard.

RNA isolation and quality control

Total RNA was isolated from intestinal scrapings using TRIzol reagent (Life Technologies) according to the manufacturer's instructions. Concentrations and purity of RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). RNA quality was verified with an Agilent 2100 Bioanalyzer (Agilent Technologies) by using 6000 Nano Chips (Agilent Technologies) according to the manufacturer's instructions.

qRT-PCR

Single-stranded cDNA was synthesized from 1 µg of total RNA by using the First Strand cDNA Synthesis Kit (Fermentas Life Sciences) according to the supplier's protocol. qRT-PCR was performed on a CFX384 Real-Time PCR Detection System (Bio-Rad) by using SensiMix SYBR No-ROX (Bioline). Primers were designed in Beacon Designer 7.6 by using sequences obtained from the ENSEMBL pig database. Specificity of the amplification was verified by melt curve analysis and evaluation of efficiency of PCR amplification. The primer sequences are listed in **Supplemental Table 2**. Samples were analysed in duplicate and mRNA expression of all genes reported was standardized to *RPLP0* gene expression.

Plasma measurements

All plasma measurements were performed in duplicate. Glucose was measured by using an enzymatic glucose assay (Glucose PAP SL; Elitech Group). Triglyceride (TG) and cholesterol concentrations were determined by using the enzymatic methods (Triglycerides Liquicolor, Cholesterol Liquicolor; INstruchemie). Insulin and PYY concentrations were measured by EIA [insulin: (porcine/canine) EIA; ALPCO Diagnostics; peptide YY (3-36): (rat, mouse, porcine, canine) EIA kit; Phoenix Pharmaceuticals]. GLP-1 was analysed with ELISA [Glucagon-like Peptide-1 (active) ELISA Kit; Millipore].

Statistical methods

Results are expressed as means ± SEM. The significance of differences between the 2 treatment groups of the variables determined at the end of the experimental period was evaluated by Student's *t* test. ANOVA was used to test for differences in RS content in the small intestine, caecum and colon. Significance of differences of plasma variables, which were measured before and after the experimental period, was determined by linear mixed-model analysis treating pig as a random effect. Analyses were performed in GraphPad Prism, version 5.04, and IBM SPSS Statistics, version 19. Differences were considered significant if $P < 0.05$.

Multivariate analysis was applied for DGGE and PITChip data interpretation. To relate changes in total bacterial community composition to environmental variables, redundancy analysis (RDA) was used as implemented in the CANOCO 4.5 software package (Biometris). RDA is the canonic form of principle component analysis and is a multivariate linear regression method in which several response variables are related to the same set of environmental variables and in which the estimated matrix of regression coefficients is of reduced rank [299]. The relative abundance of bands on DGGE gels and signal intensities for 144 genus-level phylogenetic groups of PITChip were used as responsive variables. Treatment class (DS or RS), SCFA concentration and gene expression values of *GCG* and *SLC16A1* were introduced as environmental (explanatory) variables. The latter were included because these variables were significantly different between the 2 treatment groups in the caecum and colon (see Results). RDA was performed by focusing on intersamples correlation, and the Monte Carlo permutation test was applied to evaluate whether treatment class, SCFA concentration and gene expression had significant influence on the microbial composition [228, 255]. Because the experiment had a randomized design, we used the unrestricted permutation option that yields completely random permutations. Treatment class or other environmental variables were considered to significantly affect microbial composition with *P* values <0.05. Diagrams were plotted as biplots for DGGE data and as triplots for PITChip data by using CanoDraw (Biometris).

Univariate testing of differences for individual microbial groups was processed by using a Mann–Whitney *U* signed-rank test. *P* values were corrected for multiple testing by using Benjamini-Hochberg's approach [300].

Results

Longitudinal distribution of gene expression

In the pilot experiment, the expression of several genes involved in luminal uptake and

sensing of nutrients and metabolites was measured in mucosal scrapings of 3 female pigs (**Supplemental Fig. 1**). As expected, the expression of apical sodium-dependent bile salt transporter (*SLC10A2*) was restricted to terminal ileum [301]. mRNA levels of fatty acid transport protein 4 (*SLC27A4*), liver-type fatty acid binding protein (*FABP1*) and intestine-type fatty acid binding protein (*FABP2*) were highest in the proximal jejunum and resembled the pattern observed in mice [302]. Furthermore, the gene expression pattern of glucagon (*GCG*; in the intestine the precursor for GLP-1 and GLP-2) and PYY (*PYY*) in the small intestine closely resembled the peptide concentrations found in pig intestine [303]. Taken together, these data show the validity of our gene expression measurements in the gastrointestinal tract of pigs. Moreover, we found that the distal part of the small intestine, the caecum, and the colon showed the highest expression of genes involved in SCFA uptake (*SLC16A1*, *SLC5A8)*, SCFA sensing (*FFAR2*, *FFAR3*) and satiety (*GCG*, *PYY*). Therefore, these segments were sampled in the main experiment described in this chapter.

Anthropometric variables

Body weight and fat depth were measured in all pigs at the end of the experimental period. The lengths of the small intestine and colon were determined at section. No significant differences were found between the treatment groups with respect to body weight, fat depth, and the length of the small intestine and colon (**Supplemental Table 3**).

Degradation of RS

The amount of RS was measured in the intestinal content collected at section (5 h postprandially). In RS-fed pigs, 20-40 times more RS was found in the small intestine compared with that in DS-fed pigs (**Fig. 1**). Furthermore, in the caecum of RS-fed pigs, the concentration of RS was significantly lower compared with that in the small intestine, whereas in the colon, RS concentrations were significantly lower than in the caecum and were comparable to background concentrations, i.e., those measured in DS-fed pigs (**Fig. 1**). From these observations we conclude that RS was fully degraded in the caecum.

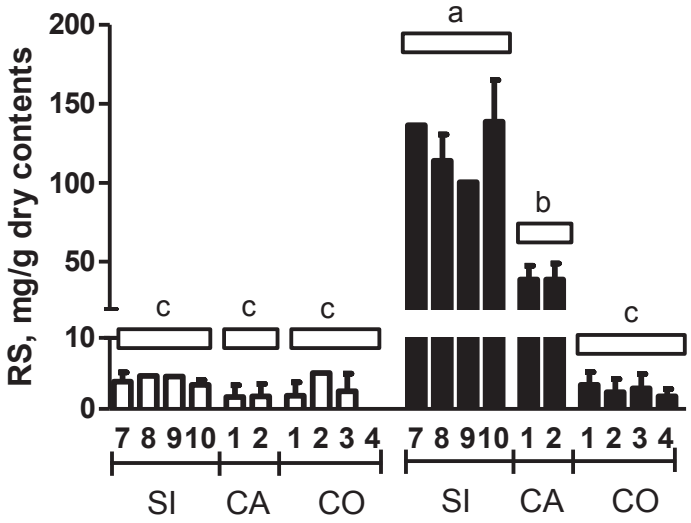

Figure 1. RS concentrations in luminal content of pigs fed the DS or RS diet for 2 wk. Values are means ± SEM, *n* = 1-4 pigs per treatment. Different letters indicate that segments differ significantly from each other, $P < 0.01$ (a > b > c). From *left* to *right*, bars represent areas of the gastrointestinal tract from proximal to distal. The small intestine was divided into 10 equal parts; parts 7-10 refer to the 4 most distal segments of the small intestine, whereas the caecum and colon were divided into 2 respectively 4 segments of equal length. White horizontal bars indicate the intestinal parts that were combined for statistical analysis to determine differences between anatomic locations. CA, caecum; CO, colon; DS, digestible starch; RS, resistant starch; SI, small intestine.

Microbiota analysis

Multivariate analysis of the DGGE data revealed a significant effect of RS treatment on the composition of the microbiota in the caecum ($P = 0.016$) and colon ($P = 0.002$), whereas no significant effect of treatment was found in the small intestine ($P = 0.18$) (**Supplemental Fig. 2**).

Because treatment effects were observed in the caecum and colon, the luminal contents of the proximal part of caecum (P1 caecum) and colon (P1 colon) were selected for further analysis using the superior PITChip technique. RDA of the PITChip data showed a visual treatment effect on the microbiota in both caecum and colon. Caecal samples of RS-fed pigs were separated from those of DS-fed pigs except for pig 24 (**Fig. 2A**). The treatment-centered separation was more obvious for the colonic samples (**Fig. 2B**). Moreover, Monte Carlo Permutation testing showed that, of the environmental variables, only luminal propionate concentration significantly ($P = 0.018$) contributed to explaining the observed variation in microbiota composition in the colon.

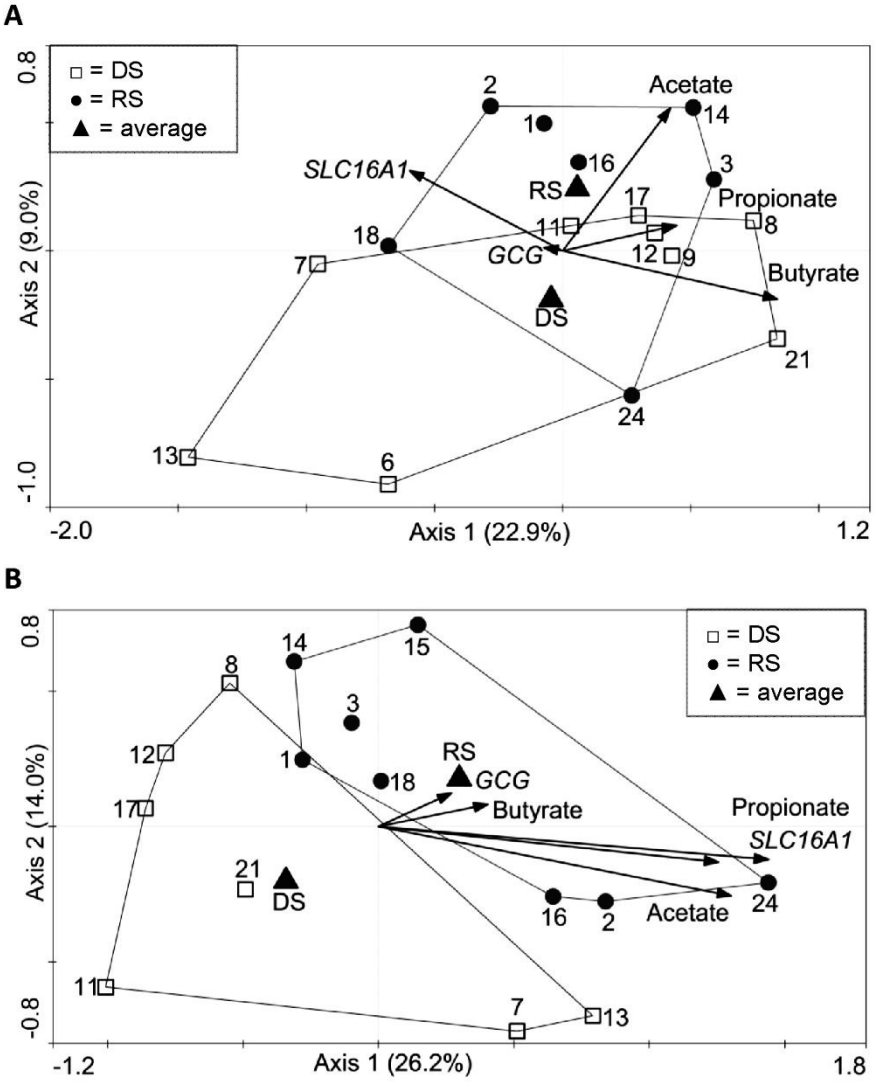

Figure 2. Triplots of RDA results, representing the principal component analysis of the microbiota composition as measured by the mean hybridization signals for 144 genus-level phylogenetic groups in the luminal content of caecum (A) and colon (B) of pigs fed the DS or RS diet for 2 wk. Samples are grouped by treatment class. Each symbol represents 1 pig, and numbers represent pig identifiers. The average of the nominal environmental variables DS and RS are represented by triangles. GCG (expression), SLC16A1 (expression), acetate, propionate and butyrate concentrations are also included as environmental variables. For clarity, the microbial groups that contributed to the first 2 principal components used as explanatory axes were omitted in these triplots. Combined, both axes explain 30.9% of the total variance in the data set for (A) and 41.6% for (B). DS, digestible starch; RS, resistant starch.

To determine which microbial groups were changed by RS treatment, univariate analysis was employed (**Tables 1** and **2**). In the caecum, we found that microbial groups of *Actinobacteria, Bacilli, Clostridium* cluster IV and XIVa, *Alphaproteobacteria, Betaproteobacteria* and *Spirochaetes* changed in relative abundance, albeit at corrected *P* values of 0.81 (**Table 1**). More specifically, *Streptococcus intermedius*-like group, *Streptococcus salivarius*-like group, *Streptococcus suis*-like group and *Neisseria*-like group decreased by the RS treatment, whereas the uncultured *Clostridia* cluster IV and *Rhodobacter*-like microorganisms increased in relative abundance (**Table 1**).

Table 1. Phylogenetic groups in the luminal content of the P1 caecum of pigs fed the DS or RS diet for 2 wk that were significantly affected by diet according to univariate analysis of PITChip data[1].

			ARC[3]		
Phylogenetic group	**Corr. *P* value[2]**	***P* value**	**DS**	**RS**	**Effect[4]**
Actinobacteria					
Actinobacteria					
Bifidobacterium	0.81	0.071	0.390 ± 0.037	0.355 ± 0.031	-
Olsenella et rel.	0.81	0.091	0.007 ± 0.002	0.010 ± 0.004	+
Firmicutes					
Bacilli					
Streptococcus intermedius et rel.	0.81	0.042	0.399 ± 0.190	0.251 ± 0.077	-
Streptococcus salivarius et rel.	0.81	0.031	0.374 ± 0.178	0.244 ± 0.085	-
Streptococcus suis et rel.	0.81	0.016	0.353 ± 0.111	0.245 ± 0.047	-
Uncultured *Bacilli*	0.81	0.091	1.273 ± 0.056	1.331 ± 0.071	+
Clostridium cluster IV					
Uncultured *Clostridia* IV	0.81	0.042	1.961 ± 0.358	2.218 ± 0.283	+
Clostridium cluster XIVa					
Butyrivibrio crossotus et rel.	0.81	0.071	0.851 ± 0.108	0.763 ± 0.094	-
Clostridium sphenoides et rel.	0.81	0.091	0.820 ± 0.094	0.971 ± 0.095	+
Eubacterium rectale et rel.	0.81	0.091	0.746 ± 0.060	0.690 ± 0.060	-
Ruminococcus ganvus et rel.	0.81	0.055	0.854 ± 0.086	0.937 ± 0.080	+
Proteobacteria					
Alphaproteobacteria					
Rhodobacter et rel.	0.81	0.042	1.263 ± 0.128	1.383 ± 0.118	+
Betaproteobacteria					
Bordetella et rel.	0.81	0.091	1.052 ± 0.093	0.974 ± 0.095	-
Neisseria et rel.	0.81	0.042	0.904 ± 0.071	0.817 ± 0.081	-
Spirochaetes					
Spirochaetes					
Brachyspira	0.81	0.091	0.009 ± 0.003	0.011 ± 0.003	+
Leptospira	0.81	0.071	0.642 ± 0.071	0.728 ± 0.082	+

[1] DS, digestible starch; P1, part 1; PITChip, Pig Intestinal Tract Chip; RS, resistant starch.
[2] Corr. *P* value indicates the *P* value corrected for multiple testing according to the procedure of Benjamini-Hochberg.
[3] ARC is the Average Relative Contribution of a microbial group. Values are means ± SDs, $n = 9$ for DS-fed pigs and $n = 7$ for RS-fed pigs.
[4] "+" or "-" indicates whether the average relative contribution of the microbial group increased or decreased by the RS treatment.

In the colon, 30 microbial groups were significantly changed by the RS treatment (corrected *P* value <0.05), whereas a trend toward significance was observed for 13 additional microbial groups (corrected *P* value between 0.05 and 0.07) (**Table 2**). Members of the *Actinobacteria*, *Weissella*-like group, *Clostridium* cluster IV, IX, XV, XVI and XVII, *Mollicutes*, *Fusobacteria* and *Betaproteobacteria* increased in relative abundance in pigs fed the RS diet. In contrast, groups within the classes of *Bacilli* (e.g. *Allofustis, Lactobacillus acidophilus*-like group and *Lactobacillus plantarum*-like group), *Clostridium*

cluster XI and XIVa, *Deltaproteobacteria* and *Gammaproteobacteria* decreased upon RS consumption (**Table 2**).

Despite the change in relative abundance of several microbial groups, treatment with RS for 14 d did not significantly alter the microbial diversity in caecum and colon as indicated by Shannon's and Simpson's indices for diversity (data not shown).

Table 2. Phylogenetic groups in the luminal content of the P1 colon of pigs fed the DS or RS diet for 2 wk that were significantly affected by diet according to univariate analysis of PITChip data[1].

			ARC[3]		
Phylogenetic group	**Corr. *P* value[2]**	***P* value**	**DS**	**RS**	**Effect[4]**
Actinobacteria					
Actinobacteria					
Eggerthella et rel.	0.045	0.009	0.731 ± 0.033	0.823 ± 0.081	+
Microbacterium	0.040	0.006	0.785 ± 0.057	0.911 ± 0.076	+
Micrococcus et rel.	0.045	0.009	0.806 ± 0.048	0.897 ± 0.069	+
Propionibacterium	0.069	0.021	1.258 ± 0.081	1.403 ± 0.093	+
Tonsillophilus	0.058	0.014	0.694 ± 0.035	0.791 ± 0.082	+
Firmicutes					
Bacilli					
Allofustis	0.058	0.014	0.694 ± 0.043	0.622 ± 0.052	-
Lactobacillus acidophilus et rel.	0.069	0.021	0.956 ± 0.151	0.736 ± 0.173	-
Lactobacillus plantarum et rel.	0.040	0.006	2.083 ± 0.178	1.835 ± 0.124	-
Weissella et rel.	0.045	0.009	0.660 ± 0.032	0.735 ± 0.052	+
Clostridium cluster IV					
Faecalibacterium et rel.	0.020	0.001	1.402 ± 0.188	1.748 ± 0.137	+
Faecalibacterium prausnitzii et rel.	0.020	0.001	1.648 ± 0.232	2.062 ± 0.134	+
Ruminococcus bromii et rel.	0.069	0.021	0.294 ± 0.049	0.367 ± 0.049	+
Sporobacter termitidis et rel.	0.015	0.001	3.300 ± 0.364	4.358 ± 0.283	+
Uncultured *Clostridia* IV	0.021	0.002	1.799 ± 0.107	2.232 ± 0.294	+
Clostridium cluster IX					
Phascolarctobacterium faecium et rel.	0.015	<0.001	0.896 ± 0.098	1.134 ± 0.068	+
Veilonella	0.015	0.001	0.602 ± 0.088	0.793 ± 0.066	+
Clostridium cluster XI					
Anaerovorax et rel.	0.045	0.009	1.078 ± 0.150	0.851 ± 0.111	-
Clostridium cluster XIVa					
Bryantella et rel.	0.045	0.009	2.022 ± 0.110	1.810 ± 0.172	-
Dorea et rel.	0.069	0.021	0.913 ± 0.106	0.760 ± 0.105	-
Eubacterium plexicaudatum et rel.	0.045	0.009	0.371 ± 0.033	0.324 ± 0.024	-
Eubacterium ventriosum et rel.	0.058	0.014	0.363 ± 0.032	0.321 ± 0.022	-
Roseburia intestinalis et rel.	0.040	0.006	1.047 ± 0.112	0.898 ± 0.065	-
Ruminococcus ganvus et rel.	0.045	0.009	0.934 ± 0.110	0.769 ± 0.079	-
Uncultured *Clostridia* XIVa	0.040	0.006	2.002 ± 0.127	1.744 ± 0.141	-
Clostridium cluster XV					
Eubacterium et rel.	0.015	0.001	0.604 ± 0.088	0.793 ± 0.066	+
Clostridium cluster XVI					
Eubacterium biforme et rel.	0.021	0.002	0.732 ± 0.048	0.833 ± 0.053	+
Clostridium cluster XVII					
Catenibacterium	0.021	0.002	0.274 ± 0.021	0.333 ± 0.024	+
Mollicutes					
Acholeplasma et rel.	0.045	0.009	0.690 ± 0.039	0.778 ± 0.072	+
Bulleidia moorei et rel.	0.021	0.002	0.336 ± 0.035	0.407 ± 0.032	+
Erysipelothrix	0.015	0.001	0.715 ± 0.119	0.995 ± 0.081	+
Solobacterium moorei et rel.	0.040	0.006	0.525 ± 0.091	0.714 ± 0.149	+
Fusobacteria					
Fusobacteria					
Fusobacterium	0.045	0.009	0.077 ± 0.003	0.090 ± 0.013	+
Proteobacteria					
Betaproteobacteria					
Bordetella et rel.	0.069	0.021	0.932 ± 0.105	1.076 ± 0.081	+
Neisseria et rel.	0.069	0.021	0.795 ± 0.094	0.916 ± 0.083	+

Oxalobacter et rel.	0.069	0.021	0.723 ± 0.091	0.832 ± 0.058	+
Sutterella wadsorthia et rel.	0.058	0.014	0.822 ± 0.098	0.943 ± 0.070	+
Deltaproteobacteria					
Uncultured *Deltaproteobacteria*	0.021	0.002	0.094 ± 0.019	0.067 ± 0.009	-
Gammaproteobacteria					
Escherichia coli et rel.	0.040	0.006	1.423 ± 0.108	1.161 ± 0.161	-
Pseudomonas et rel.	0.020	0.001	1.306 ± 0.154	0.975 ± 0.161	-
Psychrobacter et rel.	0.021	0.002	1.022 ± 0.062	0.870 ± 0.099	-
Uncultured *Gammaproteobacteria*	0.015	<0.001	1.314 ± 0.136	1.027 ± 0.134	-
Vibrio et rel.	0.058	0.014	0.673 ± 0.077	0.537 ± 0.098	-
Spirochaetes					
Spirochaetes					
Uncultured *Spirochaetes*	0.069	0.021	0.518 ± 0.043	0.456 ± 0.048	-

[1] DS, digestible starch; P1, part 1; PITChip, Pig Intestinal Tract Chip; RS, resistant starch.
[2] Corr. *P* value indicates the *P* value corrected for multiple testing according to the procedure of Benjamini-Hochberg.
[3] ARC is the Average Relative Contribution of a microbial group. Values are means ± SDs, $n = 7$ for DS-fed pigs and $n = 8$ for RS-fed pigs.
[4] "+" or "-" indicates whether the average relative contribution of the microbial group increased or decreased by the RS treatment.

SCFA concentrations

SCFA concentrations were measured in the intestinal contents collected at section (5 h postprandially). Total SCFA concentration was highest in the caecum and gradually decreased along the colon (**Fig. 3A**). In the caecum and colon, SCFA concentrations were significantly higher in RS-fed pigs compared with DS-fed pigs (Fig. 3A). The most abundant SCFAs in the intestine were acetate, propionate and butyrate. The increase in total SCFA concentration on RS could mainly be attributed to higher concentrations of acetate and propionate (**Fig. 3B**, **C**). Butyrate concentration was significantly higher only in RS-treated pigs in the colon part 2 (P2 colon) (**Fig. 3D**). Although concentrations of valerate were low, the values for caecum and colon were significantly higher in RS-fed pigs (**Fig. 3E**). However, in the caecum we found significantly higher concentrations of isobutyrate and isovalerate in DS-fed pigs (**Fig. 3F**, **G**).

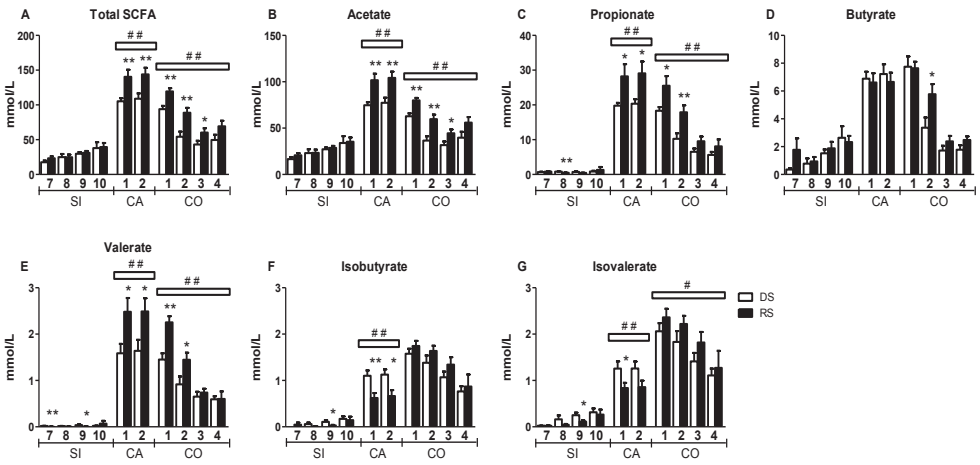

Figure 3. Total SCFAs (A), acetate (B), propionate (C), butyrate (D), valerate (E), isobutyrate (F) and isovalerate (G) concentrations in luminal contents of pigs fed the DS or RS diet for 2 wk. Values are means ± SEM, n = 6-10 pigs per treatment. *, ** DS and RS differ within the intestinal segment, P <0.05 and P <0.01, respectively. # , ## DS and RS differ within the total caecum or colon, P <0.05 and P <0.01, respectively. From left to right, bars represent areas of the gastrointestinal tract from proximal to distal. The small intestine was divided into 10 equal parts; parts 7-10 refer to the 4 most distal segments of the small intestine, whereas the caecum and colon were divided into 2 respectively 4 segments of equal length. White horizontal bars indicate the intestinal parts that were combined for statistical analysis to determine differences between treatments. CA, caecum; CO, colon; DS, digestible starch; RS, resistant starch; SI, small intestine.

Differential gene expression mucosal scrapings

The expression of several genes was determined in the mucosal scrapings collected at section (5 h postprandially). We observed a significant increase in *GCG* gene expression in RS-fed pigs in the caecum (**Fig. 4A**), whereas *PYY* gene expression was not modulated (**Fig. 4B**). In the most distal part of the small intestine, an increase in angiopoietin-like 4 (*ANGPTL4*) expression was found with RS (**Fig. 4C**). In the caecum and P2 colon, expression of *SLC16A1* was higher in RS-fed pigs compared with DS-fed pigs (**Fig. 4G**). However, the expression of the SCFA receptors *FFAR2*, *FFAR3*, G protein-coupled receptor 119 (*GPR119*) and the SCFA transporter *SLC5A8* was not different between the pigs fed RS or DS (**Fig. 4D**, **E**, **F** and **H**, respectively).

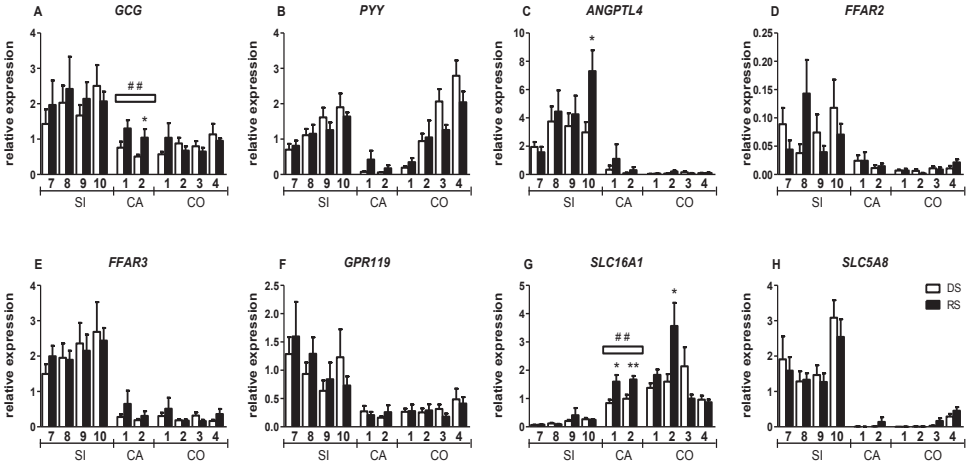

Figure 4. Relative gene expression of *GCG* (**A**), *PYY* (**B**), *ANGPTL4* (**C**), *FFAR2* (**D**), *FFAR3* (**E**), *GPR119* (**F**), *SLC16A1* (**G**) and *SLC5A8* (**H**) in mucosal scrapings along the proximal-distal axis of the intestine of pigs fed the DS or RS diet for 2 wk, as determined by qRT-PCR. Messenger RNA levels were standardized to *RPLP0*. Values are presented as means ± SEM, *n* = 6-9 pigs per treatment. *,** DS and RS differ within the intestinal segment, *P* <0.05 and *P* <0.01, respectively; $^{\#\#}$ DS and RS differ within the total caecum, *P* <0.01. From *left* to *right*, bars represent areas of the gastrointestinal tract from proximal to distal. The small intestine was divided into 10 equal parts; parts 7-10 refer to the 4 most distal segments of the small intestine, whereas the caecum and colon were divided into 2 respectively 4 segments of equal length. White horizontal bars indicate the intestinal parts that were combined for statistical analysis to determine differences between treatments. CA, caecum; CO, colon; DS, digestible starch; RS, resistant starch; SI, small intestine.

Plasma analyses

Blood samples were taken from all pigs, both before and after the intervention. Glucose, insulin, cholesterol and GLP-1 concentrations were not affected by dietary treatment (**Supplemental Fig. 3A**, **B**, **D** and **E**, respectively). However, at the end of the intervention period, plasma TG concentrations were significantly higher in RS-fed pigs compared with pigs fed DS (**Supplemental Fig. 3C**). In addition, in DS-fed pigs, plasma PYY was significantly lower at the end of the experimental period than at the start (**Supplemental Fig. 3F**).

Discussion

In the set of experiments reported in this chapter we investigated the effects of a diet high in RS in pigs. We found that RS was completely degraded in caecum, changed caecal and colonic microbiota composition, increased caecal and colonic SCFA concentrations, and increased the expression of *SLC16A1* and *GCG* in caecum. RS had no effect on plasma concentrations of GLP-1 and PYY measured 5 h postprandially.

Our data on RS contents in intestinal segments show that RS was completely degraded in caecum, which is in line with observations on the degradation of inulin in the gastrointestinal tract of pigs [304]. As a result, the composition of the microbiota differentially changed in porcine caecum and colon, as also has been reported for inulin [305]. However, the RS-induced changes in the gut microbiota depend on the initial composition of an individual's gut microbiota [96], which could explain why RS-fed pigs 24 (caecum) and 1 (colon) were not clearly separated from the DS-fed pigs on the RDA triplots. Moreover, specific bacterial groups can be selectively affected by a certain type of RS [86, 94] and even by different crystalline polymorphism of the same type of RS [306] in different locations of the intestine. Young *et al.* [95] fed type 2 RS (RS2) to rats and found blooms of *Bacteroidetes* and *Actinobacteria* in colonic digesta. However, Martínez *et al.* [94] reported that type 4 RS but not RS2 significantly induced *Bacteroidetes* and *Actinobacteria* while decreasing *Firmicutes* at the phylum level. At the species level, type 4 RS increased *Bifidobacterium adolescentis* and *Parabacteroides distasonis*, whereas RS2 significantly raised the proportions of *Ruminococcus bromii* and *Eubacterium rectale*. In a study on RS3, the relatives of *R. bromii* (R-ruminococci) and *E. rectale* were found to be increased in the colon of most volunteers [96]. Moreover, when the effects of structural variation of RS3 on fermentability by human gut microbiota were studied, it was found that retrograded RS3 that formed a B-type pattern induced *Bifidobacterium* spp., whereas A-type pattern RS3 induced *Atopobium* spp. [306].

In our study, the pigs were fed RS3, and we observed a clear change in caecal and colonic

microbiota composition compared with pigs fed DS. The relative abundance of *R. bromii* was increased in the colonic samples of RS-fed pigs, which is consistent with previous research [96]. However, *E. rectale* was not significantly changed in our study and its relative contribution in the RS group even decreased slightly. Interestingly, in addition to *E. rectale*, other bacterial groups that belong to *Clostridium* cluster XIVa showed either a significant decrease or a trend toward a decrease in RS-fed pigs, whereas bacterial groups belonging to *Clostridium* cluster IV , IX, XV, XVI and XVII increased in RS-fed pigs. This may be due to a low competitiveness of *Clostridium* cluster XIVa, because previous research indicated the *Roseburia/E. rectale* group was particularly dependent on residual dietary carbohydrate and pH to maintain its competitiveness in the colon [307]. It may also be related to the fact that we could not detect a significant effect of the RS diet on butyrate concentration in P1 colon, because several members of this group have been shown to produce butyrate [308]. In turn, we found increased relative abundance of populations related to the butyrate-producing *Faecalibacterium prausnitzii*, previously suggested as a health-promoting bacterium [309]. Furthermore, we found an increase in several propionate-producing microorganisms, including members of the genera *Propionibacterium*, *Veilonella* and *Phascolarctobacterium*. Propionate has previously been indicated as another health-promoting metabolite being produced in the large intestine [288]. In contrast, several groups of potentially pathogenic taxa within the *Gammproteobacteria*, including *Escherichia coli* and *Pseudomonas* spp., were found to be decreased in relative abundance in the colon.

Currently, most studies focus on the colonic and faecal microbiota [94-96], whereas research on the effect of RS on caecal microbiota is limited. This study is the first to our knowledge to use comprehensive microarray-based profiling to detect the RS effect on caecal microbiota in an animal model. We observed major differences between caecum and colon with respect to microbial changes, possibly due to the change in the chemical structure of RS in colon compared with caecum, which determined the RS accessibility by groups of bacteria [94].

We found that a diet high in RS increased SCFA concentrations in the luminal content compared with a DS diet, as was also observed previously in pigs [90]. However, because >95% of the SCFAs are rapidly absorbed from the colonic lumen and metabolized by the host, the total production of SCFAs is difficult to determine [310, 311]. Butyrate is almost entirely used by colonocytes as their preferred energy substrate, whereas acetate and propionate move to the liver via the portal vein. Propionate is metabolized by the liver and used for gluconeogenesis, whereas acetate is a substrate for cholesterol synthesis and lipogenesis. In addition, acetate is taken up by muscle and adipose tissue [310, 312]. It has been observed that RS especially results in an increased production of butyrate [73]. However, our experimental setup did not allow us to quantify total butyrate production, for

which preferably isotope dilution studies or direct measurements of arteriovenous differences in SCFA concentrations across the gut are required [310, 311]. Moreover, it has been suggested that butyrate is taken up by the colonocytes more rapidly than acetate and propionate [310]. This might explain why we did not observe a very profound increase in butyrate concentrations in pigs fed RS.

We found that *SLC16A1* was induced in pigs consuming the RS diet. This observation can be explained by the increased intestinal SCFA concentrations [313, 314]. Induction of *SLC16A1* gene expression was also observed by Zhou et al [290], who measured gene expression in epithelial cells of the gastrointestinal tract from rats fed a DS or an RS diet for 4 wk. They found that *SLC16A1*, *PYY* and *GCG* gene expression was induced in the caecum and colon. The fact that our study did not show an increase in *GCG* or *PYY* gene expression with RS consumption could be due to the different animal model used and the different duration of the dietary treatment.

The presence of SCFA-activated FFA receptors in the intestinal mucosa could provide a link between intestinal SCFAs and appetite and energy homeostasis. FFAR2 immunoreactivity was found to be almost completely colocalized with GLP-1 in terminal ileum, caecum and colon of rats [315]. Furthermore, it was shown in proximal colon that the densities of FFAR2-immunoreactive enteroendocrine cells and GLP-1-producing cells were increased >2-fold by fermentable fibre supplementation compared with control [315]. In addition, the activation of FFAR2 by SCFAs is suggested to facilitate or modify PYY secretion [316]. In this study, no significant differences in FFAR2 and FFAR3 gene expression were observed between the 2 diet groups. However, this observation does not exclude the possibility that the receptors were activated in RS-fed pigs, because gene expression levels do not necessarily reflect protein levels. Our experiment showed that GPR119, like GCG, is most abundantly expressed in the distal small intestine. GPR119 is expressed in intestinal endocrine L-cells and has been shown to stimulate the release of GLP-1 [317, 318]. However, in our study the expression of GPR119 and GCG was not modified upon RS feeding.

TG concentrations in plasma were found to be higher in RS-fed pigs compared with control pigs. Two mechanisms might be responsible for this increase. At first, acetate resulting from fermentation of RS in the intestine is taken up by the liver, where it can serve as a substrate for TG synthesis [310]. A second explanation is that RS increases plasma ANGPTL4 concentrations, because we observed increased ANGPTL4 gene expression in the distal part of the small intestine of RS-fed pigs. Because ANGPTL4 is an inhibitor of lipoprotein lipase, increased concentrations of ANGPTL4 result in increased plasma TG concentrations [319].

In conclusion, we showed that a diet high in RS modulates microbiota composition, SCFA concentrations and gene expression in pig intestine. These findings provide a detailed insight on the interaction between diet, microbiota, and host and may provide leads for designing functional food strategies that aim to reduce the risk of obesity and type 2 diabetes in humans. However, additional investigation is required to further elucidate the underlying molecular mechanisms and the link to satiety.

Acknowledgements

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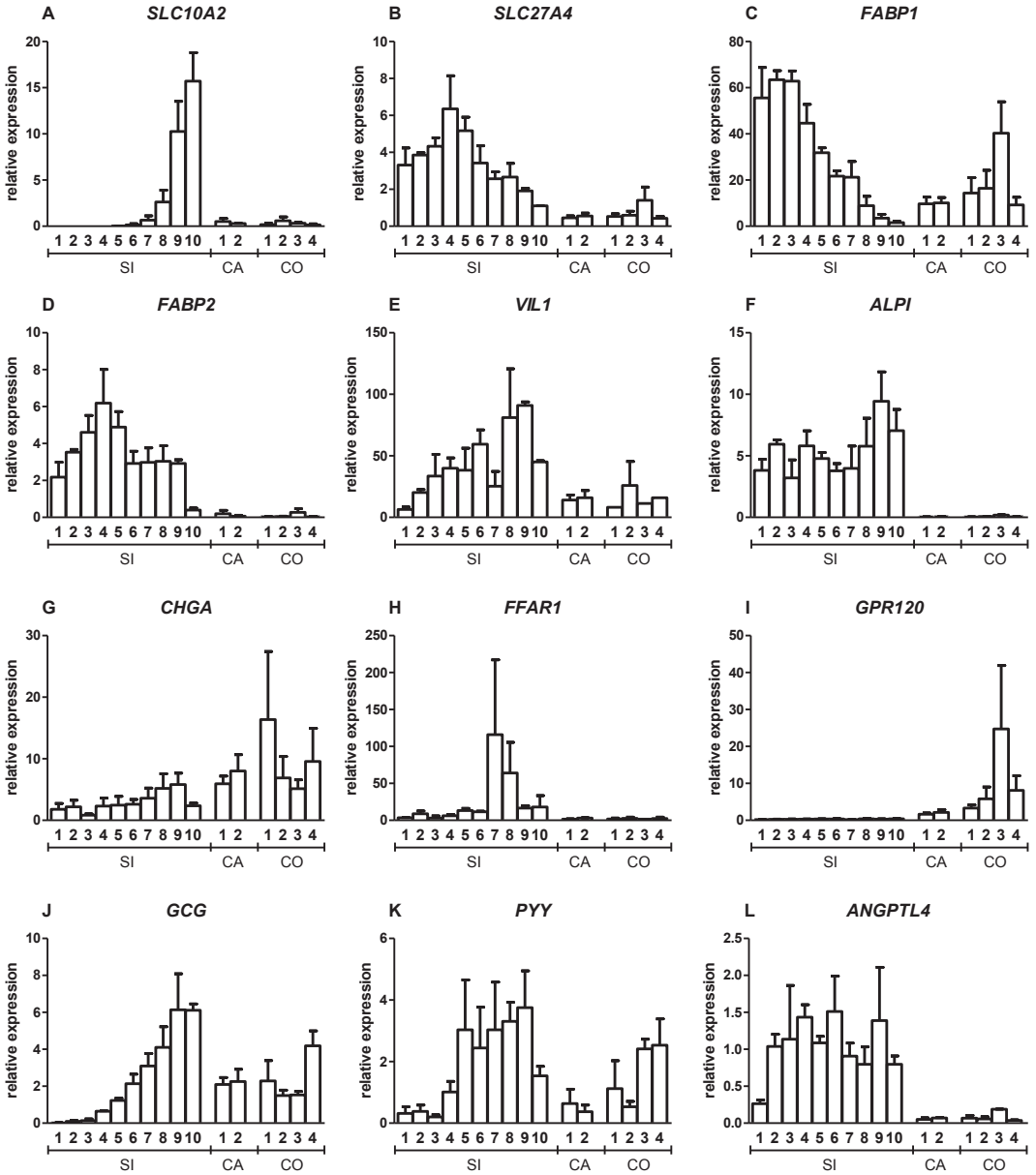
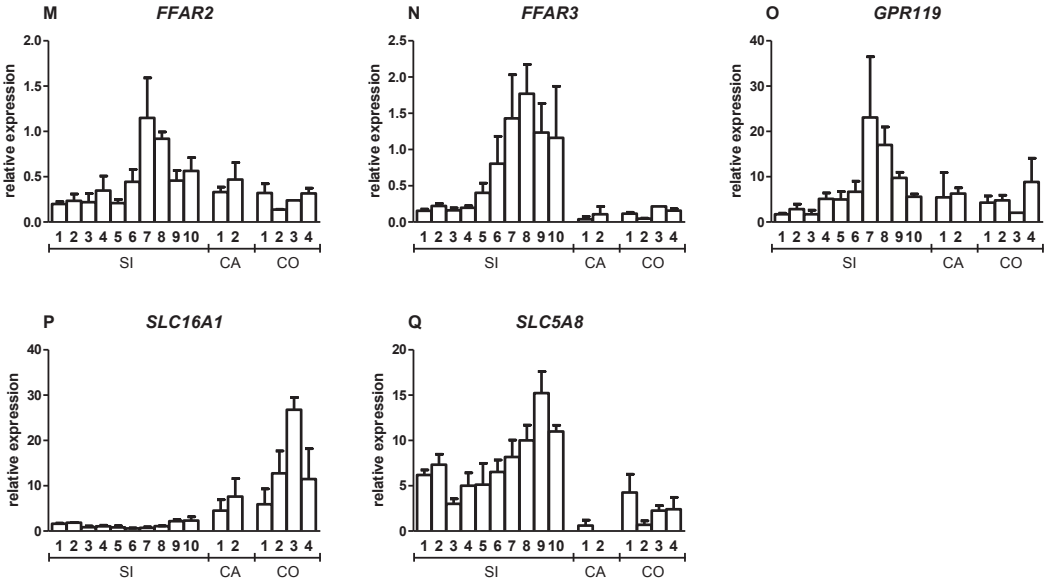
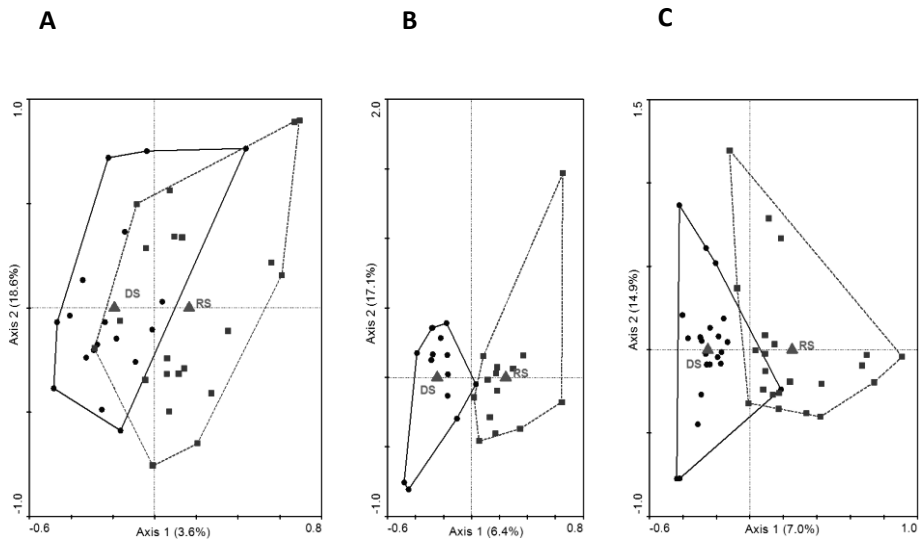
Supplemental tables and figures

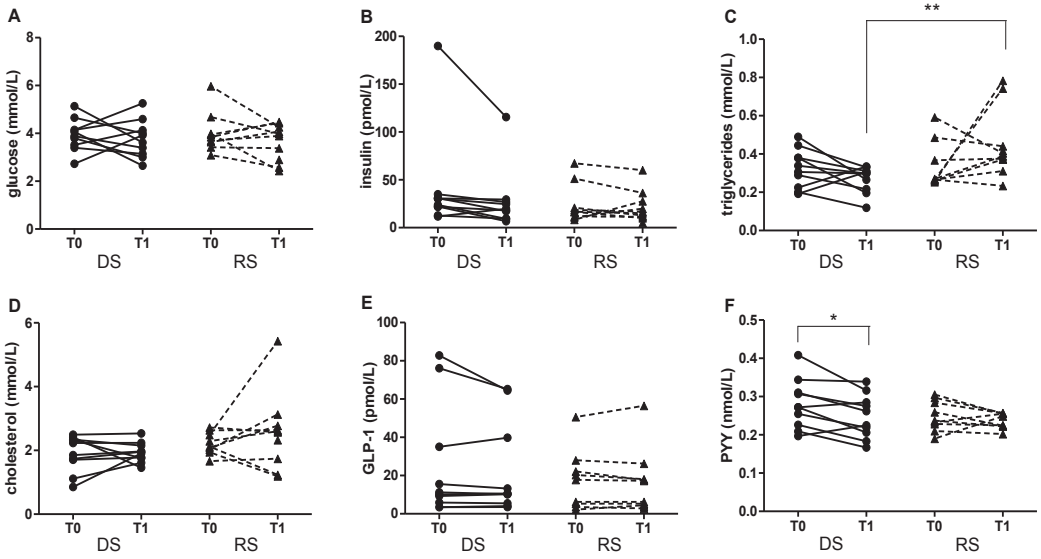




Supplemental Figure 1. Relative gene expression of Apical sodium-dependent bile salt transporter, *SLC10A2* (**A**), Fatty acid transport protein 4, *SLC27A4* (**B**), Liver-type fatty acid binding protein, *FABP1* (**C**), Intestine-type fatty acid binding protein, *FABP2* (**D**), Villin 1, *VIL1* (**E**), Intestinal alkaline phosphatase, *ALPI* (**F**), Chromagranin A, *CHGA* (**G**), Free fatty acid receptor 1, *FFAR1* (**H**), G protein-coupled receptor 120, *GPR120* (**I**), Glucagon, *GCG* (**J**), Peptide YY, *PYY* (**K**), Angiopoietin-like 4, *ANGPTL4* (**L**), Free fatty acid receptor 2, *FFAR2* (**M**), Free fatty acid receptor 3, *FFAR3* (**N**), G protein-coupled receptor 119, *GPR119* (**O**), Monocarboxylate transporter 1, *SLC16A1* (**P**) and Sodium-coupled monocarboxylate transporter 1, *SLC5A8* (**Q**) in mucosal scrapings along the proximal-distal axis of the intestine of adult female pigs, as determined by qRT-PCR. Messenger RNA levels were standardized to *RPLP0*. Values are presented as means ± SEM, $n = 3$ pigs. From *left* to *right*, bars represent areas of the gastrointestinal tract from proximal to distal. The small intestine was divided into 10 equal parts, whereas the caecum and colon were divided into 2 respectively 4 segments of equal length. CA, caecum; C, colon; SI, small intestine.



Supplemental Figure 2. RDA analysis of DGGE profiling data of samples from small intestine (**A**), caecum (**B**) and colon (**C**). Nominal environmental variables DS and RS are represented by triangles (▲). Samples are grouped by treatment: RS (■) and DS (●). Each symbol represents 1 pig. Combined both axes explain 22.2% of the total variance in the dataset for (A), 23.5% for (B) and 21.9% for (C). DS, digestible starch; RS, resistant starch.



Supplemental Figure 3. Glucose (**A**), insulin (**B**), TG (**C**), cholesterol (**D**), GLP-1 (**E**) and PYY (**F**) concentrations in plasma of pigs fed the DS or RS diet before the start of the treatment (T0) and on d12 of the treatment (T1). n = 10 pigs per treatment; *,** DS and RS differ, P <0.05 and P <0.01, respectively. DS, digestible starch; RS, resistant starch.

Supplemental Table 1. Composition of the DS and RS diet, expressed as g/kg diet[1].

Ingredient	DS	RS
Native potato starch (Paselli WA4)	350.0	-
Retrograded tapioca starch (Actistar, Cargill)	-	338.8
Wheat	249.0	249.0
Barley	150.0	150.0
Corn gluten meal	100.0	100.0
Potato protein (Protastar)	50.0	50.0
Soy bean oil	15.0	15.0
Animal fat	15.0	15.0
Vitamin mineral premix[2]	10.0	10.0
$CaCO_3$	17.0	17.0
Monocalcium phosphate	11.0	11.0
KCI	10.0	10.0
$NaHCO_3$	15.0	15.0
NaCI	5.0	5.0
L-lysine HCI	1.5	1.5
Flavouring (Luctarom Advance Cherry Honey)	1.5	1.5
Total	1000.0	988.8

[1] DS, digestible starch; RS, resistant starch.
[2] Provided the following per kg food (of the DS diet): retinol: 3.0 mg; cholecalciferol: 50 µg; α-tocopherol: 25 mg; menadione: 1.0 mg; thiamine: 0.75 mg; riboflavin: 4.0 mg; niacin: 20 mg; pyridoxine: 1.0 mg; cyanocobalamin: 15µg; panthothenic acid: 13 mg; choline chloride: 300 mg; folic acid: 2.5 mg; biotin: 0.1 mg; Fe: 80 mg ($FeSO_4.H_2O$); Cu: 10 mg ($CuSO_4.5H_2O$); Mn: 30 mg (MnO); Zn: 60 mg ($ZnSO_4.H_2O$); Co: 0.20 mg ($CoSO_4.7H_2O$); I: 0.75 mg (KI); Se: 0.20 mg (Na_2SeO_3).

Supplemental Table 2. Primer sequences used for qRT-PCR.

Gene symbol	Primer sequence (Forward)	Primer sequence (Reverse)
ALPI	CTACACATTGCGTGGAAG	ATGGAGGTATATGGCTTGA
ANGPTL4	AGATTCAGCAACTCTTCC	AATTCTGGATTCTCAAGTG
CHGA	AAGAAACAGAGCAGTTATGA	CCTCCTTCAACTCAGTCT
FFAR1	CTTGTCCTCTGTCACCTG	AGGGAGCTGGTAGTATTG
FFAR2	CGTGTTCATCGTTCAGTA	GAAGTTCTCATAGCAGGTA
FFAR3	TGGAGACCTTACGTGTTG	CGAGGATGAGAAGTAGTAGAT
GCG (glucagon)	CAAGAGGAACAAGAATAACAT	AAGAACTTACATCACTGGTA
GPR119	TATAGGCAGAAGGAGGTA	AGAGAAGGAGGAGGAATG
GPR120	TGGGATGTGTCGTTTGTT	CCTTGATGCCTTGGTGAT
FABP1(L-FABP)	TGAACTCAACGGTGACATA	ATTCTCTTGCTGATTCTCTTG
FABP2 (I-FABP)	AGATAGACCGCAATGAGA	TCCTTCTTGTGTAATTATCAGT
PYY	AGATATGCTAATACACCGAT	CCAAACCCTTCTCAGATG
RPLP0	CTTTAGGCATCACCACTA	TGTCTCCAGTCTTAATCAG
SLC5A8 (SMCT1)	CGCAGATTCCTACTAACC	GATTGTCAGTTCCACCAT
SLC10A2 (ASBT)	TGCCTCTTAATCTATACCA	GGACACAGGAACAATAAG
SLC16A1 (MCT1)	CATCAACTACCGACTTCTG	TACTGGTCTCCTCCTCTT
SLC27A4 (FATP4)	GGTTCTGGGATGATTGTG	TGGTTGAGGAGGTATCTG
VIL1	TATTATTGGTGTTCGTGCTA	TCTGGAGGAATAGGATACTAA

Supplemental Table 3. General characteristics of pigs fed the DS or RS diet for 2 wk. Values are presented as means ± SEM, n = 10 pigs per treatment[1].

	Diet		
Measurement	DS	RS	*P* value
Body weight (kg)	274 ± 6.3	271 ± 5.0	0.68
Fat depth (mm)	29.0 ± 1.4	29.0 ± 1.1	0.98
Length small intestine (m)	16.4 ± 1.6	16.9 ± 0.8	0.77
Length colon (m)	5.8 ± 0.2	5.8 ± 0.2	1.00

[1] DS, digestible starch; RS, resistant starch

Chapter 7

Resistant starch induces catabolic but suppresses immune and cell division pathways and changes the microbiome in the proximal colon of male pigs

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Abstract

Consumption of resistant starch (RS) has been associated with various intestinal health benefits, but knowledge on its effects on global gene expression in colon is rather limited. The main objective of the current study was to identify genes affected by RS in the proximal colon to infer which biological pathways were modulated. Ten 17-wk-old male pigs, fitted with a cannula in the proximal colon for repeated collection of tissue biopsies and luminal content, were fed a digestible starch (DS) diet, or a diet high in RS (34%) for two consecutive periods of 14 days in a crossover design. Analysis of the colonic transcriptome profiles revealed that upon RS feeding oxidative metabolic pathways, such as TCA cycle and beta-oxidation, were induced whereas many immune response pathways, including adaptive and innate immune system, as well as cell division were suppressed. The nuclear receptor PPARG was identified as a potential key upstream regulator. RS significantly ($P < 0.05$) increased the relative abundance of several butyrate-producing microbial groups, including the butyrate producers *Faecalibacterium prausnitzii* and *Megasphaera elsdenii*, and reduced the abundance of potentially pathogenic members of the genus *Leptospira* and the phylum of Proteobacteria. Concentrations in carotid plasma of the three main short-chain fatty acids acetate, propionate and butyrate were significantly higher with RS consumption compared to DS consumption. Overall, this study provides novel insights on effects of RS in proximal colon, and contributes to our understanding of a healthy diet.

Introduction

Dietary resistant starch (RS) is a complex polysaccharide that resists digestion and absorption in the small intestine. Resistant starches occur for a variety of reasons including milling, thermal processing and chemical modifications such as cross bonding and acylation [320]. The effects and potential health benefits of RS have been extensively studied [73, 79, 81, 321]. Beneficial effects have been reported for large intestine, i.e. cecum and colon, where RS is highly fermentable by microbiota, resulting in among others the well-known formation of short-chain fatty acids (SCFAs), but also of a variety of phytochemical metabolites that have only be partially characterized [322]. Compared to other polysaccharides, RS preferentially favors the production of butyrate in humans [321, 323, 324].

With regard to pigs, several studies showed that RS increases cecal, colonic and faecal concentrations of total SCFAs and the main individual SCFAs acetate, propionate and butyrate, concomitant with changes in microbiota composition [90, 101, 325-327]. Concentrations of SCFAs gradually decrease along the colon in pigs fed RS, with the lowest concentrations present in the distal part [101, 326], and these regional differences have been linked to the pathogenesis of colorectal cancer and inflammatory bowel disease [73, 321, 326]. Studies in rodents corroborated the findings that RS may play a role in the prevention of colorectal cancer [92, 328] and inflammatory bowel disease [329, 330].

Genome-wide transcriptional profiling, or transcriptomics, is extensively used to study how cells respond to certain stimuli or to diagnose and predict clinical outcomes [331, 332]. Similarly, there is a major interest in characterizing the genes and networks that are regulated by food components, since this contributes to our understanding of a healthy diet [333, 334]. Remarkably, data on the genome-wide effects of RS in the intestinal tract is scarce. It has only been recently reported that differential gene expression due to consumption of type 2 RS for 4 weeks suggested improvement of structure and function of the GI tract in rats compared to a cornstarch diet with the same energy density [335]. In addition, the effect of colonic butyrate administration on gene expression profile in distal colonic mucosa has been investigated, showing that butyrate affects fatty acid metabolism, electron transport, oxidative stress and apoptosis pathways in healthy humans [336].

We previously showed in pigs that 2-wk-consumption of a diet high in RS changed caecal and colonic microbiota composition, increased caecal and colonic SCFA concentrations and modified the mucosal gene expression of SLC16A1 (monocarboxylate transporter) and GCG (in intestine precursor for GLP-1 and -2) in caecum [101]. However, genome-wide transcriptional profiling was not performed in that study. Thus, extending these findings, the aim of the current study was to identify genes and corresponding biological pathways

that are modified by RS in the mucosa of the proximal colon (pCO), together with alterations in the luminal microbiome. To this end, a crossover study was performed in pigs that were fitted with a permanent cannula in the pCO for repeated collection of luminal content and tissue biopsies. The colonic gene expression profile and luminal microbiota composition were obtained by microarray techniques. Pigs were used as a model for humans, because the anatomy and physiology of the gastrointestinal tract of pigs and the pig genome bear a lot of similarities with those in humans [153, 154].

Materials and methods

Experimental design, pigs and housing

Ten male Landrace barrows (17 wk of age; initial body weight of 57.9 ±1.61 kg) from eight litters were fitted with cannulas and catheters and assigned to two dietary treatments in a crossover design. Each treatment lasted for 14d, and differed with regard to the type of starch in the diet: digestible starch (DS) or resistant starch (RS). Pigs were individually housed in metabolism pens of 2 m2, equipped with a feeder. Artificial lights were on from 0500 until 1900 and dimmed during the dark period. All experimental protocols describing the management, surgical procedures, and animal care were reviewed and approved by the Animal Care and Use Committee of Wageningen University and Research Centre (Lelystad, the Netherlands).

Diets and feeding

The 2 experimental diets used were identical except for type of starch. The main source of starch in the DS diet was pregelatinized potato starch (Paselli WA4, AVEBE), which was replaced on dry matter basis in the RS diet by retrograded tapioca starch (Actistar, Cargill). According to the supplier, this starch was at least 50% resistant to digestion in the small intestine (Megazyme RS assay, certificate of analysis Actistar, Cargill). Based on physical and chemical characteristics the RS used in this study can be classified as RS type 3 [79]. Composition of the experimental diets is presented in Supplemental Table 1. Diets were fed twice a day at 0700 and 1600 as a mash, and mixed with water (ratio water:feed = 2.5:1) in the feeders immediately before feeding. The diets were isoenergetic on gross energy (GE) basis. The daily feed allowance was adjusted to 2.8 times the energy required for maintenance (MEm = 450 kJ/kg0.75 per day) and was based on metabolic body weight (kg0.75). Pigs were weighed every week to allow the adjustment of their feeding level in accordance to metabolic body weight. All pigs had free access to water throughout the study. During the 1-week adaptation period, before surgery, pigs were fed a 50:50 mix of the DS and RS diet, and adapted to the feeding pattern and individual housing.

Surgery

In the second week after arrival, pigs underwent surgery for the placement of a cannula in the pCO and a catheter in the carotid artery. The pCO was chosen as site of investigation because of the greater fermentation and higher SCFA concentrations compared to more distal colonic regions [101, 326]. Pigs were surgically fitted with a simple T-cannula in the pCO 1.45 ±0.16 m distal from the ileocecal sphincter, as was confirmed at section. The cannula was inserted in the intestinal lumen, exteriorized through a hole, fastened to the exterior part and closed with a stopper [337]. In addition, pigs were fitted with a permanent blood vessel catheter (Tygon, Norton) as described previously [338]. The catheter for blood sampling was placed in the carotid artery, fixed firmly at the site of insertion, tunnelled subcutaneously to the back of the pig and exteriorized between the shoulder blades. See **Supplemental methods** for more details.

Colon biopsies

Biopsies from the intestinal wall were collected 300 min after the morning meal on d14 of each dietary treatment. See **Supplemental methods** for experimental details.

Digesta collection

Digesta were collected via the gut cannula on d14 of each dietary treatment, both 30 min before and 300 min after feeding. Samples were collected and stored for determination of microbiota composition, SCFA concentration, and dry matter content as described in the **Supplemental methods**. SCFA concentrations and dry matter content were determined in the luminal content as described before [101].

Blood collection

Blood was drawn from the carotid artery on d14 of each dietary treatment, 300 min after feeding. Blood was collected in 6 mL Vacutainer EDTA tubes (Becton Dickinson) supplemented with protease (Complete, EDTA-free; Roche) and dipeptidyl peptidase-4 (Millipore) inhibitors and then placed in ice water. Tubes were centrifuged for 10 min at 1300 g at 4°C within 20 min after blood collection. Plasma was aliquoted and stored at -80°C.

RNA isolation and quality control

Total RNA was isolated from biopsies using TRIzol reagent (Life Technologies) according to the manufacturer's instructions, followed by RNA Cleanup using the RNeasy Micro kit

(Qiagen). Concentrations, purity and quality of the RNA samples was determined as described in the **Supplemental methods**.

Microarray hybridization and analysis

The pCO biopsies of all 10 pigs, collected on d14 of both dietary treatments, were subjected to genome-wide expression profiling. To this end, total RNA (100 ng) was used for whole transcript cDNA synthesis using the Ambion WT expression kit (Life Technologies) and subsequently labeled using the Affymetrix GeneChip WT Terminal Labeling Kit (Affymetrix). Samples were hybridized on Porcine Gene 1.0 ST arrays (Affymetrix), washed, stained, and scanned on an Affymetrix GeneChip 3000 7G scanner. Detailed protocols for array handling can be found in the GeneChip WT Terminal Labeling and Hybridization User Manual (Affymetrix; P/N 702808, Rev. 4). Packages from the Bioconductor project [339], integrated in an online pipeline [340], were used to analyze the array data. Various advanced quality metrics, diagnostic plots, pseudoimages, and classification methods were used to determine the quality of the arrays prior statistical analysis [341]. Array data have been submitted to the Gene Expression Omnibus under accession number GSE45554.

The approximately 600,000 probes on the Porcine Gene 1.0 ST array were redefined utilizing current genome information [342]. In this study probes were reorganized based on the gene definitions as available in the NCBI Sus scrofa Entrez Gene database, build 4.1 (Sscrofa10.2 genome assembly) [343] as well as the gene predictions made by the AUGUSTUS software [344]. Since the annotation of the pig genome is still poor, the functional annotation was improved by mapping the AUGUSTUS gene predictions to the human RefSeq database [345]. Out of 17,118 pig gene predictions, 14,505 were found to have a human orthologous gene. Unless otherwise stated, the functional interpretation of the transcriptome data was performed using the human orthologs.

Normalized gene expression estimates were obtained from the raw intensity values using the robust multiarray analysis (RMA) preprocessing algorithm available in the library 'AffyPLM' using default settings [346]. Differentially expressed probe sets (genes) were identified using linear models, applying moderated t-statistics that implemented empirical Bayes regularization of standard errors (library 'limma') [347]. To adjust for both the degree of independence of variances relative to the degree of identity and the relationship between variance and signal intensity, the moderated t-statistic was extended by a Bayesian hierarchical model to define a intensity-based moderated T-statistic (IBMT) [348]. Probe sets that satisfied the criterion of $P < 0.01$ were considered to be significantly regulated.

Changes in gene expression were related to functional changes using gene set enrichment

analysis (GSEA) [234]. GSEA takes into account the broader context in which gene products function, namely in physically interacting networks, such as biochemical, metabolic, or signal transduction routes, and has the advantage that it is unbiased, because no gene selection step is used. The Enrichment Map plugin for Cytoscape was used for visualization and interpretation of the GSEA results [349]. See **Supplemental methods** for details.

Upstream Regulator Analysis in IPA (Ingenuity Systems, Redwood City; content version 14400082 released 1 Nov 2012) was used to identify the cascade of potential upstream transcriptional regulators that may explain the observed gene expression changes in the dataset, and whether they are likely activated or inhibited.

Microbiota analysis

Microbiota composition was determined in the luminal content collected from the pCO via the cannula 300 min after feeding on d14 of each dietary treatment, essentially as described before [101]. Due to technical issues, samples from only 9 of the 10 pigs could be analyzed. Samples were analyzed on the second generation Pig Intestinal Tract Chip (PITChip), an updated version of the original phylogenetic microarray [101, 227], which is comprised of more than 3,200 tiled oligonucleotides targeting the 16S rRNA gene sequences of 781 porcine intestinal microbial phylotypes. PITChip images were processed using Agilent's Feature Extraction Software version 9.5 and further processed in R (library 'microbiome') [350].

To determine relative abundance of bacterial groups, the probe-level information was summarized based on non-negative matrix factorization, which removes cross-hybridization effects based on oligo-phylotype mappings. With the non-negative matrix factorization output, relative abundance of bacterial groups defined at approximate genus level (90% 16S ribosomal RNA similarity threshold) was calculated and used for further univariate testing. Univariate testing of differences for individual microbial groups was performed using the paired Mann–Whitney U signed rank test. P values were corrected for multiple testing using a false discovery rate (FDR) method [300]. Groups that satisfied the criterion of $P < 0.05$ were considered to be significantly affected. Multivariate analysis was applied for PITChip data interpretation as described in the **Supplemental methods**.

SCFA determination by NMR spectroscopy

SCFA concentrations were determined in plasma samples obtained from the carotid artery 300 min after feeding on d14 of each dietary treatment as described in the **Supplemental methods**.

Standard statistical methods

SCFA concentrations measured in digesta were analyzed using a mixed model in SAS (version 9.1; SAS Institute). For samples derived from the pCO cannula, time and individual pigs were included as repeated measurements. The model included period, diet, time, and interaction of diet and time as fixed effects, and pig as random effect. SCFA concentrations measured in plasma were analyzed using a paired samples t-test in IBM SPSS Statistics 19. Differences were considered significant if $P < 0.05$. Results were expressed as means ± SEMs.

Results

Anthropometric variables

All pigs remained healthy during the experiment and showed normal growth and appetite. Mean body weight at the start of the experiment was 57.9 ± 1.6 kg and increased with 21.8 ± 1.1 kg during the study period. No significant effect of treatment order was found with respect to body weight development (data not shown). The mean length of the small intestine and colon, determined at section, were similar for both treatment groups (16.1 ± 0.62 m and 3.96 ± 0.18 m respectively).

Differentially expressed genes in colon

Microarray analysis was performed to identify genes that were differentially expressed in pCO by RS compared to DS. When remapping the probes to the Sscrofa 10.2 genome assembly, the expression of 748 genes was found to be significantly changed by RS ($P < 0.01$). Of these genes, 459 were induced, whereas 289 genes were suppressed by RS (Supplemental Table 2). Among the changed genes were SLC16A1 and SLC5A8, both being involved in SCFA transport. RS treatment resulted in a 1.5-fold induction of SLC16A1 expression, as we observed before [101], whereas SLC5A8 expression was reduced by 1.4-fold. The most induced gene was Intestinal-type alkaline phosphatase-like (LOC100521756), showing a 2.9-fold increase with the RS treatment, whereas the most suppressed gene was Chitinase 3-like 1 (CHI3L1), with a 3.7-fold decrease **(Supplemental Fig. 1**).

Functional implications of differential gene expression

To gain better insight into the underlying biological phenomena affected by RS, pig genes were mapped to human orthologous and GSEA was performed. Results of GSEA were summarized in an enrichment map to enhance the functional interpretation of enriched gene

sets. Using conservative significance thresholds, an enrichment map was generated that consisted of 329 nodes (gene sets), of which 57 were positively and 272 were negatively enriched (**Fig. 1**). These numbers demonstrated that the majority of the tested gene sets was suppressed with RS feeding. To enhance the interpretation of the results, functionally related modified gene sets were then manually summarized in more general categories (**Fig. 1**); a high resolution color map that includes names of all gene sets is available in **Supplemental Fig. 2**. Induced gene sets described clusters related to lipid and fatty acid metabolism, tricarboxylic acid (TCA) cycle, biological oxidation, olfactory system, and cell-cell contact. Moreover, sets containing target genes of the transcription factors peroxisome proliferator-activated receptor α (PPARA) and nuclear factor erythroid 2-related factor 2 (NRF2) were enriched with RS feeding. The suppressed gene sets were similarly interpreted (**Fig. 1 and Supplemental Fig. 2**). The enrichment map revealed a very large cluster of 154 overlapping gene sets that contained descriptors of many, if not all, aspects of the immune response, including activation of innate and adaptive immune response and proliferation of immune cells. The second largest cluster described processes related to DNA replication, DNA assembly, histone acetylation, and mitosis. Other processes that were suppressed related to nuclear export of RNA, non-coding and messenger RNA processing, post-translational protein modification, and ER stress / unfolded protein response (UPR).

Next, changes in the expression of genes that contributed to the enrichment of the 3 large clusters of processes, i.e. TCA cycle, lipid and fatty acid metabolism, and immune response were visualized (**Supplemental Fig. 3**). Most importantly, these results showed a modest yet consistent regulation of genes involved in the before-mentioned processes, supporting the robustness of the analysis. In addition, it revealed regulation of several key genes, including *PDK1* and *PDK4*; both involved in controlling and connecting glucose and fatty acid metabolism and homeostasis, *ANGPTL4*; regulating plasma TG levels, as well as *NFKB, TLR4, BCL6, ICOS* and *CR2* (that all play a role in controlling the adaptive and innate immune response).

Upstream regulators

The underlying mechanisms by which RS modulated gene expression changes are not well understood. We therefore aimed to identify potential upstream transcriptional regulators that could explain the observed shift in gene expression profile. Results of this analysis predicted the transcription factors peroxisome proliferator-activated receptor γ (PPARG) and v-ets avian erythroblastosis virus E26 oncogene homolog (ERG) to be significantly activated with RS treatment, whereas X-box binding protein 1 (XBP1) was predicted to be inhibited (**Table 1**). Because the highest Z-score (3.069) was found for PPARG, we had a closer look at the downstream PPARG target genes. Out of 21 PPARG-target genes affected

by RS, 15 genes had an expression change consistent with activation of PPARG (**Table 2**). Because *TGFBR1* and *CXCL14* are known to be down-regulated by PPARG and we indeed observed reduced expression of these target genes, this suggests that RS enhanced PPARG activation. The other 13 target genes were induced in our dataset, which corresponds with observations from literature suggesting PPARG activation.

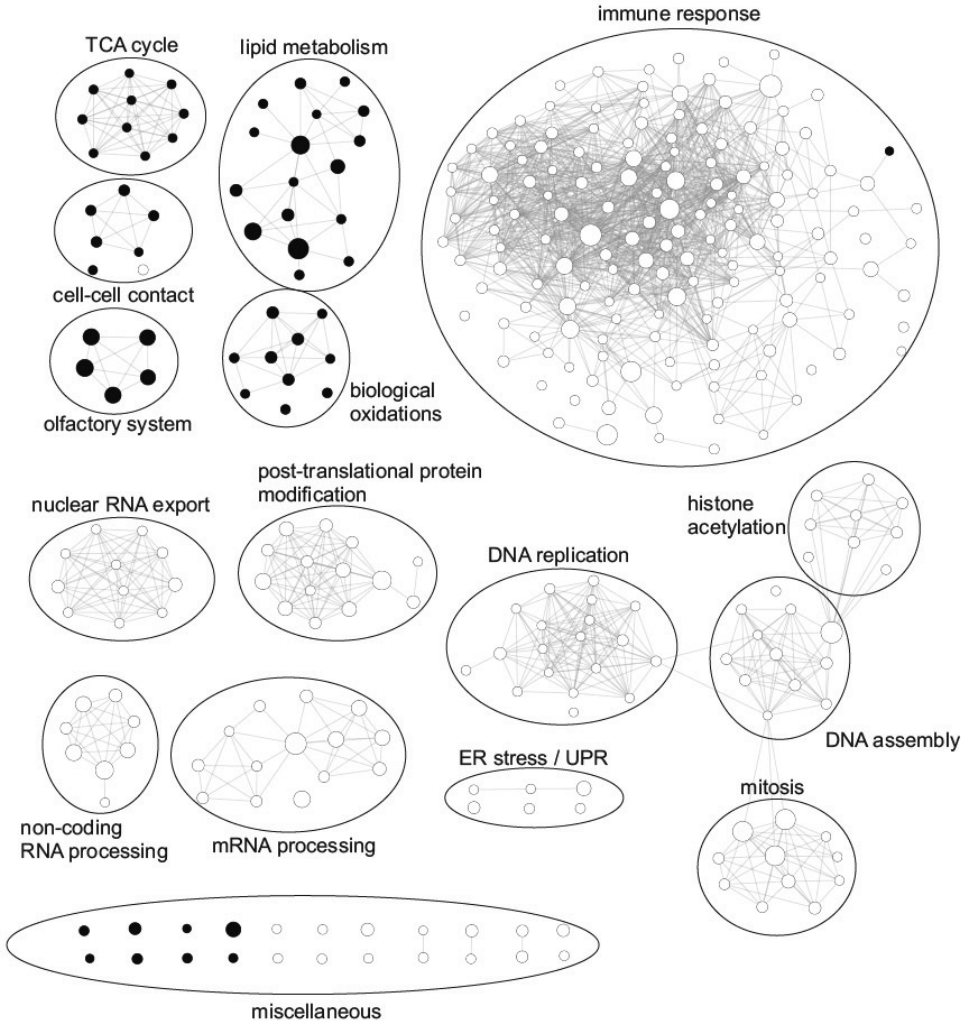

Figure 1. Enrichment map for effects of resistant starch on colonic gene expression. The map displays the enriched gene-sets in proximal colon after 2 wk consumption of the RS diet compared to 2 wk consumption of the DS diet. Nodes represent functional gene sets, and edges between nodes their similarity. Black indicates enrichment after RS consumption (i.e. induction after RS feeding), whereas white represents enrichment after DS consumption (i.e. reduction after RS feeding). Node size represents the gene set size, and edge thickness represents the degree of overlap between 2 connected gene sets. Clusters are manually grouped and labeled to highlight the

prevalent biological functions among related gene sets. See supplemental Fig. 2 for a high-resolution color version of the map that includes the names of the gene sets.

Table 1. Upstream regulators.[1]

Upstream Regulator	Molecular Type	Predicted activation state	Activation Z-score[2]	P value of overlap[3]
PPARG	Ligand-dependent nuclear receptor	Activated	3.069	3.10×10^{-4}
ERG	Transcription regulator	Activated	2.828	1.33×10^{-2}
XBP1	Transcription regulator	Inhibited	-2.041	3.96×10^{-2}

[1] Determined by Ingenuity Systems Pathway Analysis software. ERG, v-ets avian erythroblastosis virus E26 oncogene homolog; PPARG, peroxisome proliferator-activated receptor γ; XBP1, X-box binding protein 1.

[2] The activation Z-score predicts the activation state of the upstream regulator by using the gene expression pattern of its downstream genes in the data set and takes into account the consistency of target gene activation. Upstream regulators with Z-scores ≥ 2 were considered to be activated, if the Z-score was ≤ -2, the upstream regulator was considered to be inhibited.

[3] The overlap P value measures the significance of overlap between the data set genes and the genes reported to be regulated by the transcriptional activator.

Table 2. PPARG target genes[1].

Name	Gene Symbol	Prediction of activation state[2]	Observed mean log2 fold-change[3]	Literature Findings[4]
Transforming growth factor, beta receptor 1	*TGFBR1*	Activated	-0.25	Down
Chemokine (C-X-C motif) ligand 14	*CXCL14*	Activated	-0.26	Down
Carbonic anhydrase II	*CA2*	Activated	1.36	Up
Angiopoietin-like 4	*ANGPTL4*	Activated	0.58	Up
3-hydroxy-3-methylglutaryl-CoA synthase 2 (mitochondrial)	*HMGCS2*	Activated	0.49	Up
Uncoupling protein 3 (mitochondrial, proton carrier)	*UCP3*	Activated	0.39	Up
Caveolin 1, caveolae protein, 22kDa	*CAV1*	Activated	0.38	Up
Vascular endothelial growth factor A	*VEGFA*	Activated	0.37	Up
Peroxisome proliferator-activated receptor gamma	*PPARG*	Activated	0.33	Up
Solute carrier family 25 (carnitine/acylcarnitine translocase), member 20	*SLC25A20*	Activated	0.32	Up
3-hydroxybutyrate dehydrogenase, type 1	*BDH1*	Activated	0.31	Up
Lipase, hormone-sensitive	*LIPE*	Activated	0.27	Up
Diacylglycerol O-acyltransferase 1	*DGAT1*	Activated	0.25	Up
Monoglyceride lipase	*MGLL*	Activated	0.23	Up
Acyl-CoA dehydrogenase, C-4 to C-12 straight chain	*ACADM*	Activated	0.22	Up
Phosphodiesterase 3B, cGMP-inhibited	*PDE3B*	Inhibited	-0.45	Up
Serpin peptidase inhibitor, clade A, member 1	*SERPINA1*	Inhibited	-0.6	Up
Angiotensinogen (serpin peptidase inhibitor, clade A, member 8)	*AGT*	-	0.87	Regulates
Insulin-like growth factor 1 (somatomedin C)	*IGF1*	-	0.62	Regulates
Glutamic-pyruvate transaminase (alanine aminotransferase)	*GPT*	-	0.45	Regulates
Protein tyrosine phosphatase, receptor type, F	*PTPRF*	-	0.27	Regulates

[1] Determined by Ingenuity Systems Pathway Analysis software. PPARG, peroxisome proliferator-activated receptor γ.
[2] Column indicates the predicted activation state of PPARG (either activated or inhibited) based on the direction of the gene expression change in the uploaded data set.
[3] Mean $\log_2$ fold-change of the signal intensity of resistant starch compared with digestible starch.
[4] Column indicates whether literature findings support the prediction. ‘‘Down’’ and ‘‘Up’’ indicate whether the literature suggests a down- or an upregulation of the target gene by PPARG, respectively. ‘‘Regulates’’ indicates that there is insufficient support from the literature that the target gene is either up- or downregulated by PPARG.

Microbiota analysis

To take full advantage of this study, we also determined microbiota composition in the luminal content of the pCO using second generation PITChip. RS did not significantly change the microbial diversity as indicated by Shannon's index for diversity (data not shown). Principal response curves analysis showed that diet (DS or RS) explained 15.7% of the total variation in microbiota, while period and the interaction of period and diet only explained 4.8% and 6.8% respectively (data not shown). This indicated that diet was the main factor driving the microbial variation. Partial RDA of the PITChip data confirmed that diet had a significant effect on microbial variation ($P < 0.05$) (**Fig. 2**).

At the phylum level, we observed a significant increase in the relative abundance of Bacteroidetes ($P = 0.036$) (**Supplemental Fig. 4A)**. As a result, we found a significantly lower Firmicutes/Bacteroidetes ratio ($P = 0.017$) in RS-fed pigs (10.3 ± 2.0) compared to DS-fed pigs (20.1 ± 3.4) (**Supplemental Fig. 4B**).

At the approximate genus level, the relative abundance of several groups that include known butyrate-producing bacteria, including those related to *Faecalibacterium prausnitzii* and *Megasphaera elsdenii*, was increased upon RS feeding compared to DS feeding ($P < 0.05$) (**Table 3**). Other groups that increased in relative abundance comprised *Prevotella melaninogenica*-like, uncultured *Prevotella*, *Enterococcus*-like, *Dialister*-like, *Mitsuokella multacida*-like and *Clostridium ramosum*-like bacteria. On the other hand, *Eggerthella*-like, uncultured *Bacteroidetes*, *Turicibacter*-like, *Clostridium perfringens*-like, *Anaerovorax*-like, *Peptoniphilus*-like, uncultured *Clostridia* XIVa, uncultured *Planctomycetacia*, as well as a range of potentially pathogenic microbial groups, including *Leptospira*, and several facultative bacterial groups within the Proteobacteria, including *Sphingomonas*-like, uncultured *Betaproteobacteria*, *Actinobacillus indolicus*-like, *Aeromonas*, *Alishewanel*-like, *Halomonas*-like, *Pasteurella*-like, *Pseudomonas*-like, *Psychrobacter*-like, *Ruminobacter amylophilus*-like, *Vibrio*-like, and *Xanthomonas*-like, were found to be decreased on the RS diet ($P < 0.05$) (**Table 3**).

SCFA concentrations

Total SCFA concentration in the luminal content of pCO, collected 30 min before and 300 min after feeding, was not affected by dietary treatment (**Supplemental Fig. 5A**). However, the percentage of branched-chain fatty acids (BCFAs), i.e. isobutyrate and isovalerate, of total SCFAs was significantly lower in pigs fed RS (**Supplemental Fig. 5B**). Acetate, propionate, and butyrate concentrations determined in carotid plasma collected 300 min after feeding were significantly higher with RS consumption compared to DS consumption (**Fig. 3**).

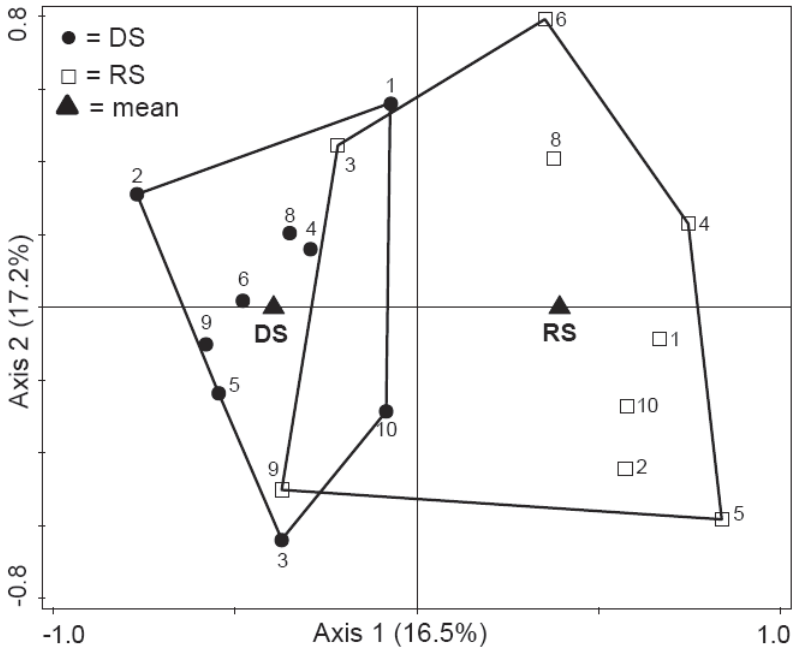

Figure 2. Biplot of partial RDA results, representing the principal component analysis of the microbiota composition as measured by the mean hybridization signals for 151 genus-level phylogenetic groups in the luminal content of proximal colon of pigs fed the DS or RS diet for 2 wk. Samples are grouped by diet. Each symbol represents 1 pig, with numbers indicating pig identifiers. The mean of the nominal environmental variables DS and RS are represented by triangles. DS, digestible starch; RS, resistant starch.

Table 3. Phylogenetic groups in the luminal content of proximal colon of pigs fed DS or RS diet for 2 wk, which were significantly affected by diet according to univariate analysis of PITChip data.

Phylogenetic group	*P* value	FDR	ARC[2]		Effect[3] (RS-DS)
			DS	RS	
Actinobacteria					
Actinobacteria					
Eggerthella et rel.	0.008	0.08	0.109 ± 0.050	0.033 ± 0.043	-
Microbacterium et rel.	0.055	0.22	0.586 ± 0.100	0.709 ± 0.193	+
Bacteroidetes					
Bacteroidetes					
Parabacteroides distasonis et rel.	0.055	0.22	0.411 ± 0.278	0.622 ± 0.278	+
Prevotella melaninogenica et rel.	0.012	0.09	0.056 ± 0.055	3.079 ± 3.194	+
Uncultured *Bacteroidetes*	0.039	0.20	0.605 ± 0.934	0.080 ± 0.241	-
Uncultured *Prevotella*	0.008	0.08	0.836 ± 0.531	2.501 ± 1.679	+
Firmicutes					
Bacilli					
Enterococcus et rel.	0.039	0.20	2.480 ± 0.576	3.025 ± 0.657	+
Lactobacillus amylovorus et rel.	0.055	0.22	1.094 ± 0.963	0.400 ± 0.458	-
Lactobacillus salivarius et rel.	0.055	0.22	0.926 ± 0.356	1.267 ± 0.426	+
Turicibacter et rel.	0.004	0.08	0.197 ± 0.069	0.062 ± 0.036	-
Clostridium cluster I					
Clostridium perfringens et rel.	0.008	0.08	2.398 ± 1.763	0.403 ± 0.810	-
Clostridium cluster IV					
Faecalibacterium prausnitzii et rel.	0.020	0.12	0.969 ± 0.398	1.925 ± 1.142	+
Uncultured *Clostridia* IV	0.055	0.22	2.404 ± 0.863	1.697 ± 1.005	-
Clostridium cluster IX					
Dialister et rel.	0.004	0.08	0.453 ± 0.248	0.852 ± 0.328	+
Megasphaera elsdenii et rel.	0.008	0.08	0.850 ± 0.210	1.386 ± 0.600	+
Mitsuokella multacida et rel.	0.008	0.08	1.084 ± 0.301	1.776 ± 0.743	+
Clostridium cluster XI					
Anaerovorax et rel.	0.004	0.08	1.811 ± 0.681	0.495 ± 0.973	-
Clostridium cluster XIII					
Peptoniphilus et rel.	0.008	0.08	0.601 ± 0.350	0.176 ± 0.364	-
Clostridium cluster XIVa					
Uncultured *Clostridia* XIVa	0.039	0.20	2.412 ± 0.828	1.657 ± 0.928	-
Clostridium cluster XVIII					
Clostridium ramosum et rel.	0.008	0.08	0.213 ± 0.057	0.296 ± 0.101	+
Planctomycetes					
Planctomycetacia					
Uncultured *Planctomycetacia*	0.012	0.09	0.159 ± 0.114	0.037 ± 0.048	-
Proteobacteria					
Alphaproteobacteria					
Rhizobium et rel.	0.055	0.22	0.552 ± 0.085	0.382 ± 0.153	-
Sphingomonas et rel.	0.027	0.17	0.214 ± 0.203	0.059 ± 0.154	-
Betaproteobacteria					
Uncultured *Betaproteobacteria*	0.012	0.09	0.329 ± 0.046	0.246 ± 0.066	-
Gammaproteobacteria					
Actinobacillus indolicus et rel.	0.008	0.08	0.332 ± 0.058	0.227 ± 0.079	-
Aeromonas	0.020	0.12	0.671 ± 0.147	0.389 ± 0.268	-
Alishewanella et rel.	0.020	0.12	0.472 ± 0.093	0.255 ± 0.176	-
Escherichia coli et rel.	0.055	0.22	0.397 ± 0.190	0.166 ± 0.196	-
Halomonas et rel.	0.004	0.08	0.747 ± 0.204	0.448 ± 0.225	-
Pasteurella et rel.	0.012	0.09	0.145 ± 0.041	0.093 ± 0.037	-
Pseudomonas et rel.	0.008	0.08	0.697 ± 0.253	0.279 ± 0.340	-
Psychrobacter et rel.	0.008	0.08	0.629 ± 0.126	0.408 ± 0.173	-
Ruminobacter amylophilus et rel.	0.039	0.20	0.211 ± 0.022	0.153 ± 0.057	-
Thiocapsa et rel.	0.055	0.22	0.675 ± 0.163	0.450 ± 0.202	-
Vibrio et rel.	0.008	0.08	0.690 ± 0.149	0.438 ± 0.207	-
Xanthomonas et rel.	0.020	0.12	0.852 ± 0.063	0.682 ± 0.140	-
Spirochaetes					
Spirochaetes					
Leptospira	0.012	0.09	0.717 ± 0.172	0.402 ± 0.208	-

[1] ARC, average relative contribution; DS, digestible starch; et rel., and related species; FDR, false-discovery rate; PITChip, Pig Intestinal Tract Chip; RS, resistant starch.
[2] ARC is the mean relative contribution (%) of a microbial group. Values are means±SDs, n = 9/treatment.
[3] "+" or "-" indicates whether the mean relative contribution of the microbial group increased or decreased with the RS treatment.

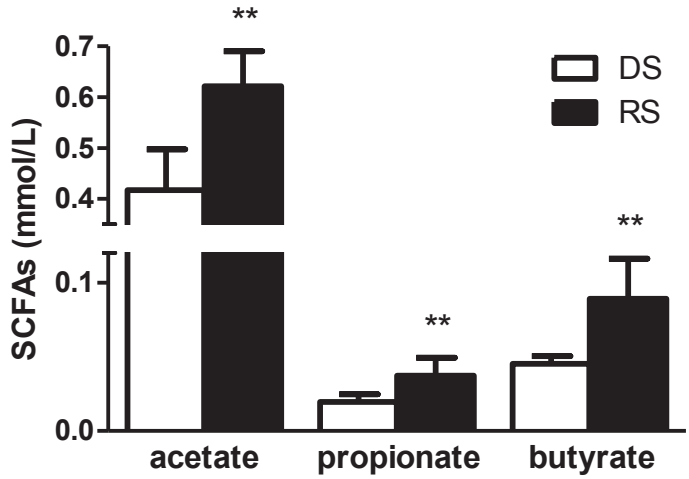

Figure 3. Acetate, propionate, and butyrate concentrations in peripheral plasma of pigs fed the DS or the RS diet for 2 wk. Samples were collected 300 min after feeding. Values are means ± SEM, n = 10 for DS-fed pigs and n = 9 for RS-fed pigs. ** indicates P <0.01. DS, digestible starch; RS, resistant starch.

Discussion

In the present study, we examined the effects of 2-wk-consumption of a diet high in RS on the mucosal transcriptome, and luminal microbiota composition and SCFA concentrations in proximal colon of pigs. Our results suggested that compared to a DS diet, the RS diet shifted colonic gene expression profile of the host from immune regulation and cell division towards oxidative metabolic pathways and reduced the abundance of several potentially pathogenic bacteria in the colonic lumen. In addition, plasma SCFA concentrations increased on the RS diet. The nuclear receptor PPARG was identified as a potential key upstream regulator.

Functional implications in the colon

Genome-wide expression profiling revealed that RS modified many biological pathways. Regarding the metabolic pathways, we observed increased expression in RS-fed pigs of genes involved in fatty acid beta-oxidation and TCA cycle, two catabolic pathways responsible for generating energy through the oxidization of acetyl-CoA. This is to be expected since SCFAs, and especially butyrate, are known to serve as energy source for colonocytes as precursor to the TCA cycle and electron transport chain [351, 352]. Since the generation of ATP from NADH and succinate results in the formation of reactive oxygen species [353], this may explain the ostensible activation of gene sets describing oxidative processes, including NRF2 antioxidant response pathways, that comprises

induction of *HMOX, NQO1* and *GST* levels [354]. Interestingly, other parts of lipid metabolism, i.e. absorption and metabolism of triglycerides, were also induced with RS feeding. However, because these processes typically occur in small intestine, the functional implications for colon remain to be demonstrated. In contrast, the induction of genes involved in cell-cell contact may be related to the reported improved intestinal barrier function observed upon RS consumption in rodents [329, 355].

The array data also showed that RS suppressed genes involved in both the innate and the adaptive immune response. This implies that the colon of RS-fed pigs is less immuno-active, which we believe is partially due to reduced exposure to potential pathogens, as was substantiated by microbiota analysis of luminal content that showed that especially members of the Proteobacteria were reduced in relative abundance in the RS group. Alternatively, this suppression may be directly due to the increase in SCFA concentrations [356-358]. In any case, this observation is in line with previous studies on RS in relation to immune regulation, showing amelioration of inflammatory bowel disease upon RS feeding in rodents [330, 355] and reduction of colonic and systemic immune reactivity in pigs [359]. However, it should also be kept in mind that immune signaling may affect microbiota composition, as was shown by altered gut microbiota in mice lacking toll-like receptor 5, an essential protein for pathogen recognition and activation of innate immunity [360]. Other large clusters of gene sets suppressed by RS functionally represent processes related to DNA replication and assembly, histone modification, mitosis, RNA processing and post-translational protein modification, i.e. the full machinery of gene transcription and translation. Suppression of these gene sets are in line with observations in pigs that RS inhibits cell growth and proliferation of mucosal epithelium and intraepithelial immune cells [359].

Our analysis identified three potential upstream regulators that may mediate the effects of RS on gene expression. PPARG and ERG were predicted to be activated by RS, whereas XBP1 was predicted to be inhibited. PPARG is a member of the peroxisome proliferator-activated receptor (PPAR) subfamily of nuclear receptors, and is well expressed in colon [361]. PPARG has been implicated in the pathology of numerous diseases, including the inhibition of inflammatory bowel disease in mice [362], pigs [363], and humans [364]. Moreover, PPARG activation suppresses the activity of NF-κB, thereby blocking pro-inflammatory gene transcription [365]. Since PPARG has recently been shown to be activated by SCFAs, especially butyrate [366], this provides strong evidence for a molecular mechanism by which RS may modulate colonic gene expression. ERG is a member of the erythroblast transformation-specific (ETS) family of transcriptions factors. All members of this family are key regulators of cell proliferation, differentiation, angiogenesis, inflammation, and apoptosis [367]. The physiological function of ERG is largely unknown, but it has been demonstrated to play a role in the specification of

lymphocytes to the T lineage [368]. Our data point to a role for ERG in the suppression of the T-cell mediated adaptive immune response by RS. XBP1 is a transcription factor known to regulate multiple processes. XBP1 is required for the transcription of class II major histocompatibility genes and the differentiation of immunoglobulin secreting plasma B cells [361]. More recently, XBP1 has been shown to have an important function in the unfolded protein response (UPR), a process that intersects with many different inflammatory and stress signaling pathways, including the NF-κB pathway [361, 369]. Importantly, the UPR is required for maintenance of normal epithelial function, and disturbances in the UPR have been linked to the etiology of IBD and other intestinal inflammatory disorders [370]. The results of our study hint to a reduced UPR with RS feeding, which may have important physiological and immunological implications. The exact way RS feeding realized this remains to be investigated.

Microbiota composition

Evidence suggesting that the gut microbiome is highly important in the regulation of energy homeostasis and fat storage is accumulating [343, 371]. Supporting this view, the Firmicutes/Bacteroidetes ratio has been linked to adiposity in humans, because obese individuals were found to have fewer Bacteroidetes compared to lean controls. In addition, the relative abundance of Bacteroidetes increased while the abundance of Firmicutes decreased with weight loss in obese participants [215]. Because we found a significantly lower Firmicutes/Bacteroidetes ratio in RS-fed pigs compared to DS-fed pigs, the microbial profile might have shifted towards a more healthy phenotype in RS-fed pigs. Although the relative abundance of Firmicutes slightly, albeit not significantly, decreased (**Supplementary Fig. 2**), we observed a significant increase in the relative abundance of several microbial groups previously shown to produce butyrate, including *Faecalibacterium prausnitzii* and *Megasphaera elsdenii* [308, 344, 345]. Other microbial populations stimulated by RS included fermenting microorganisms such as members of the *Parabacteroides, Prevotella, Mitsuokella multacida*, and lactic acid bacteria, that produce organic acids such as acetate, lactate, and succinate that are in turn used as main substrates for the production of propionate and butyrate [308]. Furthermore, the RS diet also led to the decrease in relative abundance of several potentially pathogenic microorganisms, including members of the genus *Leptospira* [350], as well as a range of facultative anaerobes within the phylum of Proteobacteria, including for example *Escherichia coli*, *Pseudomonas* spp., *Actinobacillus* spp. [372] and *Pasteurella* spp. [373]. Thus, despite major differences between our previous study and the current study regarding, among others, pigs' sex (gilts vs. barrows) , age (22 vs. 5 mo) and body weight (270 vs. 60 kg), the microbial changes were very similar to what we observed before [101], demonstrating the consistent modulation of the microbiota by RS .

Fermentation products

In contrast with our previous study in pigs consuming the same RS diet , in the current study no significant difference in SCFA concentration was observed in the pCO digesta 300 min after feeding. However, the reduction in percentage of BCFAs in colonic digesta confirms the applied dietary contrast. These branched-chain products are formed by amino acid fermenting microbial species that metabolize undigested and endogenous proteins, peptides, and amino acids [374], particularly when carbohydrates as preferential energy source are absent. The lower percentage of BCFAs thus reflects the use of RS as energy source by microbiota whereas in the DS diet the microbiota used proteinaceous energy sources due to the digestion and fermentation of starch in the upper gastrointestinal tract and the cecum. Moreover, plasma SCFA concentrations are known to be a more reliable measure of colonic SCFA production [375]. As expected, we observed increased plasma SCFA concentrations upon RS feeding.

Conclusion

We demonstrated that compared with an iso-caloric DS diet, a diet high in RS provoked major changes in colonic gene expression, that represent induction of oxidative metabolic pathways, and suppression of immune response and cell division pathways. We also showed that RS favored the growth of microbial populations producing organic acids, and inhibited a range of potentially pathogenic microbial groups. Our results provide a comprehensive overview on effects of RS in colon, that emphasize the resilience of the colon and contributes to our understanding of a healthy diet. Because pigs are known to be a good model for humans, our study outcomes are highly relevant to human health.

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Supplemental methods

Surgery

After an overnight fast, all pigs were sedated with intramuscular Ketamine 10 mg/kg (Ketamine; Alfasan) and Midazolam 0.75 mg/kg (Dormicum; Roche) and anesthesia was intravenously induced with the anodyne Sufentanil 1 µg/kg (Sufenta; Janssen-Cilag). Pigs were intubated and anaesthesia was maintained by inhalation of 2% Sevoflurane (Abbott) combined with 40% oxygen and nitrous oxide. A Sufentanil infusion was maintained at 1 µg/(kg·h).

The first 3 d after surgery, pigs were fed a restricted amount of the 50:50 mix of the DS and RS diet, i.e. 25%, 50% and 75% of their daily feed allowance on d 1, 2 and 3 respectively, to allow a gradual recovery and to avoid problems with the gut cannula. Pigs were habituated to digesta collection from the cannula and blood sampling in the first week after surgery. After 4 to 6 d of postsurgical recovery, pigs were gradually switched to one of two dietary treatments (DS and RS).

Colon biopsies

Pigs were acutely sedated by i.v. injection with propofol (Alfasan). The stopper was unscrewed from the cannula and digesta was removed to expose the mucosal wall of the pCO. An endoscope (OES Colonofiberscope, Olympus CF type ITIOL/1, Olympus Optical Co; LTD) was inserted via the permanent cannula into the lumen of the pCO, the intestinal wall was illuminated (OES Halogen light source with flash, Model CLE-F10, Olympus Optical Co; LTD) and visualized on a monitor (Endovision 538; Karl Storz). Biopsies were taken by an endoscopic biopsy forceps (Olympus FB-28U-1, 2 mm, 225 cm, Olympus Optical Co; LTD) and collected in screw cap tubes, after which they were immediately frozen in liquid nitrogen and stored at -80°C until further analysis.

Digesta collection

To determine microbiota composition, digesta were collected in 1.5 mL Eppendorf tubes, after which the tubes were immediately frozen in liquid nitrogen and stored at -80°C until further analyses. In addition, digesta was collected in pre-weighed 2 mL Eppendorf tubes with 0.75 mL H3PO4 for determining SCFA concentrations. These tubes were weighed again, thoroughly mixed on a vortex and stored at -20°C until further analysis. For measuring dry matter content, digesta were collected in empty pre-weighed Eppendorf tubes and stored at -20°C until further analysis.

RNA quality control

Concentrations and purity of colonic RNA samples were determined on a NanoDrop ND-1000 spectrophotometer (Isogen Life Science). RNA quality was verified on an Agilent 2100 Bioanalyzer (Agilent Technologies) by using 6000 Nano Chips (Agilent Technologies) according to the manufacturer's instructions. RNA was judged as suitable for array hybridization only if samples exhibited intact bands corresponding to the 18S and 28S ribosomal RNA subunits, and displayed no chromosomal peaks or RNA degradation products (RNA Integrity Number >8.0).

GSEA analysis

GSEA was used to find enriched gene sets in the induced or suppressed genes. Genes were ranked based on the paired IBMT-statistic and subsequently analyzed for over- or underrepresentation in predefined gene sets derived from Gene Ontology, KEGG, National Cancer Institute, PFAM, Biocarta, Reactome and WikiPathways pathway databases. Only gene sets consisting of more than 15 and fewer than 500 genes were taken into account. Statistical significance of GSEA results was determined using 1,000 permutations.

Enrichment map

Results from GSEA were displayed in an enrichment map. Only gene sets passing conservative significance thresholds (p-value <0.001, False Discovery Rate (FDR) <2%) were selected for visualization in the enrichment map, resulting in 329 total gene sets (out of 4,293) significantly enriched after RS feeding (57 induced and 272 suppressed gene sets, respectively). The cutoff for the overlap coefficient was set at 0.5

Multivariate analysis microbiota

To relate changes in total bacterial community composition to diet (DS or RS), period, and the interaction of period and diet, redundancy analysis (RDA) and Principal response curves were used as implemented in the CANOCO 5 software package (Biometris, Wageningen, the Netherlands). RDA is the canonical form of principle component analysis and is a multivariate linear regression method where several response parameters are related to the same set of environmental (explanatory) variables. The signal intensities for 151 genus-level phylogenetic groups of PITChip were used as responsive variables. Partial RDA was employed to analyze the effect of diet on microbiota. RDA was performed by centering the species and samples, using freely exchangeable whole-plot permutations.

SCFA determination by NMR spectroscopy

Plasma samples were diluted 1:1 in a 75 mM phosphate buffer (pH 7.4) and filtered using Nanosep centrifugal devices with Omega Membrane (Pall Corporation). The molecular weight cut-off of the filter was 10K. Subsequently, 200 μL of the eluate was transferred to a 3 mm NMR tube (Bruker match system). Samples were stored at -20 ^{0}C until analysis using NMR spectroscopy. Before NMR measurements, samples were slowly warmed up to room temperature and measured at 310 K (calibrated temperature) in an Avance III NMR spectrometer operated at 600.13 MHz. After transfer of each sample into the magnet, the sample was equilibrated at 310 K for 5 min. Subsequently automated locking, automated shimming and automated 90 degree pulse angle determination was performed. 1H NMR NOESY datasets were acquired for each sample. In addition, each dataset was automatically processed and aligned using the alanine signal (upfield resonance of the alanine doublet signal) at 1.49 ppm. From the aligned spectra, integrals for resonances of the metabolites of interest were selected and quantified. Concentrations of metabolites were calculated based on the number of hydrogens for each metabolite selected.

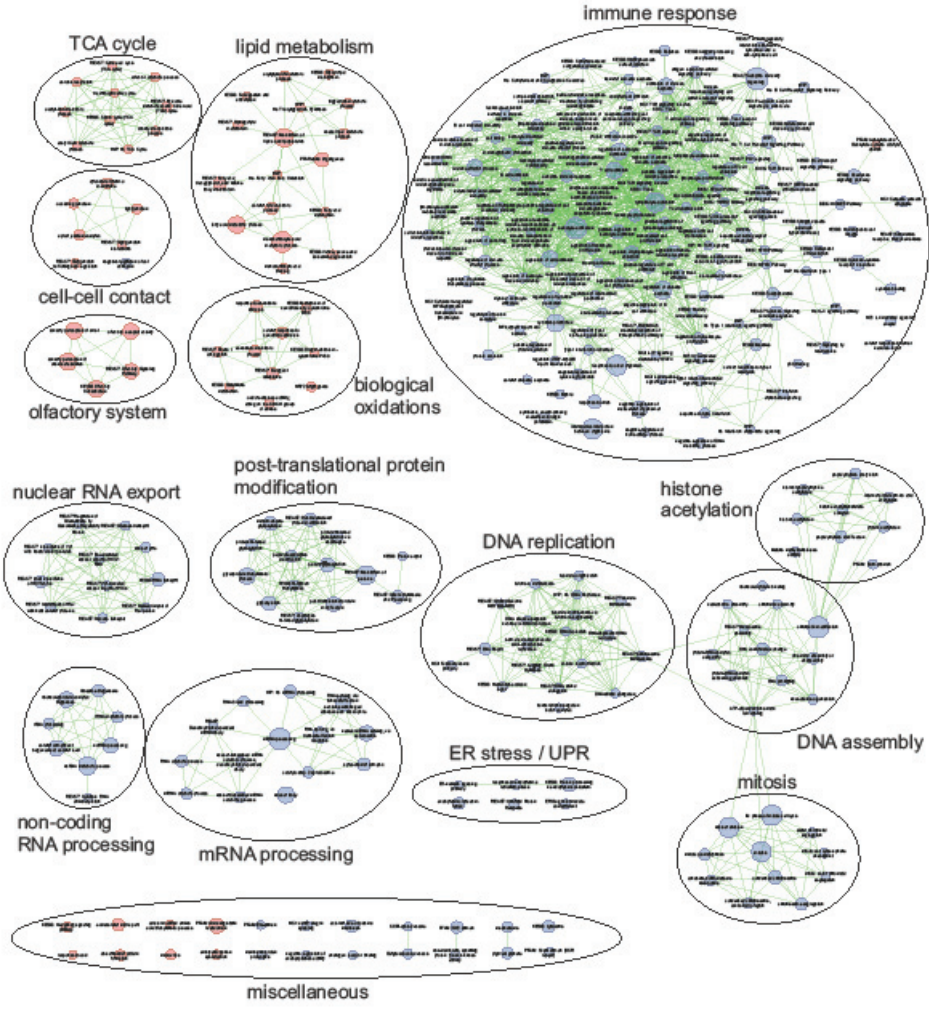
Supplemental tables and figures

Top induced genes				Individual log^2 FC
Gene Description	**Gene symbol**	**Entrez ID**	**Mean log^2 FC**	**1 2 3 4 5 6 7 8 9 10**
Intestinal-type alkaline phosphatase-like	LOC100521756	100521756	1.55	
Carbonic anhydrase II	CA2	100154873	1.35	
Uncharacterized LOC100621113	LOC100621113	100621113	1.01	
Hydroxysteroid (17-beta) dehydrogenase 2	HSD17B2	100312973	1.00	
Transmembrane 4 L6 family member 20-like	LOC100513630	100513630	0.96	
Uncharacterized protein C5orf4 homolog	LOC100525263	100525263	0.95	
Solute carrier family 30, member 10	SLC30A10	100623097	0.92	
Angiotensinogen-like	LOC100157073	100157073	0.86	
Carbonic anhydrase 12-like	LOC100152749	100152749	0.83	
Carcinoembryonic antigen-related cell adhesion molecule 7-like	LOC100524810	100524810	0.81	
ATP-binding cassette, sub-family A (ABC1), member 6	ABCA6	100520861	0.79	
Agmatinase, mitochondrial-like	LOC100519548	100519548	0.74	
ADAM metallopeptidase domain 23	ADAM23	100518044	0.74	
Claudin 10	CLDN10	100153752	0.73	
Thiopurine S-methyltransferase	TPMT	100157630	0.72	
Ubiquitin carboxyl-terminal hydrolase 2-like	LOC100520041	100520041	0.72	
Transient receptor potential cation channel subfamily M member 6-like	LOC100157775	100157775	0.71	
Transmembrane protein 117	TMEM117	100524623	0.71	
Poly (ADP-ribose) polymerase family, member 15	PARP15	100520273	0.70	
Serine palmitoyltransferase, long chain base subunit 3	SPTLC3	100519280	0.68	
Collagen alpha-6(VI) chain-like	LOC100516642	100516642	0.67	
Guanylate cyclase activator 2A (guanylin)	GUCA2A	100301560	0.66	
EGF containing fibulin-like extracellular matrix protein 1	EFEMP1	100512046	0.66	
Pancreatic lipase-related protein 2	PNLIPRP2	100462755	0.66	
Olfactory receptor 9K2-like	LOC100737562	100737562	0.66	

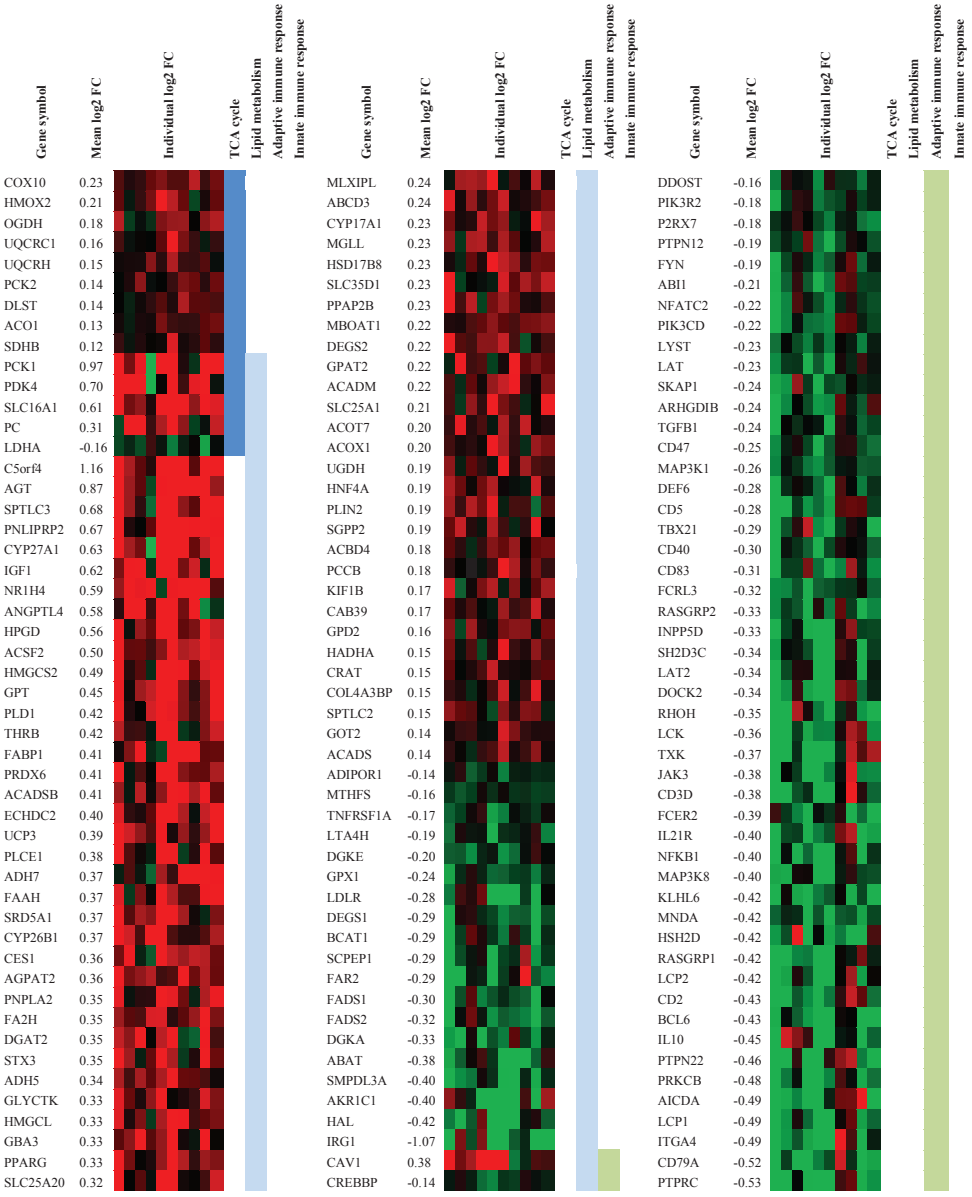
Top suppressed genes				Individual log^2 FC
Gene Description	**Gene Symbol**	**Entrez ID**	**Mean log^2 FC**	**1 2 3 4 5 6 7 8 9 10**
Chitinase 3-like 1 (cartilage glycoprotein-39)	CHI3L1	396865	-1.88	
C4b-binding protein alpha chain-like	LOC100520761	100520761	-1.42	
Secretory leukocyte peptidase inhibitor	SLPI	396886	-1.13	
C-type lectin domain family 7, member A	CLEC7A	100038025	-0.94	
Immunoresponsive 1 homolog (mouse)	IRG1	100524951	-0.93	
Chemokine (C-X-C motif) receptor 4	CXCR4	396659	-0.89	
P2Y purinoceptor 13-like	LOC100524766	100524766	-0.86	
BPI fold containing family B, member 2	BPIFB2	100113424	-0.84	
Tryptophan hydroxylase 1	TPH1	100511002	-0.80	
Chromosome 1 open reading frame 162 ortholog	C4H1orf162	100627962	-0.78	
SLAM family member 7-like	LOC100154053	100154053	-0.78	
Placenta-specific gene 8 protein-like	LOC100525175	100525175	-0.78	
Antileukoproteinase-like	LOC100512873	100512873	-0.74	
Rho GTPase activating protein 15	ARHGAP15	100520808	-0.74	
Monocarboxylate transporter 7-like	LOC100739042	100739042	-0.71	
Lymphoid enhancer-binding factor 1	LEF1	100170126	-0.70	
Transmembrane protein 156-like	LOC100525349	100525349	-0.69	
Membrane-spanning 4-domains, subfamily A, member 1	MS4A1	100627952	-0.68	
SLAM family member 6	SLAMF6	100156912	-0.67	
Sorting nexin-10-like	LOC100520876	100520876	-0.66	
Acyloxyacyl hydrolase (neutrophil)	AOAH	100522290	-0.64	
Clusterin	CLU	397025	-0.63	
CD1B antigen	PCD1B	100038007	-0.62	
Interleukin 2 receptor, gamma	IL2RG	397156	-0.60	
A-kinase anchor protein 5-like	LOC100153460	100153460	-0.60	

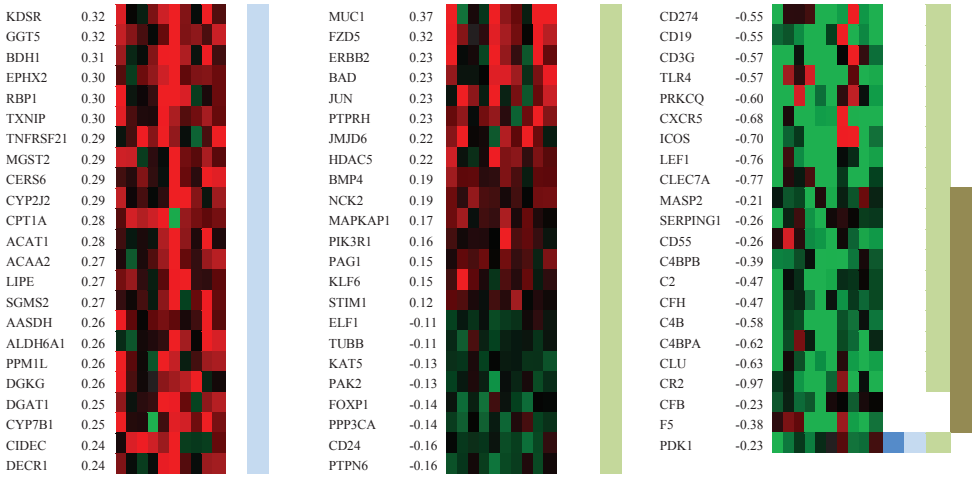
Supplemental Figure 1. Top 25 induced genes and bottom 25 suppressed genes observed in pigs after 2 wk consumption of the RS diet compared to 2 wk consumption of the DS diet, based on the gene definitions of the

NCBI Sus scrofa Entrez Gene database, build 4.1. Mean log2 fold changes of the signal intensity of RS compared with DS were determined from the individual response of the 10 pigs, which are expressed as a heatmap. The intensity of the red and green color indicates the degree of induction or suppression per pig, respectively.

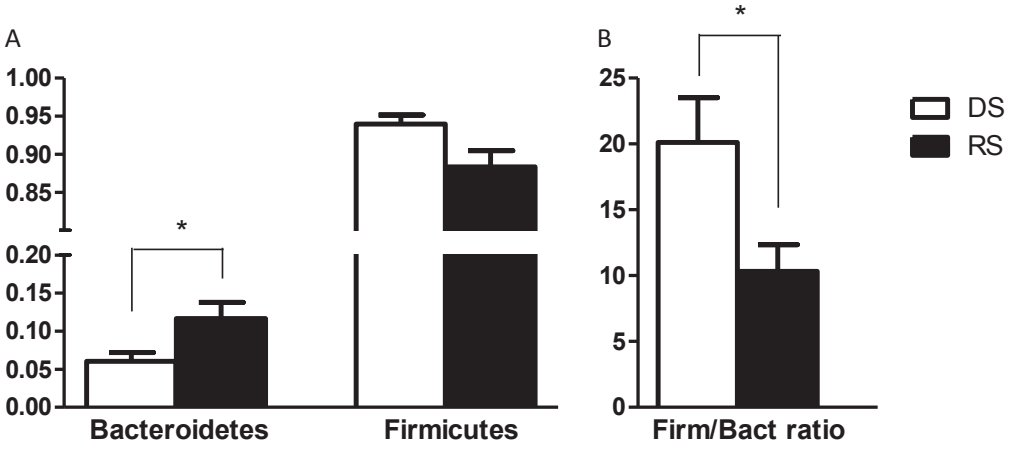


Supplemental Figure 2. High resolution color enrichment map for effects of resistant starch on colonic gene expression. The map displays the enriched gene-sets in proximal colon after 2 wk consumption of the RS diet compared to 2 wk consumption of the DS diet. Nodes represent functional gene sets, and edges between nodes their similarity. Red node color indicates enrichment after RS consumption (i.e. induction after RS feeding), whereas blue represents enrichment after DS consumption (i.e. reduction after RS feeding). Node size represents the gene set size, and edge thickness represents the degree of overlap between 2 connected gene sets. Clusters are manually grouped and labeled to highlight the prevalent biological functions among related gene sets. The abbreviation before the name of the gene set indicates the database the gene set was derived from: no abbreviation: Gene Ontology; KEGG: Kyoto Encyclopedia of Genes and Genomes; BIOC: Biocarta; REACT: NCI: National Cancer Institute; Reactome, WiP: WikiPathways, PFAM: protein family.

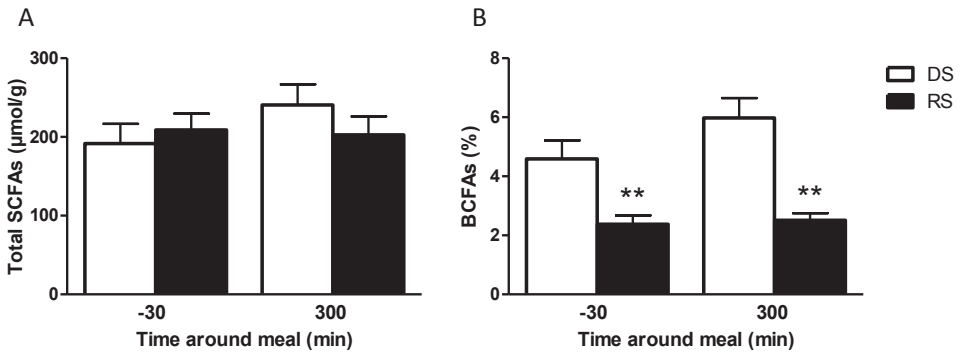




Supplemental Figure 3. Heatmap of significantly regulated genes (P <0.05) present in the positively enriched gene sets describing TCA cycle or lipid metabolism, or present in the negatively enriched gene sets describing processes involved in adaptive or innate immune response. Mean and individual log2 fold changes are presented. The intensity of the red and green color indicates the degree of induction or suppression per pig, respectively, and ranged from -0.65 (green) to 0.65 (red). Pigs are ranked based on their identifier. The vertical color bars indicate gene set membership; dark blue: TCA cycle, light blue: lipid metabolism, olive green: adaptive immune response, tan: innate immune response.



Supplemental Figure 4. The abundance of the phyla Bacteroidetes and Firmicutes (**A**) and the ratio Firmicutes/Bacteroidetes (**B**) in pigs fed DS or RS for 2 wk, as determined by PITChip. Data are presented as means ± SEM, $n = 9$ per treatment. * indicates $P < 0.05$. DS, digestible starch; RS, resistant starch.



Supplemental Figure 5. Total SCFAs (**A**) and percentage of BCFAs from total SCFAs (**B**) measured in luminal content from proximal colon, collected 30 min before feeding (-30) and 300 min after feeding in pigs fed the DS and the RS diet. Data are presented as means ± SEM, n = 10 per treatment. ** indicates P <0.01. BCFAs, branched-chain fatty acids; DS, digestible starch; RS, resistant starch.

Supplemental Table 1. Ingredient and nutrient composition of the experimental diets.

	DS	RS
Ingredient composition (g/kg)		
Pregelatinized purified potato starch[1]	**350.0**	0.0
Retrograded tapioca starch[2]	0.0	**342.6**
Soy oil	29.2	29.5
Wheat	200.0	202.3
Beet pulp (sugar<100 g/kg)	50.0	50.6
Barley	150.0	151.7
Wheat gluten meal	60.0	60.7
Potato protein[3]	100.0	101.1
Premix[4]	10.0	10.1
$CaCO_3$	13.5	13.7
$Ca(H_2PO_4)_2$	11.0	11.1
NaCl	3.0	3.0
L-lysine HCl	2.2	2.2
L-tryptophan	0.2	0.2
MgO (80%)	0.4	0.4
$NaHCO_3$	14.0	14.2
KCl	3.0	3.0
TiO_2	2.0	2.0
Flavor[5]	1.5	1.5
Nutrient (g/kg dry matter)		
Dry matter (g/kg)	894.5	910.0
Organic matter	941.4	941.9
Crude protein (N x 6.25)	190.9	194.3
Crude fat	16.1	28.6
Starch	524.7	477.1
Sugar	13.1	69.4
TiO_2	1.6	1.6
Energy content (MJ/kg)		
GE	16.48	16.78

DS, digestible starch diet; RS, resistant starch diet.
[1]PaselliTM WA4, Avebe Food, Veendam, the Netherlands.
[2]C*Actistar 11700, Cargill, Amsterdam, the Netherlands.
[3]Protostar, Avebe Food, Veendam, the Netherlands.
[4]Provided the following per kg of feed: vitamin A: 7500 IU; vitamin D3: 1500 IU; vitamin E: 60 mg; vitamin K3: 1.0 mg; vitamin B1: 1.0 mg; vitamin B2: 4.0 mg; vitamin B6: 1.0 mg; vitamin B12: 20 µg; niacin: 20 mg; calcium-D pantothenate: 10.5 mg; choline chloride: 100 mg; folic acid: 0.4 mg; Fe: 120 mg (FeSO4.H2O); Cu: 15 mg (CuSO4.5H2O); Mn: 60 mg (MnO); Zn: 75 mg (ZnSO4.H2O); I: 4.0 mg (KI); Se: 0.30 mg (Na2SeO3); anti-oxidant: 75 mg.
[5]Luctarom Advance Cherry Honey, Lucta S.A., Barcelona, Spain.

Supplemental Table 2. All significantly regulated genes (P <0.01) observed in pigs after 2 wk consumption of the RS diet compared to 2 wk consumption of the DS diet. See **Appendices**.

Chapter 8

General discussion

Main findings of the research

The central objective of this research was to determine the effects of antibiotic treatment, microbial exposure and diet on the development of intestinal microbiota, focusing on the pig as an important production animal as well as a model for humans. To achieve this objective, a series of experiments were performed both in piglets and adult pigs to determine the impact of maternal antibiotic treatment (**Chapter 3**), early antibiotic administration (**Chapter 4**), and early microbial exposure (**Chapter 5**) on piglets' intestinal microbiota (**Chapter 3, 4 and 5**) and mucosal tissue gene expression (**Chapter 4 and 5**), as well as the effect of a diet high in resistant starch (RS) on microbiota, short-chain fatty acid (SCFA) concentration and host gene expression in the large intestine of adult pigs (**Chapter 6 and 7**). The approaches taken included analysis of the microbiota in intestinal content and faeces by using a phylogenetic microarray specific for pigs, determination of the metabolites in samples taken at various locations in the gut, and in some cases global gene expression of the pig epithelial cells (**Table 1, 2 and 3**).

Table 1. Overview of microbiota analysis in this thesis**.**

Study	Animals	Treatment	Treatment duration	Sample	Sampling time	Method
Chapter 3	Sow	Amoxicillin	10 days before the estimated farrowing date till 21 days after farrowing	Faeces	Start/End of treatment	PITChip 1.0
	Piglet(Offspring)	-	-	Ileal/Colonic content	14/21/28/42 day after birth	
Chapter 4	Piglet	Tulathromycin	At day 4 after birth	Jejunal content	8 day after birth	PITChip 2.0
Chapter 5	Piglet	Group 1: tri-partite microbiota[1]	Day 1 to 4 after birth[2]	Faeces	3/5/7/14/28 day after birth	PITChip 1.0
		Group 2: tri-partite microbiota & Diluted faeces of an adult sow		Jejunal/ileal content	16 day after birth	PITChip 2.0
Chapter 6	Pig (4 month)	RS	14 days	Caecal/colonic content	14 days after RS treatment	PITChip 1.0
Chapter 7	Pig (22 month)	Cross feeding of RS and DS	14 days each	Content from proximal colon	14 days after each treatment	PITChip 2.0

[1]Tri-partite microbiota1, a mixture of *Lactobacillus amylovorus*, *Clostridium glycolicum*, *and Parabacteroides* sp. ASF519
[2] From day 1 to 3 after birth, all piglet received the tri-partite microbiota, at day 4 and 5, piglets of group 1 received a placebo inoculant, piglets of group 2 received diluted faeces of an adult sow

Table 2. Main analysis in this thesis focusing on host gene expression.

Study	Sample	Method
Chapter 4	Mucosal scrapings of jejunum	Porcine Agilent microarray slides, G2519F *Sus scrofa*
	Mucosal scrapings of ileum	
	Blood	
Chapter 5	Mucosal tissue of jejunum	Porcine Agilent microarray slides, G2519F *Sus scrofa*
	Mucosal tissue of ileum	
Chapter 6	Mucosal scraping of proximal colon	qRT-PCR
	Mucosal scraping of Caecum	
	Mucosal scraping of Colon	
Chapter 7	Biopsies in proximal colon	Porcine Genen 1.0ST arrays (Affymetrix)

Table 3. Main analysis focusing on SCFA concentration.

Study	Location	Method
Chapter 6	Distal small intestine	Gas chromatography
	Caecum	
	Colon	
Chapter 7	Distal small intestine	Gas chromatography
	Caecum	
	Proximal colon[1]	
	Colon[2]	
	Peripheral Plasma	NMR spectroscopy

[1]SCFAs measured in luminal content collected from the location of the cannula.
[2]SCFAs measured in luminal content collected at section.

There are three main findings that can be derived from the work described in this thesis (**Fig.1**).

Firstly, administration of antibiotics to either sows or piglets impacted the intestinal microbiota of piglets at early age. In **Chapter 3**, antibiotic treatment of sows during the perinatal period drastically impacted the sows' faecal microbiota, and caused a decrease in relative abundance of bacteria related to the genera *Lactobacillus*, *Staphylococcus* and *Streptococcus*. This maternal antibiotic treatment furthermore influenced the gut microbiota of the piglets, especially that present in the ileum, leading to increased relative abundance of Proteobacteria mainly driven by a stimulation of *Escherichia coli* and *Pseudomonas*

during administration (at day 14 and 21 of age). As described in **Chapter 4,** early antibiotic administration to piglets affected the composition and diversity of gut microbiota and reduced the mucosal tissue expression of host genes associated with a large number of immune-related processes.

Secondly, exposure of newborn piglets to a complex microbiota at early age drastically affected the piglet's intestinal microbiota and mucosal tissue gene expression, when compared to association with a simple tri-partite microbiota composed of *Lactobacillus amylovorus*, *Clostridium glycolicum*, and *Parabacteroides* sp. ASF519. Gene sets linked to immune system development were significantly enriched in the ileum of piglets exposed to complex microbiota (**Chapter 5**). The complex microbial association led to increased relative abundance of microbial groups that are known to have beneficial effects such as members of the genus *Lactobacillus* and butyrate producing members of *Clostridium* clusters, whereas it reduced the relative contribution of potential pathobionts, albeit differently in jejunum and ileum. The complex microbial association furthermore induced enrichment of immune related gene sets in the ileal mucosa, and immune modulatory genes were negatively correlated with the abundance of pathobionts in ileal digesta.

Thirdly, a diet high in resistant starch (RS) modulated the microbiota composition, short chain fatty acid (SCFA) concentration and mucosal tissue gene expression in the large intestine of adult pigs. The RS diet induced the stimulation of health associated butyrate-producing bacteria, whereas potentially pathogenic members were reduced in relative abundance (**Chapter 6 and 7**). In **Chapter 6**, caecal and colonic SCFA concentrations were significantly higher in RS-fed pigs, and caecal expression of genes encoding for monocarboxylate transporter 1 (SLC16A1) and glucagon (GCG) was induced by RS. As reported in **Chapter 7**, concentrations in carotid plasma of acetate, propionate, and butyrate were significantly higher following RS consumption. RS induced oxidative metabolic pathways, whereas many immune response pathways and cell division were suppressed.

Effect of antibiotics on the development of intestinal microbiota at early age

Antibiotics are commonly used to control bacterial infection of humans and animals in hospitals and on farms. Administration of antibiotics can strongly affect the composition of the intestinal microbiota of both adult pigs (**Chapter 3**) and piglets (**Chapter 4**). **In Chapter 3**, when amoxicillin was administrated to the sows, it inhibited bacteria related to *L.acidophilus*, *L. delbrueckii*, *L. gasseri*, *L. plantarum*, *S. aureus*, *S. bovis*, *S. intermedius* and *S.suis*. This result is in line with previous reports showing that amoxicillin commonly had an inhibiting effect on *Lactobacillus*, *Streptococcus* and *Staphylococcus* [59, 208-210].

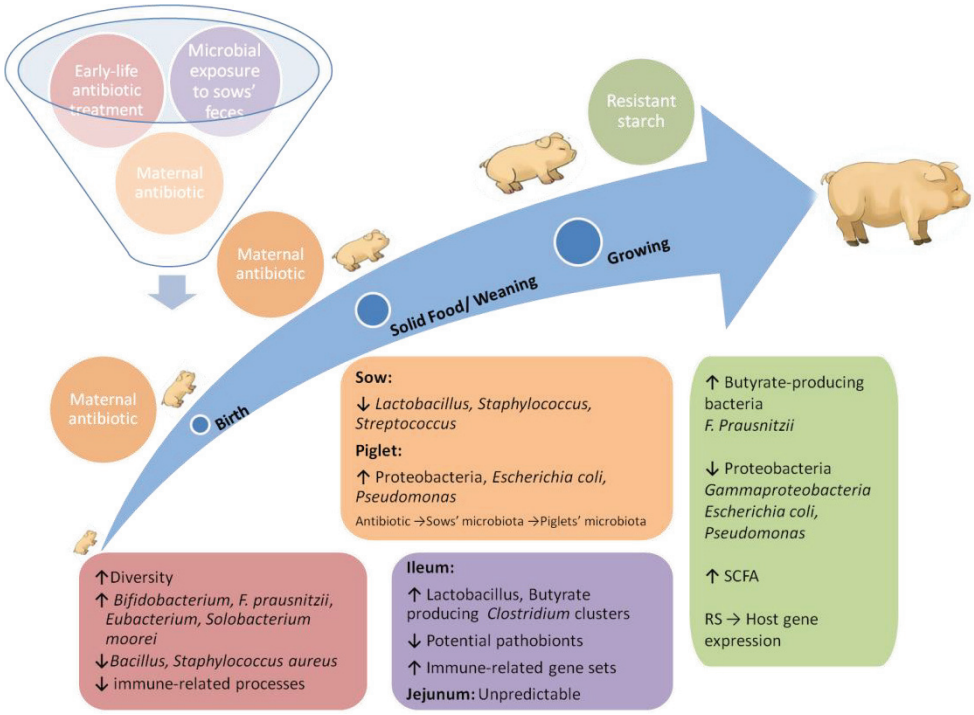


Figure 1. Overview of the main findings described in this thesis. This thesis determined the impact of maternal antibiotic treatment (**Orange**), early antibiotic administration (**Red**), and early microbial exposure to sows' faeces (**Purple**) on piglets' intestinal microbiota and mucosal tissue gene expression at early age, as well as resistant starch (RS, **Green**) on microbiota, short-chain fatty acid (SCFA) concentration and host gene expression in the large intestine of adult pigs. Antibiotic treatment of sows during the perinatal period (**Orange pies**) caused a decrease in relative abundance of bacteria related to *Lactobacillus*, *Staphylococcus* and *Streptococcus* of sows' faeces, and led to increased relative abundance of Proteobacteria in piglets' faeces, mainly driven by a stimulation of *Escherichia coli* and *Pseudomonas* during administration (**Orange plate**). Early antibiotic administration to piglets (**Red pie**) affected composition and diversity of gut microbiota and reduced the mucosal tissue expression of host genes associated with a large number of immune-related processes (**Red plate**). Exposure of caesarian derived newborn piglets to sows' faeces (**Purple pie**) affected the piglet's intestinal microbiota and enriched ileal mucosal gene sets linked to immune system development, whereas effects on jejunal microbiota and gene expression was less clear (**Purple plate**). RS (**Green pie**) modulated the microbiota composition, short chain fatty acid (SCFA) concentration and mucosal tissue gene expression in the large intestine of adult pigs (**Green plate**). The RS diet induced the stimulation of health associated butyrate-producing bacteria, whereas potentially pathogenic members were reduced in relative abundance. Caecal and colonic SCFA concentrations were significantly higher in RS-fed pigs, and caecal expression of genes encoding for monocarboxylate transporter 1 (SLC16A1) and glucagon (GCG) was induced by RS. Concentrations in carotid plasma of acetate, propionate, and butyrate were significantly higher following RS consumption. RS induced oxidative metabolic pathways, whereas many immune response pathways and cell division were suppressed.

However, in the study described here, such inhibiting effect was not observed in the intestinal microbiota of piglets. Moreover, microbial groups that were affected both in faeces of amoxicillin-treated sows and ileal content of their offspring, showed an opposite direction of change. For example, amoxicillin caused an increased relative abundance of bacteria related to *Alistipes* in treated sows' faeces, whereas this population was decreased

in the ileal content of the offspring at the age of 14 days. These opposing findings may suggest that the maternal amoxicillin treatment may indirectly affect the gut microbiota of the offspring through disturbing the maternal microbiota. Since maternal microbiota can be transferred to the offspring (see **Chapter 1**), the impact will be manifested in the next generation. Another possible explanation is that amoxicillin-resistance genes or bacteria harbouring these have been selected and transferred from the mothers to their offspring. Therefore, these genes or bacteria that harbour them in the piglet gut showed increased relative abundance, whereas sensitive bacteria were reduced by the maternal amoxicillin. However, the present study is not conclusive on the main mechanism by which maternal amoxicillin affected the intestinal microbial of the offspring. Further analysis should be performed to characterize the milk microbiota as well as determine potential amoxicillin residues directly transferred from the mothers to their offspring.

Chapter 4 describes that when the antibiotic was directly administered to the piglets at an early age, it caused an increase of the microbial diversity as well as the relative abundance of *Bifidobacterium*. This finding differs from the conclusion of most previous human infant studies summarized in **Chapter 1.** One possible reason could be that all the previous infant studies determined the effect of antibiotics in faecal samples, whereas in the current piglet study we analyzed the microbiota in jejunal content. Therefore, our piglet study can not directly be compared to the previous human infant studies. On the other hand, the used antibiotics also differ. In our present study, piglets received an injection (subcutaneously in the neck) with 0.1 ml tulathromycin at day 4 after birth. Tulathromycin is a triamilide macrolide antibiotic found to be safe and effective against respiratory bacterial pathogens in cattle and swine. However, the effect of tulathromycin on the gut microbiota has not been studied in detail up to now. This thesis is the first report that tulathromycin impacts the gut microbiota of piglets. As shown in **Chapter 1**, most of the human infant studies evaluate β-Lactam antibiotics such as amoxicillin, cefalexin and ampicillin. Considering the various targets, doses, and durations of antibiotic treatment, it is not surprising that gut microbial communities respond to different antibiotics in different ways or even in opposite ways.

Microbial exposure at early age

Microbial exposure at early age is probably the most complicated factor to determine when studying the development of intestinal microbiota. Just like the individual variation of each person's or animal's gut microbiota, the microbial exposure at early age for each individual can also strongly vary, due to the fact that microbial exposure of infants and young animals seems to happen everywhere. Starting from birth, the neonates are exposed to either their mothers' vaginal and faecal bacteria or a more complex indoor microbial environment which includes their mothers' skin microbiota, mostly depending on the mode of delivery [31-33]. Later on, the infants and young animals may continue to be exposed to their

mother's milk, skin and faecal microbiota during daily contact [37, 38, 191, 207, 376]. At the same time, infants and young animals are exposed to a broad range of indoor and outdoor microbial environments, such as hospital and day care for infants and farms for animals [46, 48-50]. It seems that the microbial exposure at early age is to some extent accidental, and earliest colonization events are determined by opportunistic colonization by bacteria to which an infant or a piglet is exposed in its environment [13]. Such opportunistic colonization can be one of the reasons that explain the individual variation of the intestinal microbiota.

Impact of external factors on the intestinal microbiota differs along the intestine

The impact that external factors have on the development of intestinal microbiota at early age differs along the intestine. In the present reaserch, different microbial groups were affected by the maternal amoxicillin treatment in the ileum and colon of the newborn piglets (**Chapter 3**). Similarly, most microbial groups that were affected by the complex microbial association were different when comparing jejunal and ileal digesta (**Chapter 5**). Such site-specific microbiota responses to external factors were also found in the study of adult pigs. Most microbial groups that changed in their relative abundance in response to inclusion of RS in the caecum showed no significant change in the colon (**Chapter 6**). These findings may be a result of the interaction of external factors and internal gut physiological conditions. The function and architecture of the gut differs along its length [74]. Moreover, several physiological parameters, such as pH, peristaltic movement of the tract, desquamation of epithelial cells and mucosal flow, also influence the microbiota distribution in the gut [261]. Consequently, bacterial diversity and composition differs from the small intestine to large intestine (**Chapter 2**), which can explain the site-specific responses of microbial communities that were induced by the external factors in the studies described in this thesis. Therefore, future studies should consider these site-specific effects of external factors on the intestinal microbiota before drawing conclusions. To date, faecal sampling has been extensively used to characterize intestinal microbial communities of humans, especially in infant studies. However, this approach may lead to inaccurate assessments of the intestinal microbiota of newborns. A recent study of human neonates showed that site-specific microbial communities established in faecal and mucosal samples [377]. Interestingly, in contrast to adult studies, intestinal bacterial diversity was higher in mucosal tissue than in faecal samples. This finding questions the reliance on faecal microbiota as a proxy for the developing intestinal microbiota of human infants and animals, and also reinforces the need for a proper model for studying the infant intestinal microbiota. Pigs and mice are the most common models used for study the human microbiota (**Chapter 2)**. Recently, human gut microbiota has been successfully transplanted into germ-free piglets and mice, resulting in a human-like microbial community [156, 378]. Moreover, the

microbial shifts occurring in these models due to a certain treatment are likely to take place in humans as well [152]. For instance, the microbiota of two human twins discordant for obesity was studied in mice, where the impact of the microbiota was linked to the phenotype of the human donor [132]. Therefore, such "humanized" neonatal piglets or mice seem a promising model to be applied in studies addressing infant intestinal microbiota.

Effect of RS on microbiota in the large intestine of adult pigs

Many studies have been done to study the effects of RS on the intestinal microbiota. However, most of these studies either only focused on the numbers of certain bacteria of the intestinal microbiota using culture methods, or assessed the effect of RS on intestinal microbiota with *in vitro* models. Only few studies have addressed the impact of RS on the intestine *in vivo* with culture-independent methods as reviewed in **Chapter 1.** The present study provides a detailed analysis of the RS effects on the intestinal microbiota, SCFA concentration and host gene expression data in adult pigs by using microarrays (**Chapter 6 and 7**). The 2-week feeding of RS was found to modulate the microbial composition in the large intestine. However, since no longitudinal sampling was performed, it was impossible to predict in which order colonic changes occurred. In addition, determining the main driving force of observed microbial changes, either RS itself or the SCFA produced by its microbial fermentation, is difficult based on current observations, and should be addressed in further studies that provide improved spatio-temporal resolution of measurements. Finally, it should be noted that research findings in pigs can be used to improve the welfare of restrictedly fed animals, such as adult pigs, which may suffer from hunger and related welfare problems [379], and for which acceleration of satiation through dietary means could provide innovative avenues towards alleviating such issues.

The pig as a model

In this thesis, the pig was selected as study model, because the pig is an important production animal as well as a model for humans. As pig is an important livestock for meat production, this animal is studied intensively with the object of optimizating production. In recent years, due to the ban on the application of in-feed antibiotics as growth promotors in many countries, more studies have focused on identifying alternatives to feeding antibiotics, including the inclusion of prebiotics and probiotics in the feed [380] to improve the pig health. As shown here, manipulation of microbial colonisation of the intestinal tract at early age is also an alternative. It can be a powerful tool to strengthen intestinal barrier function, robustness and immunological competence of young piglets, and furthermore contribute to improved pig health and thereby providing new avenues towards more sustainable animal production. The output of this thesis, development of microbiota at early life (**Chapter 3, 4 and 5**) as well as the interaction between early microbial colonization and the development

of gut function **(Chapter 5)**, provides basic knowledge and clues for manipulation of the gut microbiota. Such knowledge is useful for providing a sound basis for the design of innovative management strategies as alternatives to in-feed antibiotics, especially for low-input farming systems.

In addition to its role as an important livestock, the pig is also regarded as a good model for human studies because of the high similarities to human beings in physiology, anatomy and nutrition (see **Chapter 2**) [153-155]. In human microbiota research, invasive sampling from the intestine of large numbers of healthy individuals is not feasible for both practical and ethical reasons. In contrast, the pig model can provide an easier way for collecting many (invasive) samples along relevant spatio-temporal gradients, and also allow multiple comparisons and regulating the genotype background. In addition, the pig model allows to study the interaction between the host and the microbiota, as well as provide an alternative way to study the *in vivo* responses to beneficial, commensal and pathogenic microorganisms in the intestinal tract. These advantages make the pig essential as model for human intestinal studies. However, the pig as a model for human studies has some limitations notably as there are differences in the intestinal tract, diet and behaviour (**Chapter 2**) between pigs and humans. These should be taken into account when translating the knowledge gained from pig studies to the human situation [109, 139]. On the other hand, the long history of pig breeding has improved pig meat production and growth rate [381]. The pig breeding may also impact the pig genetics and furthermore the interaction of the pig with its gut microbiota, for example, gaining more energy from microbial fermention in the large intestine to adapt a fast growth. To this end, it is interesting to note that marked differences were found in microbial composition and function when comparing lean and obese pig genotypes [382, 383]. In spite of the above-mentioned limitations, promising pig models such as gnotobiotic pigs and humanized pigs may be even more helpful for translating the output from the pig research to human studies. As introducted in **Chapter 2**, gnotobiotic pigs have been used to study various human intestinal pathogens and recently been successfully used as an animal model to study the microbial colonization. With respect to humanized pigs, transplantation of human gut microbiota produced a donor-like microbial community in the piglet gut, and the microbial succession with aging of such humanized piglets was similar to that observed in humans [156]. Taken all together, gnotobiotic and humanized pigs could be appropriate models for studying the human intestinal microbiota, especially human infant microbiota.

Outlook and future perspectives

Antibiotic useage

Since the first antibiotic, penicillin, became available, the antibiotic usage has always been a heavily debated issue. Although antibiotic usage improves our lives and that of farm animals by preventing and treating disease, its collateral effects on the mammalian gut microbiome should not be ignored [42]. The present research suggested a negative effect of the maternal amoxicillin treatment during pregnancy and lactation with an increase of *E.coli* in the offspring intestine (**Chapter 3**). However, the main mechanism, by which maternal amoxicillin affects the intestinal microbial of the offspring, is still not clear. The findings in **Chapter 3** indicated that the maternal amoxicillin treatment may indirectly affect the gut microbiota of offspring through disturbing the maternal microbiota and the transfer of maternal microbiota to offspring. Most antibiotics are either injected or administered orally, thereby circulating throughout the mother's system and potentially affecting the entire mother's microbiota [42]. Therefore, antibiotic effects on maternal milk and skin microbiota should also be considered when determining the maternal antibiotic effects. Especially, more studies should be performed to determine the effects of antibiotic treatment on the microbiota of breast milk, since breast milk is the main food for breast-feeding neonate humans and animals. Currently, the analysis of microbiota in breast milk is quite limited, and no reports on the antibiotic effects on the breast milk microbiota are available. The knowledge of antibiotic effects on the breast milk microbiota will be important for both doctors and mothers taking decisions of choosing antibiotics during lactation. To this end, knowledge regarding antibiotic resistance genes and their distribution throughout the microbiome will also be important [384, 385].

Long-term effects of pre-and post-natal factors on the intestinal microbiota

As introduced in **Chapter 1,** many factors are involved in the intestinal microbiota development, including both prenatal factors and postnatal factors. There is no doubt that the introduction of solid foods and weaning will significantly affect the gut microbiota and may drive the microbiota into an adult type [2, 13, 18, 19]. Considering the drastic effects caused by dietary factors later in life, it is possible that pre-and post-natal influences can be at least partially be overcome by later colonization events. For example, Matsumiya *et al.* investigated mother-to-newborn infant transmission of *Lactobacillus* species, and found that the acquired lactobacilli from mother do not last in the intestine of the infant long-term, but rather, are replaced by other populations derived from milk or unknown sources after birth [31]. Moreover, the RS studies described in this thesis have shown that already two weeks of RS treatment can modify the intestinal microbiota of adult pigs (**Chapter 6 and 7**). This finding further questions how long the effects of pre-and post-natal factors can last.

The present research only provides evidences on the impact of early-life events on the intestinal microbiota of piglets either before weaning or at early post weaning. The knowledge of the long-term effects of these early-life events is lacking. Therefore, longitudinal studies are required for determining the influences of early-life events on the intestinal microbiota and gut function in later life. To this end it is interesting to note that, using the pig model described in **Chapter 3** of this thesis, differences in intestinal enzyme activities were found in pigs born to mothers that were treated with amoxillin before birth after being fed a high fed diet between 140 and 169 days of age [197].

Systems biology approaches including the analysis of microbial function

Recently developed techniques addressing the functionality of the intestinal microbiota beyond compositional analyses, including metatranscriptomic analyses, have recently become available using extreme throughput next generation technology sequencing to determine the function of certain bacteria within a complex microbial community [386]. To this end, efficient bioinformatic pipelines have been developed to deal with large amounts of functional genomic data derived from largely uncultured microorganisms such as those found in the gut, and application has recently provided a first glimpse of the interacive microbial networks responsible for carbohydrate metabolism in the human small intestinal tract [125, 387]. This approach will be further applied for intestinal content that we collected in the animal trial in **Chapter 7.** This approach will allow to collect information on the microbial activity responsible for RS metabolism, in addition to the microbial composition that we have already known. This approach could help answering our current questions in **Chapter 7,** for instance, the cross effects of diet, and the main factor, either RS itself or its production SCFA, driving the microbial change. The output of this approach will be more reliable and supports the general believe that fermentable fibres have a beneficial impact on intestinal health.

Studies described in this thesis, together with recent breathtaking conceptual and technological advances in the field of gut microbiomics and systems biology [388], have further reinforced the notion that future studies will have to focus on integrating datasets on host factors, intestinal microbiota and environmental influences to understand the complexity of host-microbe interactions.

Appendices

Supplemental Table 2 in Chapter 7. All significantly regulated genes (P <0.01) observed in pigs after 2 wk consumption of the RS diet compared to 2 wk consumption of the DS diet.

					Change per pig									
Abbreviation	Name	Entrez ID	Average log2 Fold Change	IBMT P-value	1	2	3	4	5	6	7	8	9	10
LOC100521756	intestinal-type alkaline phosphatase-like	100521756	1.55	0.00037	3.23	1.64	0.34	0.75	3.04	3.47	-0.14	0.67	1.32	1.19
CA2	carbonic anhydrase II	100154873	1.35	0.00002	0.97	1.68	1.33	0.34	2.03	3.21	1.02	0.98	0.74	1.18
LOC100621113	uncharacterized LOC100621113	100621113	1.01	0.00004	1.34	0.49	0.33	0.14	2.07	1.83	1.34	0.50	0.91	1.12
HSD17B2	hydroxysteroid (17-beta) dehydrogenase 2	100312973	1.00	0.00238	1.72	0.82	-0.12	-0.35	2.48	2.70	1.20	0.51	0.86	0.18
LOC100513630	transmembrane 4 L6 family member 20-like	100513630	0.96	0.00406	1.59	0.43	0.44	0.17	2.09	3.09	-0.33	0.09	0.62	1.40
LOC100525263	uncharacterized protein C5orf4 homolog	100525263	0.95	0.00056	1.42	0.40	0.05	0.21	2.14	2.34	0.97	0.33	0.63	0.97
SLC30A10	solute carrier family 30, member 10	100623097	0.92	0.00021	0.80	0.88	0.89	0.23	1.85	1.40	0.86	-0.44	0.84	1.85
LOC100157073	angiotensinogen-like	100157073	0.86	0.00150	1.67	0.35	0.23	-0.26	0.59	2.65	1.12	1.03	0.83	0.35
LOC100152749	carbonic anhydrase 12-like	100152749	0.83	0.00024	1.19	0.08	0.55	0.19	1.43	2.04	1.07	0.24	0.39	1.11
LOC100524810	carcinoembryonic antigen-related cell adhesion molecule 7-like	100524810	0.81	0.00015	1.27	0.47	0.61	0.32	2.19	0.82	0.72	0.05	0.93	0.73
ABCA6	ATP-binding cassette, sub-family A (ABC1), member 6	100520861	0.79	0.00400	0.38	-0.12	-0.04	0.19	0.49	2.35	1.83	1.58	0.95	0.23
LOC100519548	agmatinase, mitochondrial-like	100519548	0.74	0.00022	1.52	0.10	0.45	0.19	1.57	1.23	0.45	0.16	0.77	1.00
ADAM23	ADAM metallopeptidase domain 23	100518044	0.74	0.00333	-0.05	0.33	0.41	0.47	0.57	2.18	-0.37	1.56	1.49	0.82
CLDN10	claudin 10	100153752	0.73	0.00001	0.58	0.75	0.46	0.35	1.09	1.36	0.92	0.18	0.94	0.66
TPMT	thiopurine S-methyltransferase	100157630	0.72	0.00137	0.50	0.43	-0.19	0.40	1.90	1.89	1.06	0.28	0.51	0.45
LOC100520041	ubiquitin carboxyl-terminal hydrolase 2-like	100520041	0.72	0.00051	0.99	0.21	-0.18	0.53	1.12	2.02	0.54	0.74	0.88	0.37
LOC100157775	transient receptor potential cation channel subfamily M member 6-like	100157775	0.71	0.00009	1.06	0.44	0.46	0.01	0.56	1.80	0.94	0.40	0.74	0.74
TMEM117	transmembrane protein 117	100524623	0.71	0.00153	0.90	0.18	0.14	-0.06	1.15	2.13	0.36	0.14	1.12	1.07
PARP15	poly (ADP-ribose) polymerase family, member 15	100520273	0.70	0.00014	0.94	0.55	0.14	0.21	0.78	1.21	0.27	0.87	1.69	0.31
SPTLC3	serine palmitoyltransferase, long chain base subunit 3	100519280	0.68	0.00228	1.16	0.34	0.13	-0.29	1.45	1.94	0.31	0.22	0.82	0.73
LOC100516642	collagen alpha-6(VI) chain-like	100516642	0.67	0.00187	0.63	0.11	2.10	-0.14	0.85	1.21	0.33	0.58	0.76	0.23
GUCA2A	guanylate cyclase activator 2A (guanylin)	100301560	0.66	0.00197	0.46	0.59	0.42	-0.42	1.37	1.72	1.13	0.10	0.10	1.17
EFEMP1	EGF containing fibulin-like extracellular matrix protein 1	100512046	0.66	0.00777	0.42	1.01	1.36	-0.38	0.25	2.48	0.27	-0.01	0.88	0.36
PNLIPRP2	pancreatic lipase-related protein 2	100462755	0.66	0.00012	0.77	0.08	0.00	0.22	1.29	1.05	1.18	0.58	0.87	0.59
LOC100737562	olfactory receptor 9K2-like	100737562	0.66	0.00450	1.67	1.49	0.87	-0.04	1.66	0.03	0.40	0.69	-0.09	-0.09
LOC100514834	uncharacterized LOC100514834	100514834	0.65	0.00040	0.67	0.71	0.78	0.18	0.83	1.77	0.15	-0.11	0.69	0.82
CNNM2	cyclin M2	100155831	0.65	0.00033	1.24	0.28	0.16	0.01	0.94	1.58	0.45	0.28	0.82	0.71
TN-X	tenascin-X	445520	0.64	0.00160	0.69	0.68	0.96	0.24	0.67	1.55	-0.60	0.37	1.46	0.42

LOC100621356	protein-arginine deiminase type-2-like	100621356	0.63	0.00172	1.60	0.25	0.08	0.22	1.23	1.50	0.05	0.05	0.86	0.49
IGF1	insulin-like growth factor 1 (somatomedin C)	397491	0.62	0.00074	0.35	0.68	0.57	0.02	1.23	1.74	0.15	0.53	0.08	0.89
SLC16A1	solute carrier family 16, member 1 (monocarboxylic acid transporter 1)	100127159	0.62	0.00015	1.00	0.27	0.23	0.06	0.92	1.56	0.62	0.37	0.63	0.51
RETNLB	resistin like beta	100037943	0.61	0.00403	0.97	-0.19	0.50	0.06	1.82	1.24	0.72	-0.26	1.08	0.20
RBP1	retinol binding protein 1, cellular	100156666	0.61	0.00031	0.89	0.35	1.05	0.34	0.54	1.39	0.86	0.21	-0.11	0.55
ANGPTL4	angiopoietin-like 4	397628	0.60	0.00427	0.21	1.77	1.15	0.18	0.79	1.23	0.34	0.79	-0.42	0.00
LOC100626580	ankyrin repeat domain-containing protein 40-like	100626580	0.60	0.00103	1.11	-0.17	0.13	0.53	1.42	0.72	0.36	0.01	1.09	0.78
LOC100738347	zinc finger protein 821-like	100738347	0.59	0.00001	0.78	0.44	0.13	0.32	0.99	0.74	0.68	0.23	0.72	0.84
LOC100515779	dual specificity tyrosine-phosphorylation-regulated kinase 2-like	100515779	0.59	0.00001	0.66	0.46	0.45	0.23	1.09	0.72	0.21	0.45	1.02	0.57
LOC100511032	prolargin-like	100511032	0.58	0.00863	0.45	0.12	0.37	-0.32	0.63	2.46	0.52	0.80	0.44	0.34
TRPV3	transient receptor potential cation channel, subfamily V, member 3	100515051	0.58	0.00218	1.18	-0.08	0.22	0.11	1.31	0.86	0.14	0.04	1.34	0.69
LOC100519087	neurexin-1-alpha-like	100519087	0.58	0.00544	0.62	-0.04	0.26	-0.60	0.74	1.92	0.79	0.81	0.78	0.50
LOC100516689	uncharacterized protein C1orf115-like	100516689	0.57	0.00060	1.15	0.51	0.19	0.03	1.10	1.37	0.17	0.10	0.60	0.52
HPGD	hydroxyprostaglandin dehydrogenase 15-(NAD)	100156186	0.57	0.00056	0.68	-0.10	0.31	0.23	1.06	1.07	0.24	0.30	1.43	0.49
LOC100515222	UDP-glucuronosyltransferase 2C1-like	100515222	0.57	0.00027	0.92	0.41	0.62	0.08	0.78	1.39	0.76	-0.15	0.52	0.36
LOC100516366	dermatopontin-like	100516366	0.57	0.00608	1.16	0.07	0.13	0.04	0.80	1.91	-0.09	0.47	1.14	0.03
LOC100152498	fibroblast growth factor 12-like	100152498	0.57	0.00149	0.40	0.56	0.34	0.63	0.97	1.18	0.00	-0.39	0.73	1.25
TM6SF1	transmembrane 6 superfamily member 1	100155238	0.56	0.00513	1.02	0.17	-0.32	-0.16	1.77	0.85	0.11	0.53	1.06	0.59
ID4	inhibitor of DNA binding 4, dominant negative helix-loop-helix protein	100144508	0.55	0.00026	1.06	0.49	-0.24	0.25	1.06	0.51	0.60	0.25	0.86	0.66
LOC100515463	multiple epidermal growth factor-like domains protein 11-like	100515463	0.55	0.00373	-0.56	1.04	0.61	0.64	0.83	1.52	0.35	0.70	0.06	0.28
RAI14	retinoic acid induced 14	100145886	0.54	0.00422	0.97	0.39	0.43	0.45	1.73	0.60	0.21	-0.24	-0.19	1.02
PID1	phosphotyrosine interaction domain containing 1	100302508	0.53	0.00057	0.81	0.36	0.82	0.30	0.53	1.33	0.31	0.75	-0.28	0.38
LOC100624891	UDP-glucuronosyltransferase 2B31-like	100624891	0.53	0.00432	0.82	0.11	1.14	-0.21	0.45	1.67	0.83	-0.09	0.26	0.32
PLEKHG6	pleckstrin homology domain containing, family G (with RhoGef domain) member 6	100520565	0.53	0.00012	0.57	0.01	0.36	0.53	1.24	0.88	0.31	0.19	0.59	0.62
LOC100739060	protein PTHB1-like	100739060	0.53	0.00937	0.79	0.73	0.56	0.20	0.12	2.06	-0.13	-0.07	0.90	0.11
LOC100520019	rho-related GTP-binding protein RhoC-like	100520019	0.52	0.00036	0.91	0.45	0.46	0.91	1.27	0.56	0.14	0.07	0.03	0.45
MAOA	monoamine oxidase A	414424	0.52	0.00200	0.78	-0.25	0.24	0.02	0.99	1.54	0.50	0.24	0.67	0.46
SCNN1A	sodium channel, non-voltage-gated 1 alpha subunit	396608	0.52	0.00001	0.64	0.54	0.16	0.16	0.79	0.83	0.32	0.29	0.76	0.70
LOC100626928	uncharacterized LOC100626928	100626928	0.52	0.00018	0.33	0.59	0.28	0.06	0.97	1.27	0.30	0.00	0.62	0.45
LOC100152247	tumor protein p53-inducible nuclear protein 2-like	100152247	0.52	0.00600	0.93	0.32	-0.19	0.02	1.57	0.98	0.35	-0.21	0.31	1.07
LOC100521401	transmembrane and immunoglobulin domain-containing protein 1-like	100521401	0.51	0.00372	0.93	-0.22	-0.33	0.05	0.63	1.33	0.71	0.32	1.08	0.57
COX7A1	cytochrome c oxidase polypeptide	399685	0.51	0.00690	0.76	0.11	-0.21	-0.26	1.49	1.35	0.67	0.15	0.46	0.55

	VIIa-muscle/heart											
LOC1005		100521		0.00	0.7	0.2	0.3	0.1	1.2	0.6	0.5	0.0	0.6	0.3
21274	protein FAM131C-like	274	0.51	027	8	8	2	6	6	5	9	6	6	0
	uveal autoantigen with coiled-coil domains and													
LOC1005	ankyrin repeats	100523		0.00	0.7	0.0	0.3	0.2	0.9	1.3	0.4	-0.	0.1	0.9
23347	protein-like	347	0.51	203	6	3	5	2	7	0	6	18	6	9
		100155		0.00	0.9	0.4	0.1	0.5	0.8	0.9	0.5	-0.	0.5	0.3
RNF152	ring finger protein 152	834	0.50	019	6	2	7	0	5	0	2	18	1	7
	NADPH oxidase, EF-hand	100156		0.00	0.1	0.5	1.3	-0.	-0.	1.5	0.1	0.5	0.6	0.3
NOX5	calcium binding domain 5	068	0.50	820	4	1	9	08	22	5	1	1	9	6
	acyl-CoA synthetase	100512		0.00	0.7	0.2	0.2	0.1	0.3	1.2	0.4	0.4	0.6	0.5
ACSF2	family member 2	859	0.50	010	7	3	6	6	0	3	1	5	5	1
	ectonucleoside triphosphate	100154		0.00	0.8	0.2	0.2	-0.	0.6	1.4	0.3	0.2	0.7	0.4
ENTPD5	diphosphohydrolase 5	506	0.50	107	3	4	0	25	6	0	7	8	6	6
	adenosylhomocysteinase-li	100514		0.00	0.9	-0.	0.2	0.2	1.3	0.7	-0.	0.3	0.8	0.5
AHCYL2	ke 2	931	0.49	233	1	33	6	8	8	9	06	8	0	3
	nuclear receptor subfamily	100153		0.00	0.2	0.6	0.4	0.6	-0.	0.5	0.8	0.9	0.7	0.1
NR1H4	1, group H, member 4	960	0.49	076	9	9	8	1	35	2	9	3	4	4
	3-hydroxy-3-methylglutar yl-CoA synthase 2			0.00	0.7	0.1	0.2	-0.	0.7	1.3	0.5	0.2	0.5	0.5
HMGCS2	(mitochondrial)	397673	0.49	076	5	0	1	15	6	9	7	6	0	6
	acyl-Coenzyme A													
LOC1001	dehydrogenase family,	100153		0.00	0.6	0.3	0.2	-0.	0.8	1.7	0.3	0.0	0.6	0.4
53698	member 11	698	0.49	451	4	1	6	28	1	1	1	1	6	9
LOC1007	transmembrane protease	100739		0.00	0.7	0.3	0.4	0.4	0.6	0.6	0.1	0.3	0.7	0.3
39411	serine 2-like	411	0.49	000	0	3	8	6	5	7	8	8	3	4
				0.00	1.1	0.3	0.4	0.4	0.7	0.8	0.0	-0.	0.5	0.5
IYD	iodotyrosine deiodinase	403124	0.49	053	4	1	8	3	8	1	4	19	6	3
	gastric intrinsic factor	100514		0.00	0.7	-0.	0.5	-0.	1.3	0.6	0.2	0.5	0.7	0.9
GIF	(vitamin B synthesis)	273	0.49	879	7	41	2	52	4	0	6	4	9	9
LOC1005	uncharacterized	100511		0.00	1.1	0.1	-0.	-0.	0.1	1.4	0.6	0.5	0.8	0.6
11514	LOC100511514	514	0.49	641	0	9	25	40	8	0	8	5	3	1
	cartilage intermediate layer protein, nucleotide			0.00	0.8	0.3	0.7	-0.	0.5	-0.	0.8	0.5	1.3	0.2
CILP	pyrophosphohydrolase	397672	0.49	293	0	5	8	10	4	37	2	1	1	3
	bifunctional 3'-phosphoadenosine													
LOC1001	5'-phosphosulfate synthase	100156		0.00	0.6	-0.	0.3	0.1	1.5	0.7	0.7	-0.	0.2	0.8
56262	2-like	262	0.49	327	3	24	7	2	3	8	1	04	1	0
	ras homolog family	100154		0.00	0.2	0.1	0.1	1.0	1.1	0.5	1.0	-0.	0.1	0.7
RHOV	member V	565	0.48	396	7	5	6	5	1	3	4	37	5	5
	peptidase M20 domain	100627		0.00	0.7	0.2	0.2	-0.	0.7	1.5	0.5	0.1	0.0	0.6
PM20D1	containing 1	595	0.48	395	7	3	4	19	0	9	7	8	8	4
LOC1005	leucine-rich repeat	100520		0.00	1.2	-0.	0.4	0.8	1.5	-0.	-0.	0.2	0.3	0.4
20161	neuronal protein 3-like	161	0.48	957	4	22	1	3	9	04	05	3	5	6
	TOX high mobility group	100519		0.00	0.9	0.2	0.4	0.5	1.1	0.1	-0.	0.3	0.4	0.5
TOX3	box family member 3	713	0.48	049	7	8	7	7	5	9	17	6	3	5
LOC1005	organic solute transporter	100512		0.00	0.9	0.6	0.2	-0.	-0.	1.4	0.3	0.4	0.2	0.5
12372	alpha	372	0.48	236	4	9	2	09	11	7	6	7	9	6
LOC1001	uncharacterized protein	100152		0.00	0.4	0.2	0.5	-0.	1.5	0.7	-0.	0.2	1.1	0.4
52200	C6orf222-like	200	0.48	818	1	8	1	39	8	1	15	6	3	4
TMPRSS	transmembrane protease,	100514		0.00	0.8	0.3	-0.	0.1	0.9	0.4	0.2	0.3	0.7	0.7
4	serine 4	419	0.47	017	6	8	09	9	4	3	1	9	0	4
LOC1006	uncharacterized	100622		0.00	0.6	0.1	0.2	0.5	0.9	0.6	0.8	-0.	0.7	0.1
22224	LOC100622224	224	0.47	018	1	3	6	1	2	4	3	01	0	5
	disintegrin and metalloproteinase													
LOC1001	domain-containing protein	100157		0.00	0.8	0.4	0.2	0.4	0.2	0.2	0.3	0.4	0.8	0.6
57954	7-like	954	0.47	005	4	7	0	3	7	7	2	2	4	4
LOC1005	ADP-ribosylation	100515		0.00	0.9	0.3	-0.	0.3	1.0	1.1	-0.	0.2	0.3	0.6
15798	factor-like protein 14-like	798	0.47	242	5	8	04	3	4	1	31	8	2	5
	glycerophosphodiester phosphodiesterase													
LOC1006	domain-containing protein	100626		0.00	0.6	0.5	-0.	0.0	0.3	1.6	-0.	0.4	0.9	0.2
26512	1-like	512	0.46	609	4	6	04	0	9	1	22	8	6	7
		100155		0.00	0.1	0.5	0.8	0.6	0.7	1.1	-0.	0.3	0.2	0.4
THBS3	thrombospondin 3	108	0.46	285	9	4	2	3	6	8	49	4	5	1
	dimeric dihydrodiol			0.00	0.4	0.1	0.7	0.1	0.3	1.1	0.6	0.0	0.6	0.3
SUS2DD	dehydrogenase	397337	0.46	038	9	5	3	7	4	6	4	1	3	1
	ankyrin 3, node of Ranvier	100154		0.00	0.7	-0.	0.1	-0.	1.2	0.9	0.5	-0.	0.6	0.7
ANK3	(ankyrin G)	687	0.46	588	9	04	8	46	2	7	3	02	8	9
				0.00	0.9	0.5	0.1	0.1	0.9	0.1	0.2	-0.	0.9	0.5
IHH	Indian hedgehog	397174	0.46	087	7	1	8	2	9	3	7	05	2	8

GGT1	gamma-glutamyltransferase 1	397095	0.46	0.00292	0.55	0.39	0.08	-0.53	0.47	1.07	0.73	0.83	0.22	0.80
SLC9A2	solute carrier family 9, subfamily A (NHE2, cation proton antiporter 2), member 2	100101921	0.46	0.00002	0.58	0.19	0.32	0.10	0.65	0.86	0.60	0.34	0.38	0.54
LOC100622888	transmembrane protein 150C-like	100622888	0.45	0.00726	0.09	0.35	0.48	-0.22	0.29	1.72	0.72	0.31	0.07	0.73
CDHR2	cadherin-related family member 2	100525685	0.45	0.00164	0.81	0.23	-0.01	0.03	1.03	0.91	0.07	0.11	0.96	0.40
LOC100151985	H(+)/Cl(-) exchange transporter 4-like	100151985	0.45	0.00157	0.84	-0.02	-0.01	0.45	1.31	0.66	0.29	0.30	0.44	0.26
LOC100516309	glycerophosphoinositol inositolphosphodiesterase GDPD2-like	100516309	0.45	0.00401	0.66	0.20	0.06	-0.25	1.35	0.72	0.27	0.10	0.53	0.88
SELENBP1	selenium binding protein 1	100152724	0.45	0.00128	0.65	-0.07	0.30	-0.11	0.75	1.21	0.71	0.08	0.44	0.55
LOC100621791	protein DEPP-like	100621791	0.45	0.00072	0.51	0.36	0.05	0.25	1.01	1.11	0.18	0.24	0.16	0.61
LOC100522223	mesoderm induction early response protein 3-like	100522223	0.45	0.00041	0.42	0.05	0.05	0.35	1.13	0.64	0.10	0.40	0.75	0.57
LOC100525560	cyclin-dependent kinase 4 inhibitor D-like	100525560	0.44	0.00006	0.89	0.56	0.28	0.44	0.64	0.44	0.47	0.29	0.25	0.48
PRDX6	peroxiredoxin 6	399538	0.44	0.00119	0.77	-0.07	0.18	0.10	1.00	1.10	0.33	0.25	0.28	0.43
NBEAL1	neurobeachin-like 1	100514254	0.44	0.00037	0.71	0.11	-0.11	0.36	0.61	0.93	0.50	0.21	0.84	0.26
COL14A1	collagen, type XIV, alpha 1	100158059	0.44	0.00204	0.64	0.30	0.66	-0.04	0.27	1.35	0.12	-0.02	0.80	0.35
RCAN1	regulator of calcineurin 1	100511902	0.44	0.00374	0.95	0.45	0.24	-0.24	0.84	1.31	-0.08	0.37	0.22	0.34
LOC100156972	zinc finger protein 664-like	100156972	0.44	0.00144	0.47	0.42	0.36	-0.22	0.39	1.35	0.62	0.06	0.38	0.60
LOC100739604	axin-2-like	100739604	0.44	0.00230	0.93	0.28	-0.02	0.09	0.57	1.03	0.88	0.59	-0.02	0.05
LOC100524044	sodium bicarbonate cotransporter 3-like	100524044	0.44	0.00092	0.20	0.26	0.30	-0.13	0.94	1.13	0.52	0.15	0.54	0.46
LOC100524618	alanine aminotransferase 1-like	100524618	0.43	0.00020	0.55	0.00	0.43	0.47	0.89	0.69	0.29	0.08	0.32	0.62
SEMA6A	sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	100522008	0.43	0.00000	0.54	0.42	0.40	0.19	0.59	0.34	0.61	0.22	0.66	0.37
SHROOM3	shroom family member 3	100512481	0.43	0.00012	0.62	0.20	0.24	0.44	0.99	0.44	0.09	0.26	0.85	0.48
ADAMTS19	ADAM metallopeptidase with thrombospondin type 1 motif, 19	100518181	0.43	0.00922	0.18	-0.07	0.42	0.01	0.31	1.53	0.05	0.17	0.99	0.69
LOC100514274	harmonin-like	100514274	0.43	0.00001	0.73	0.20	0.22	0.31	0.66	0.39	0.42	0.24	0.60	0.50
LOC100524831	dedicator of cytokinesis protein 1-like	100524831	0.43	0.00000	0.62	0.38	0.31	0.38	0.56	0.61	0.26	0.27	0.39	0.48
LOC100518372	tetratricopeptide repeat protein 38-like	100518372	0.42	0.00103	0.67	0.01	0.14	0.26	1.13	0.84	0.32	0.02	0.32	0.52
PTPRR	protein tyrosine phosphatase, receptor type, R	100154962	0.42	0.00804	0.30	0.51	0.06	-0.11	0.96	1.41	-0.21	0.32	0.58	0.40
LOC100518563	protein FAM160A1-like	100518563	0.42	0.00131	0.85	0.06	0.12	0.16	0.77	0.88	0.15	-0.04	0.76	0.50
LOC100515418	UPF0733 protein C2orf88 homolog	100515418	0.42	0.00079	0.75	-0.07	0.49	-0.11	0.53	0.80	0.17	0.54	0.47	0.57
LOC100737900	olfactory receptor 51G2-like	100737900	0.42	0.00319	0.07	0.18	-0.03	-0.09	1.00	0.76	0.32	0.42	0.99	0.55
LOC100523465	transmembrane protein 100-like	100523465	0.42	0.00796	0.38	0.18	1.00	0.00	0.29	1.44	0.17	-0.11	0.24	0.57
PLD1	phospholipase D1, phosphatidylcholine-specific	100519446	0.41	0.00024	0.73	0.09	0.04	0.43	0.93	0.57	0.30	0.17	0.28	0.59
WNT2B	wingless-type MMTV integration site family, member 2B	100520560	0.41	0.00088	0.94	0.24	0.32	0.40	0.15	1.12	0.38	0.36	0.12	0.10
RALGPS1	Ral GEF with PH domain and SH3 binding motif 1	100157921	0.41	0.00097	0.67	-0.14	0.67	0.16	0.63	0.34	0.56	-0.11	0.55	0.81
LOC100737624	E3 ubiquitin-protein ligase NEDD4-like	100737624	0.41	0.00973	-0.13	0.04	1.07	0.02	0.14	1.05	1.03	0.09	0.09	0.68
PLA2G16	phospholipase A2, group XVI	100512584	0.41	0.00167	0.20	0.55	0.66	0.06	0.12	0.85	0.00	0.92	0.60	0.11

LOC100739336	centrosomal protein CEP57L1-like	100739336	0.41	0.00220	0.42	0.41	0.56	-0.10	-0.03	1.19	0.35	0.60	0.33	0.34
LOC100156793	olfactory receptor 8S1-like	100156793	0.40	0.00018	-0.02	0.31	0.12	0.77	0.66	0.39	0.40	0.68	0.30	0.44
KIF3A	kinesin family member 3A	100525739	0.40	0.00285	0.46	0.50	0.22	0.03	0.24	1.44	0.37	0.43	0.24	0.13
LOC100156928	uncharacterized LOC100156928	100156928	0.40	0.00199	0.85	0.06	0.20	-0.10	0.82	0.78	-0.05	0.24	0.65	0.59
LOC100516628	UDP-glucuronosyltransferase 2B18-like	100516628	0.40	0.00872	0.53	0.72	0.26	0.92	0.93	0.48	0.57	-0.58	-0.26	0.44
LOC100516116	28S ribosomal protein S36, mitochondrial-like	100516116	0.40	0.00165	0.44	-0.19	0.18	0.25	0.06	0.86	0.70	0.34	0.69	0.67
LOC100514093	pre-B-cell leukemia transcription factor 4-like	100514093	0.40	0.00346	0.61	-0.14	0.22	0.02	0.59	1.02	0.61	-0.11	0.34	0.80
LOC100524518	ciliary neurotrophic factor receptor subunit alpha-like	100524518	0.40	0.00135	0.32	0.42	0.33	0.14	0.86	1.04	-0.05	0.03	0.50	0.36
AIFM3	apoptosis-inducing factor, mitochondrion-associated, 3	100525540	0.40	0.00903	0.64	-0.22	-0.07	0.45	1.19	0.11	0.89	-0.13	0.40	0.70
LOC100157426	stonin-2-like	100157426	0.39	0.00015	0.43	0.17	0.24	0.49	0.83	0.71	0.08	0.21	0.54	0.25
LIPE	lipase, hormone-sensitive	397583	0.39	0.00263	-0.04	0.61	0.33	-0.01	0.24	0.77	1.13	0.19	0.30	0.41
ACADSB	acyl-CoA dehydrogenase, short/branched chain	100154810	0.39	0.00113	0.33	-0.10	0.40	0.06	0.40	1.12	0.59	0.37	0.53	0.22
LOC100519847	enoyl-CoA hydratase domain-containing protein 2, mitochondrial-like	100519847	0.39	0.00049	0.51	0.11	0.07	0.22	0.87	0.71	0.42	-0.04	0.46	0.60
LOC100157995	5 nucleotidase, ecto	100157995	0.39	0.00953	0.28	0.21	0.48	0.02	0.01	1.59	-0.06	0.22	0.56	0.58
LOC100156815	small subunit of serine palmitoyltransferase A-like	100156815	0.39	0.00156	0.28	-0.12	0.41	0.25	0.72	0.80	0.58	-0.09	0.31	0.74
LOC100519130	probable carboxypeptidase PM20D1-like	100519130	0.39	0.00122	0.07	0.38	0.13	0.25	0.61	0.89	0.86	0.21	0.40	0.08
LOC100522399	uncharacterized LOC100522399	100522399	0.39	0.00148	0.81	0.21	-0.19	0.55	0.45	0.60	0.19	0.17	0.20	0.85
LOC100525112	cytochrome P450 2J2-like	100525112	0.38	0.00178	0.58	0.02	0.04	0.16	-0.11	0.92	0.80	0.21	0.54	0.48
LOC100739292	uncharacterized LOC100739292	100739292	0.38	0.00025	0.45	0.32	0.41	0.58	0.40	0.37	-0.01	0.21	0.98	0.14
ABCC2	ATP-binding cassette, sub-family C (CFTR/MRP), member 2	397535	0.38	0.00918	0.89	0.55	0.03	-0.38	0.68	0.47	0.72	-0.26	0.42	0.73
PLCE1	phospholipase C, epsilon 1	100157745	0.38	0.00872	0.95	0.18	0.04	-0.07	1.04	0.99	-0.19	0.12	0.55	0.23
ART4	ADP-ribosyltransferase 4 (Dombrock blood group)	100152653	0.38	0.00879	-0.38	0.67	0.33	0.33	0.47	1.33	0.21	0.00	0.37	0.42
UCP3	uncoupling protein 3 (mitochondrial, proton carrier)	397116	0.38	0.00009	0.66	0.48	0.36	0.20	0.59	0.06	0.35	0.12	0.55	0.67
RETSAT	retinol saturase (all-trans-retinol 13,14-reductase)	100519138	0.38	0.00184	0.52	0.02	0.08	0.04	0.52	1.13	0.63	0.08	0.42	0.35
LOC100511705	olfactory receptor 51F1-like	100511705	0.38	0.00956	0.16	-0.25	-0.34	0.24	0.83	0.93	0.55	0.86	0.47	0.36
LOC100523648	transcription factor COE4-like	100523648	0.38	0.00530	0.96	0.45	0.32	-0.20	0.85	-0.05	0.06	0.72	0.40	0.25
C20orf118	Uncharacterized protein C20orf118	100158114	0.38	0.00056	0.64	0.32	0.13	0.39	0.47	0.87	-0.12	0.09	0.48	0.48
GUCY1A2	guanylate cyclase 1, soluble, alpha 2	100522091	0.38	0.00609	-0.03	0.05	0.28	-0.10	0.63	1.27	0.48	0.50	0.32	0.37
LOC100626612	proline-rich protein 15-like protein-like	100626612	0.37	0.00282	0.60	0.37	0.13	0.12	0.44	1.28	0.03	0.25	0.55	0.26
LOC100521423	adenylate kinase isoenzyme 1-like	100521423	0.37	0.00446	0.57	0.07	0.07	0.66	1.10	0.60	0.00	0.04	0.10	0.50
LOC100624704	rap1 GTPase-activating protein 2-like	100624704	0.37	0.00069	0.77	0.41	0.10	0.07	0.69	0.67	0.10	0.23	0.44	0.21
LOC100520886	syntaxin-3-like	100520886	0.37	0.00432	0.67	0.39	-0.03	0.25	1.03	0.47	-0.21	0.03	0.81	0.28
LOC100623939	phospholemman-like	100623939	0.37	0.00735	0.28	-0.01	0.19	-0.17	0.54	1.26	0.43	0.12	0.77	0.27
LOC100157017	probable 2-ketogluconate reductase-like	100157017	0.37	0.00146	0.36	-0.13	0.32	0.39	0.57	0.96	0.11	0.23	0.54	0.32
LOC100518046	3-oxo-5-alpha-steroid 4-dehydrogenase 1-like	100518046	0.37	0.00085	0.69	0.21	0.40	0.32	0.66	0.81	0.39	0.04	-0.14	0.30

LOC1006		100621		0.00	0.5	-0.	-0.	0.5	0.4	1.0	0.1	0.1	0.9	0.1
21032	kelch-like protein 7-like	032	0.37	586	1	13	02	3	1	6	8	5	0	0
LOC1005	uncharacterized	100524		0.00	0.3	0.7	0.6	0.3	0.5	0.4	0.2	0.0	-0.	0.3
24863	LOC100524863	863	0.36	088	2	8	2	6	6	1	2	3	02	5
	1-acylglycerol-3-phosphat e O-acyltransferase 2 (lysophosphatidic acid	100170		0.00	0.6	0.2	0.4	0.4	0.2	0.1	0.6	0.3	0.4	0.1
AGPAT2	acyltransferase, beta)	768	0.36	002	2	8	1	3	2	6	2	1	0	9
	slit homolog 3	100513		0.00	-0.	0.5	0.4	0.0	0.1	0.9	0.0	-0.	0.6	0.8
SLIT3	(Drosophila)	444	0.36	428	01	7	9	3	0	6	7	06	4	3
	mitochondrial fission	100513		0.00	0.6	-0.	0.3	0.1	0.0	0.7	0.3	0.4	0.3	0.6
MFF	factor	442	0.36	063	6	11	2	4	6	7	9	0	5	4
	ankyrin repeat													
LOC1006	domain-containing protein	100627		0.00	0.6	0.0	0.2	0.2	0.7	0.6	0.1	0.1	0.2	0.3
27989	31-like	989	0.36	047	1	5	5	9	8	8	9	1	9	7
LOC1005	cytosolic phospholipase	100513		0.00	0.9	0.0	-0.	0.2	0.9	0.5	0.4	-0.	0.4	0.4
13834	A2 zeta-like	834	0.36	962	4	5	24	4	9	1	7	23	8	1
LOC1005	cytochrome P450	100521		0.00	0.5	0.4	0.2	0.6	0.7	0.1	0.1	0.0	0.1	0.4
21659	26B1-like	659	0.36	082	5	6	0	0	8	9	1	8	8	0
	BTB (POZ) domain	100156		0.00	0.6	0.1	0.0	0.2	0.6	0.7	0.2	0.1	0.5	0.2
BTBD3	containing 3	598	0.36	041	2	0	6	4	9	2	2	2	2	8
	integral membrane protein	100144		0.00	0.5	0.1	0.1	0.0	0.9	0.8	0.2	0.0	0.2	0.3
ITM2C	2C	481	0.35	145	1	4	3	6	3	5	7	2	9	5
LOC1005		100517		0.00	0.3	0.2	-0.	0.0	0.6	1.3	0.0	0.1	0.2	0.6
17092	carboxypeptidase M-like	092	0.35	950	7	6	14	3	2	3	6	5	2	1
	CDC42 binding protein													
CDC42B	kinase gamma	100520		0.00	0.6	0.4	-0.	0.5	0.6	0.0	0.3	-0.	0.6	0.3
PG	(DMPK-like)	358	0.35	151	7	5	02	1	3	0	3	02	5	0
	thiosulfate sulfurtransferase/rhodanes													
LOC1001	e-like domain-containing	100158		0.00	0.3	-0.	0.0	0.1	0.5	0.9	0.4	0.1	0.4	0.4
58075	protein 1-like	075	0.35	146	7	09	9	0	7	4	6	6	7	5
LOC1007	OCIA domain-containing	100738		0.00	0.3	-0.	0.1	-0.	1.1	0.1	0.2	0.4	0.5	0.7
38638	protein 2-like	638	0.35	893	3	03	9	23	5	7	2	3	5	1
LOC1006	heat-stable enterotoxin	100626		0.00	0.7	-0.	0.1	-0.	0.8	0.8	-0.	-0.	0.7	0.3
26467	receptor-like	467	0.35	999	6	09	6	01	7	7	08	12	7	4
	S100 calcium binding	100153		0.00	0.6	0.3	0.6	0.5	0.4	0.5	0.0	-0.	0.2	0.1
S100A14	protein A14	930	0.35	084	6	6	1	5	5	2	2	14	7	6
				0.00	0.4	-0.	0.2	0.0	0.9	0.2	0.3	0.1	0.5	0.6
FAAH	fatty acid amide hydrolase	396949	0.35	291	7	14	3	6	3	8	1	0	5	8
LOC1005		100514		0.00	0.3	-0.	0.3	0.1	0.5	0.5	0.0	0.3	0.6	0.5
14762	F-box only protein 48-like	762	0.35	069	1	07	9	0	8	8	4	1	6	6
	multidrug													
LOC1006	resistance-associated	100620		0.00	0.5	-0.	0.6	-0.	0.2	0.8	0.4	0.0	0.4	0.5
20159	protein 4-like	159	0.34	192	7	11	0	12	0	1	8	7	3	2
	signal peptide, CUB	100524		0.00	0.3	0.3	0.0	0.0	0.6	0.1	0.5	0.0	0.9	0.4
SCUBE1	domain, EGF-like 1	621	0.34	242	3	4	1	7	3	3	4	5	1	3
	patatin-like phospholipase	100049		0.00	0.5	-0.	0.1	0.0	1.1	0.5	0.2	-0.	0.4	0.5
PNPLA2	domain containing 2	704	0.34	501	5	04	3	8	4	1	4	14	2	4
		100523		0.00	0.3	0.2	0.2	0.5	0.5	0.1	0.2	0.3	0.6	0.3
FA2H	fatty acid 2-hydroxylase	966	0.34	007	6	1	0	0	0	1	2	5	7	0
	Zinc binding alcohol													
LOC1001	dehydrogenase, domain	100156		0.00	0.6	0.2	0.7	-0.	0.2	0.5	0.7	-0.	0.1	0.3
56930	containing 1	930	0.34	472	9	6	5	04	1	6	3	14	0	0
LOC1005	uncharacterized protein	100511		0.00	0.0	0.6	0.6	0.3	0.2	0.9	0.0	0.3	0.1	-0.
11991	C12orf69-like	991	0.34	306	3	0	2	9	8	3	8	7	3	01
C2H11orf	chromosome 11 open	100513		0.00	0.1	-0.	0.2	0.0	0.7	0.6	0.4	-0.	0.7	0.7
49	reading frame 49 ortholog	582	0.34	702	5	13	2	1	9	0	1	08	4	0
LOC1005		100513		0.00	-0.	0.3	0.8	0.5	0.3	0.4	0.3	0.2	0.0	0.4
13311	olfactory receptor 5K1-like	311	0.34	080	17	2	1	0	9	7	7	7	2	2
	trafficking protein, kinesin	100522		0.00	0.3	0.0	0.1	0.3	0.8	0.4	0.0	0.1	0.6	0.3
TRAK1	binding 1	277	0.34	076	1	7	7	2	8	6	3	3	5	8
LOC1006		100626		0.00	0.6	0.9	0.2	0.2	0.4	0.0	0.7	-0.	0.0	0.0
26257	UPF0632 protein A-like	257	0.34	560	9	5	1	6	4	9	2	08	9	4
LOC1005	uncharacterized	100513		0.00	0.8	0.0	0.2	0.2	1.0	0.3	0.5	-0.	0.2	0.3
13188	LOC100513188	188	0.34	973	0	1	3	1	9	1	0	34	0	8
	receptor tyrosine kinase-like orphan receptor	100520		0.00	0.7	0.0	0.3	0.2	0.6	0.4	0.3	-0.	0.3	0.3
ROR1	1	620	0.34	103	7	8	1	8	8	5	4	13	0	1
LOC1007		100736		0.00	0.3	0.1	0.5	0.2	0.5	0.8	0.0	-0.	0.5	0.4
36803	protein S100-A1-like	803	0.34	384	9	2	5	0	4	1	9	36	8	6
				0.00	-0.	0.7	0.7	0.2	0.3	0.7	0.3	-0.	-0.	0.5
PC	pyruvate carboxylase	397630	0.34	544	13	8	3	1	7	1	4	14	02	4
LOC1005	transmembrane protein	100523		0.00	0.3	-0.	0.0	0.5	0.9	0.1	-0.	0.4	0.6	0.3
23460	236-like	460	0.34	414	8	12	9	6	6	4	15	4	8	9
LOC1006	hydroxymethylglutaryl-Co	100621		0.00	0.5	0.1	0.2	-0.	0.5	0.9	0.5	0.1	0.2	0.3
21218	A lyase,	218	0.34	218	2	6	3	20	1	6	5	4	1	1

	mitochondrial-like												
LOC1006	uncharacterized	100626		0.00	0.7	0.0	0.2	0.3	-0.	0.3	0.2	0.2	0.8	0.2
26613	LOC100626613	613	0.34	165	3	7	1	8	04	7	3	9	6	8
	cadherin-related family	100514		0.00	0.8	0.1	-0.	-0.	0.8	0.6	0.1	-0.	0.5	0.5
CDHR5	member 5	561	0.34	633	1	9	06	14	5	4	0	09	5	2
	tubulin tyrosine ligase-like	100621		0.00	0.0	0.0	0.4	0.5	0.2	0.8	0.5	0.1	-0.	0.6
TTLL1	family, member 1	517	0.34	326	3	3	7	0	6	4	8	6	15	6
	ly6/PLAUR													
LOC1006	domain-containing protein	100621		0.00	0.5	0.3	0.3	0.2	0.7	0.4	0.1	-0.	0.3	0.4
21830	6B-like	830	0.34	140	3	1	1	5	9	7	5	28	8	4
LOC1001	ATP-binding cassette,	100155		0.00	0.7	0.1	-0.	-0.	0.6	0.6	0.2	-0.	0.5	0.4
55585	sub-family B, member 6	585	0.34	213	2	3	03	04	6	5	8	01	7	3
	phosphatidylinositol													
LOC1005	4,5-bisphosphate	100510		0.00	0.7	0.2	0.2	0.0	0.3	0.2	0.5	-0.	0.4	0.4
10987	5-phosphatase A-like	987	0.33	101	7	6	9	3	9	2	1	04	3	8
		100156		0.00	0.5	-0.	0.2	0.1	0.6	0.3	0.3	0.1	0.5	0.5
VIL1	villin 1	373	0.33	049	8	13	4	5	3	6	0	1	4	6
LOC1007	neurobeachin-like protein	100738		0.00	0.5	-0.	0.1	-0.	0.6	0.8	0.4	-0.	0.6	0.2
38323	1-like	323	0.33	371	7	05	2	08	3	8	4	06	0	8
	pleckstrin and Sec7	100623		0.00	0.4	0.5	0.3	0.3	0.4	0.4	0.2	0.1	0.0	0.3
PSD3	domain containing 3	956	0.33	004	5	4	0	0	2	4	7	9	9	2
	chloride channel protein 2			0.00	0.5	0.0	0.4	0.4	0.3	-0.	0.5	0.0	0.4	0.5
CLC-2	variant	397147	0.33	030	0	9	3	2	6	05	9	6	1	3
				0.00	0.0	-0.	0.3	0.4	0.8	0.3	0.3	-0.	0.4	0.6
ACY1	aminoacylase 1	396930	0.33	255	5	01	9	4	2	2	5	12	4	5
LOC1005	uncharacterized protein	100525		0.00	0.5	0.5	0.0	0.3	0.3	0.5	0.3	0.2	0.4	0.0
25667	C8orf48-like	667	0.33	051	4	4	0	5	6	2	1	5	2	3
		100523		0.00	0.7	-0.	0.1	0.1	0.8	0.4	0.2	0.1	0.5	0.2
LDHD	lactate dehydrogenase D	064	0.33	160	0	13	9	4	1	0	8	6	7	2
LOC1005	proteinase-activated	100519		0.00	0.4	0.1	0.1	0.0	0.8	0.3	-0.	0.1	0.6	0.5
19703	receptor 2-like	703	0.33	093	3	8	8	9	3	5	06	8	1	3
		100515		0.00	0.8	-0.	0.1	0.1	0.6	0.0	0.4	0.0	0.5	0.4
HOXD10	homeobox D10	344	0.33	332	5	06	5	0	9	7	6	1	8	7
LOC1005		100519		0.00	0.5	0.3	0.0	0.1	0.4	0.4	0.2	0.0	0.5	0.4
19058	frizzled-5-like	058	0.33	032	8	5	1	0	9	2	4	4	8	9
	bone morphogenetic	100157		0.00	0.4	0.9	0.3	0.2	0.3	0.1	-0.	0.4	0.6	-0.
BMP2	protein 2	103	0.33	252	3	3	0	7	7	4	12	1	2	05
	tyrosyl-DNA	100157		0.00	0.5	0.0	0.1	0.1	0.2	0.8	0.0	0.0	0.7	0.5
TDP2	phosphodiesterase 2	860	0.33	182	2	5	6	8	4	2	4	4	5	1
LOC1005	E3 ubiquitin-protein ligase	100518		0.00	0.6	0.0	0.1	-0.	0.8	0.5	0.4	0.0	0.4	0.5
18407	Praja-1-like	407	0.33	544	0	5	2	30	1	0	3	7	4	7
LOC1006	ribonucleoprotein	100623		0.00	0.6	0.0	-0.	0.0	0.6	0.9	0.1	0.1	0.4	0.2
23447	PTB-binding 2-like	447	0.33	341	3	0	03	5	0	8	3	9	6	8
	pro-neuregulin-3,													
LOC1006	membrane-bound	100622		0.00	0.0	0.0	0.7	0.7	0.4	0.3	0.4	0.2	-0.	0.4
22354	isoform-like	354	0.33	170	0	0	2	1	5	3	7	1	05	4
	leucine-rich repeats and													
	immunoglobulin-like	100154		0.00	0.6	0.5	0.0	0.0	0.3	0.6	0.1	0.2	0.5	0.0
LRIG2	domains 2	795	0.33	103	5	1	0	0	4	9	8	4	6	9
	peroxisome													
	proliferator-activated			0.00	0.7	0.2	0.0	0.0	0.4	0.5	0.4	0.1	0.3	0.3
PPARG	receptor gamma	397671	0.33	030	2	9	0	6	0	8	0	4	3	2
	V-set and immunoglobulin	100153		0.00	0.4	0.1	0.3	0.1	0.5	0.2	0.3	0.1	0.6	0.2
VSIG10	domain containing 10	531	0.32	016	9	9	1	6	0	4	6	1	3	5
LOC1005	uncharacterized	100516		0.00	0.9	0.3	0.2	0.2	0.2	0.5	0.1	0.2	-0.	0.4
16232	LOC100516232	232	0.32	449	9	6	2	1	4	1	4	3	14	7
	diphosphoinositol													
LOC1005	polyphosphate	100519		0.00	0.4	0.1	-0.	-0.	0.5	1.0	0.4	0.2	0.1	0.4
19192	phosphohydrolase 2-like	192	0.32	395	4	6	01	11	1	4	5	3	2	0
	hyaluronoglucosaminidase			0.00	0.5	0.4	0.1	0.1	0.5	-0.	0.3	0.1	0.8	0.4
HYAL1	1	404698	0.32	295	9	2	3	0	2	25	1	1	3	4
	ADAM metallopeptidase													
ADAMT	with thrombospondin type	100522		0.00	0.1	0.2	0.7	0.0	0.4	0.8	0.3	0.2	-0.	0.4
S5	1 motif, 5	952	0.32	861	7	4	2	1	9	6	9	8	38	3
LOC1005	uncharacterized	100518		0.00	0.4	0.3	0.6	0.7	0.4	0.1	-0.	0.3	0.5	0.1
18838	LOC100518838	838	0.32	524	2	3	5	1	2	0	42	2	0	7
	peptidase domain													
	containing associated with	100626		0.00	0.3	-0.	0.6	0.4	0.1	0.6	-0.	0.7	0.0	0.2
PAMR1	muscle regeneration 1	770	0.32	321	6	01	4	2	2	9	05	1	5	6
	replication factor C	100157		0.00	0.6	0.0	0.1	0.3	0.8	0.2	0.2	0.2	0.2	0.1
RFC4	(activator 1) 4, 37kDa	731	0.32	105	3	1	6	6	6	6	7	6	2	4
	alcohol dehydrogenase 5	100513		0.00	0.4	0.1	0.3	0.2	0.4	1.0	0.2	0.1	0.1	0.0
ADH5	(class III), chi polypeptide	555	0.32	162	1	7	3	1	0	2	5	9	5	3
LOC1005		100518		0.00	0.2	0.6	0.0	-0.	0.1	-0.	0.2	0.4	0.7	0.9
18535	olfactory receptor 9G1-like	535	0.32	934	8	7	7	06	7	29	8	2	0	3

LOC100155431	beta-1,3-galactosyltransferase 4-like	100155431	0.32	0.00088	0.44	0.01	0.42	0.27	0.71	0.24	0.31	0.10	0.27	0.39
LOC100522516	glycerate kinase-like	100522516	0.32	0.00567	0.71	0.17	0.07	0.25	0.83	0.24	0.04	-0.414	0.45	0.54
LOC100524216	protein FAM101B-like	100524216	0.31	0.00082	0.70	0.40	0.18	0.27	0.71	0.14	0.30	-0.08	0.24	0.30
LOC100524459	mitochondrial carnitine/acylcarnitine carrier protein-like	100524459	0.31	0.00852	0.77	-0.02	0.17	-0.04	0.62	0.89	0.04	0.02	0.16	0.53
LOC100622182	uncharacterized LOC100622182	100622182	0.31	0.00343	0.32	0.09	0.08	0.42	0.99	0.63	-0.05	0.10	0.57	0.08
ANO9	anoctamin 9	100627366	0.31	0.00056	0.35	0.25	-0.13	0.10	0.48	0.34	0.42	0.20	0.68	0.41
LOC100516303	riboflavin transporter 2-like	100516303	0.31	0.00760	0.71	0.48	-0.41	0.40	0.60	0.38	0.18	-0.12	0.58	0.31
FRRS1	ferric-chelate reductase 1	100151766	0.31	0.00598	0.58	0.16	-0.17	0.01	0.31	0.97	0.22	0.12	0.31	0.59
LOC100623545	olfactory receptor 6C3-like	100623545	0.31	0.00212	0.09	0.21	0.06	0.28	0.81	0.28	0.71	0.22	0.29	0.15
TXNIP	thioredoxin interacting protein	733688	0.31	0.00009	0.54	0.20	0.08	0.07	0.59	0.42	0.22	0.28	0.43	0.27
STYK1	serine/threonine/tyrosine kinase 1	100523437	0.31	0.00950	0.40	0.22	0.05	0.14	0.68	0.92	-0.42	0.25	0.62	0.24
LOC100152988	3-ketodihydrosphingosine reductase-like	100152988	0.31	0.00370	0.50	0.00	-0.11	0.29	0.47	0.81	0.26	-0.02	0.65	0.23
LOC100525333	mu-crystallin homolog	100525333	0.31	0.00899	0.18	0.32	0.12	-0.33	0.73	1.02	0.45	0.56	0.23	0.18
LOC100524003	taste receptor type 2 member 41-like	100524003	0.31	0.00550	0.28	0.18	0.16	-0.07	0.78	0.18	0.66	0.09	0.15	0.67
USP32	ubiquitin specific peptidase 32	100512129	0.31	0.00202	0.71	0.59	0.10	-0.01	0.11	0.22	0.36	0.50	0.42	0.08
CDKN2B	cyclin-dependent kinase inhibitor 2B (p15, inhibits CDK4)	397227	0.31	0.00655	0.86	0.07	0.21	0.33	0.74	0.56	0.02	-0.25	0.32	0.20
LOC100155473	dedicator of cytokinesis protein 1-like	100155473	0.31	0.00627	0.86	-0.28	0.19	0.19	0.55	0.59	0.11	0.14	0.13	0.60
VEGFA	vascular endothelial growth factor A	397157	0.31	0.00432	0.53	0.68	0.22	0.25	0.82	0.33	0.04	-0.15	-0.01	0.27
LOC100738721	neurexin-1-alpha-like	100738721	0.31	0.00937	0.28	0.39	0.18	-0.18	0.66	0.69	0.53	0.04	0.54	-0.15
LOC100516823	consortin-like	100516823	0.31	0.00409	0.38	0.21	0.43	-0.04	0.10	0.91	0.07	0.12	0.64	0.23
SLC9A3R1	solute carrier family 9, subfamily A (NHE3, cation proton antiporter 3), member 3 regulator 1	100233201	0.31	0.00007	0.54	0.15	0.16	0.08	0.38	0.43	0.31	0.21	0.49	0.29
LOC100521068	olfactory receptor 7E24-like	100521068	0.31	0.00255	-0.06	0.65	0.06	0.47	0.71	-0.16	0.26	0.48	0.44	0.41
LOC100515293	TOMM20-like protein 1-like	100515293	0.30	0.00542	0.63	0.56	0.32	-0.03	0.20	-0.09	0.68	0.05	0.55	0.17
SLC45A4	solute carrier family 45, member 4	100154055	0.30	0.00440	0.46	0.27	-0.21	0.22	0.98	0.36	0.25	0.14	0.29	0.30
LOC100518272	growth hormone-regulated TBC protein 1-like	100518272	0.30	0.00906	0.42	-0.16	-0.22	0.35	0.81	0.24	0.51	-0.04	0.77	0.37
LOC100621023	uncharacterized protein KIAA0146-like	100621023	0.30	0.00382	0.51	-0.10	0.28	0.22	0.27	0.90	0.01	0.23	0.22	0.50
TMEM63B	transmembrane protein 63B	100151872	0.30	0.00459	0.78	0.18	0.56	0.05	0.45	0.04	0.41	-0.16	0.11	0.61
LOC100520536	olfactory receptor 8H3-like	100520536	0.30	0.00245	-0.01	0.03	0.60	0.47	0.55	0.39	0.37	-0.20	0.23	0.60
LOC100517243	adipose most abundant gene transcript 2 protein-like	100517243	0.30	0.00667	0.09	0.30	0.56	0.07	0.61	0.81	0.01	0.20	0.03	0.28
LOC100520493	uncharacterized LOC100520493	100520493	0.30	0.00217	0.75	0.46	0.43	0.05	-0.12	0.07	0.15	0.33	0.36	0.53
LOC100522085	RELT-like protein 1-like	100522085	0.30	0.00023	0.35	0.43	0.12	0.20	0.63	0.35	0.09	0.06	0.96	0.66
COL4A5	collagen, type IV, alpha 5	100519180	0.30	0.00088	0.60	0.25	0.01	0.26	0.48	0.52	0.20	0.13	0.15	0.42
GPR39	G protein-coupled receptor 39	100158236	0.30	0.00143	0.48	0.57	-0.04	0.34	0.66	0.60	0.06	0.41	0.07	0.26
KIAA1671	Uncharacterized protein KIAA1671	100154475	0.30	0.00029	0.25	0.29	0.26	0.22	0.67	0.29	0.22	0.13	0.57	0.09
BCORL1	BCL6 corepressor-like 1	100525271	0.30	0.00487	0.64	-0.25	0.45	0.54	0.31	0.23	0.32	-0.05	0.50	0.32
ABCD3	ATP-binding cassette, sub-family D (ALD),	100626343	0.30	0.00260	0.63	0.04	0.47	-0.03	0.33	0.68	0.00	0.05	0.21	0.49

	member 3											
LHFPL2	lipoma HMGIC fusion partner-like 2	100523234	0.30	0.00081	0.33	0.29	0.43	0.46	0.73	0.26	0.23	0.02	-0.04	0.27
IGSF3	immunoglobulin superfamily, member 3	100512602	0.30	0.00095	0.59	0.42	0.28	0.27	0.21	0.47	0.05	0.10	0.11	0.46
LOC100621650	protein furry homolog	100621650	0.29	0.00307	0.52	0.20	0.18	0.22	0.33	0.37	0.18	0.04	0.06	0.40
LOC100624014	SPRY domain-containing SOCS box protein 4-like	100624014	0.29	0.00661	0.34	0.26	0.03	0.72	0.90	0.12	0.18	-0.05	0.15	0.30
LOC100157757	serine/threonine-protein kinase Sgk2-like	100157757	0.29	0.00826	0.31	-0.08	0.25	-0.10	0.38	0.80	0.13	0.01	0.39	0.85
EPHX2	epoxide hydrolase 2, cytoplasmic	414425	0.29	0.00200	0.19	-0.22	0.28	0.35	0.49	0.75	0.23	0.32	0.29	0.25
ITPK1	inositol-tetrakisphosphate 1-kinase	100152339	0.29	0.00193	0.48	0.15	0.22	0.35	0.67	0.33	0.49	-0.09	-0.13	0.45
LOC100513365	microsomal glutathione S-transferase 2-like	100513365	0.29	0.00659	0.48	0.47	-0.20	0.11	-0.03	0.95	0.27	0.21	0.40	0.22
LOC100153684	uncharacterized protein C14orf43-like	100153684	0.29	0.00139	0.86	0.38	0.17	0.05	0.21	0.28	0.28	0.34	0.30	0.09
BDH1	3-hydroxybutyrate dehydrogenase, type 1	100155725	0.29	0.00896	0.44	-0.13	-0.12	0.19	0.62	0.81	0.42	-0.12	0.59	0.19
CPT1A	carnitine palmitoyltransferase 1A (liver)	399527	0.29	0.00497	0.27	0.45	0.47	0.46	0.48	-0.51	0.37	0.33	0.25	0.28
CLDN3	claudin 3	431781	0.28	0.00383	0.79	-0.03	0.12	0.15	0.54	0.20	0.20	-0.08	0.59	0.36
LOC100513308	transmembrane 4 L6 family member 5-like	100513308	0.28	0.00849	0.55	0.21	-0.19	0.32	0.76	0.37	0.29	-0.13	0.27	0.38
LOC100512228	uncharacterized LOC100512228	100512228	0.28	0.00468	0.66	0.32	0.04	0.40	0.28	-0.26	0.30	0.27	0.56	0.26
LOC100515771	transmembrane protein 205-like	100515771	0.28	0.00819	-0.03	-0.04	0.40	-0.15	0.79	0.61	0.28	0.24	0.49	0.20
LOC100736625	protein ETHE1, mitochondrial-like	100736625	0.28	0.00283	0.17	-0.07	0.33	0.08	0.54	0.80	0.05	0.19	0.23	0.47
LOC100516533	uncharacterized LOC100516533	100516533	0.28	0.00067	0.27	0.22	0.66	0.42	-0.07	0.34	0.23	0.14	0.29	0.28
CAMK1D	calcium/calmodulin-dependent protein kinase ID	100511150	0.28	0.00232	0.55	0.30	0.33	-0.25	0.11	0.39	0.25	0.24	0.61	0.24
LOC100511570	protein FAM46B-like	100511570	0.28	0.00751	0.27	0.17	-0.13	0.20	0.71	-0.02	0.67	0.23	0.17	0.50
MYH1	myosin, heavy chain 1, skeletal muscle, adult	100125538	0.28	0.00949	0.02	0.44	0.51	0.22	0.29	-0.03	0.78	0.19	0.36	-0.21
LOC100157003	protein amnionless-like	100157003	0.28	0.00498	0.77	0.14	0.10	0.16	0.70	0.27	0.01	0.03	0.30	0.30
LOC100524283	calcium-binding mitochondrial carrier protein Aralar1-like	100524283	0.28	0.00868	0.32	-0.27	0.35	0.18	0.60	0.30	0.71	-0.10	0.08	0.58
SYBU	syntabulin (syntaxin-interacting)	100157483	0.28	0.00987	0.45	0.16	0.07	0.02	0.22	0.54	-0.06	0.25	0.95	0.16
SLC39A14	solute carrier family 39 (zinc transporter), member 14	100152468	0.28	0.00029	0.62	0.23	0.22	0.23	0.36	0.30	0.08	0.02	0.39	0.29
INPP5F	inositol polyphosphate-5-phosphatase F	100144587	0.27	0.00321	0.37	0.24	-0.15	-0.01	0.02	0.53	0.31	0.59	0.52	0.33
NGFRAP1	nerve growth factor receptor (TNFRSF16) associated protein 1	100513514	0.27	0.00860	0.37	0.27	0.46	-0.18	0.90	0.15	0.14	0.25	0.08	0.30
LOC100511910	coronin-2A-like	100511910	0.27	0.00268	0.60	-0.10	0.34	0.33	-0.09	0.39	0.26	0.20	0.61	0.21
MAGI1	membrane associated guanylate kinase, WW and PDZ domain containing 1	100520932	0.27	0.00034	0.38	0.07	0.37	0.34	0.32	0.05	0.24	0.24	0.31	0.42
RHOF	ras homolog family member F (in filopodia)	100144500	0.27	0.00368	-0.14	-0.11	0.28	0.40	0.28	0.21	0.34	0.30	0.76	0.42
PALM	paralemmin	654410	0.27	0.00522	0.52	0.41	0.15	-0.01	0.67	0.33	0.46	0.01	-0.09	0.28
CDX2	caudal type homeobox 2	100127132	0.27	0.00072	0.71	0.11	0.30	0.33	0.15	0.18	0.40	0.07	0.31	0.37
HS3ST2	heparan sulfate (glucosamine) 3-O-sulfotransferase 2	100522426	0.27	0.00273	0.35	-0.03	0.09	0.37	0.56	0.32	0.36	0.41	0.27	0.04
LOC100518697	nostrin-like	100518697	0.27	0.00716	0.23	0.04	0.50	0.21	-0.28	0.34	0.24	0.09	0.66	0.58
RNF157	ring finger protein 157	100522340	0.27	0.00427	0.35	0.13	0.28	-0.10	0.49	0.48	0.12	-0.07	0.63	0.40

LOC100520809	uncharacterized LOC100520809	100520809	0.27	0.00042	0.43	0.25	0.13	0.15	0.51	0.23	0.21	-0.01	0.51	0.31
ARHGAP42	Rho GTPase activating protein 42	100519555	0.27	0.00617	0.46	-0.03	0.05	0.10	0.43	0.77	-0.15	0.18	0.50	0.39
LOC100626073	major facilitator superfamily domain-containing protein 9-like	100626073	0.27	0.00179	0.51	0.17	0.16	0.12	0.63	0.17	0.49	0.21	0.07	0.17
LOC100521277	receptor-type tyrosine-protein phosphatase F-like	100521277	0.27	0.00654	0.82	0.35	0.24	0.00	0.50	-0.08	0.30	-0.16	0.41	0.30
LOC100524521	keratin-like protein KRT222-like	100524521	0.27	0.00904	0.23	0.24	0.04	0.28	0.59	0.20	0.11	-0.07	0.82	0.24
LOC100515686	transmembrane protein 92-like	100515686	0.27	0.00057	0.29	0.13	0.21	0.36	0.56	0.41	0.11	-0.03	0.32	0.31
ADAM15	ADAM metallopeptidase domain 15	100153483	0.27	0.00519	0.68	0.47	0.16	0.15	0.58	-0.03	0.41	-0.12	0.23	0.15
LOC100624870	myeloma-overexpressed gene 2 protein homolog	100624870	0.27	0.00055	0.34	0.28	0.19	0.30	0.42	0.15	0.03	0.33	0.56	0.07
LOC100522569	complement C1q tumor necrosis factor-related protein 9A-like	100522569	0.27	0.00621	0.29	0.18	0.04	0.46	0.69	-0.04	0.68	0.25	-0.01	0.32
LOC100622619	liprin-beta-2-like	100622619	0.27	0.00605	0.54	0.14	-0.03	0.23	0.48	0.15	0.64	-0.15	0.34	0.33
MLXIPL	MLX interacting protein-like	100170769	0.27	0.00765	-0.01	0.56	0.57	0.16	0.46	0.67	0.13	-0.08	0.48	0.03
MYOG	myogenin (myogenic factor 4)	497618	0.27	0.00849	0.35	0.03	-0.21	0.49	0.51	0.24	0.45	0.16	0.58	0.06
KLF4	Kruppel-like factor 4 (gut)	595111	0.27	0.00123	0.54	0.34	0.42	0.07	0.18	0.09	0.31	-0.11	0.53	0.28
FRY	furry homolog (Drosophila)	100155799	0.26	0.00803	0.27	0.20	0.01	0.18	0.88	0.36	-0.28	0.25	0.40	0.39
LOC100524254	NADH-cytochrome b5 reductase 3-like	100524254	0.26	0.00396	0.49	-0.02	0.23	0.20	0.58	0.65	0.05	-0.09	0.35	0.20
LOC100522555	abhydrolase domain-containing protein FAM108C1-like	100522555	0.26	0.00248	0.64	0.28	0.13	0.21	0.64	0.32	0.11	-0.05	0.14	0.19
LOC100522154	uncharacterized LOC100522154	100522154	0.26	0.00246	0.56	0.23	0.04	0.22	0.77	0.25	0.04	0.18	0.00	0.23
LOC100512084	TBC1 domain family member 2A-like	100512084	0.26	0.00471	0.68	0.43	-0.08	0.14	0.07	0.35	0.01	0.45	0.51	0.06
DNAH1	dynein, axonemal, heavy chain 1	100156016	0.26	0.00828	-0.01	0.17	-0.08	0.61	0.55	0.00	0.39	0.18	0.32	0.47
LOC100736656	calcium/calmodulin-dependent protein kinase type 1D-like	100736656	0.26	0.00722	0.31	0.17	0.01	-0.10	0.47	0.49	0.58	-0.15	0.61	0.20
LOC100522322	transmembrane protein 82-like	100522322	0.26	0.00762	0.12	0.13	0.34	0.39	0.49	0.01	0.17	-0.06	0.50	0.59
RBFOX2	RNA binding protein, fox-1 homolog (C. elegans) 2	100513594	0.26	0.00839	0.42	0.15	0.29	0.09	0.60	0.76	-0.02	-0.15	0.22	0.22
LOC100738902	interleukin-17D-like	100738902	0.26	0.00768	0.07	0.19	0.58	0.13	0.33	0.41	-0.10	0.04	0.58	0.16
LOC100627497	FH2 domain-containing protein 1-like	100627497	0.26	0.00394	0.29	0.38	0.29	0.17	0.61	-0.14	0.16	0.13	0.32	0.36
PPP2R5D	protein phosphatase 2, regulatory subunit B', delta	100134967	0.25	0.00054	0.34	0.22	0.16	0.17	0.55	0.53	0.33	0.11	0.13	0.28
THRA	thyroid hormone receptor, alpha	397387	0.25	0.00371	0.56	0.14	0.05	0.03	0.38	0.45	0.25	-0.17	0.55	0.30
LOC100621118	abhydrolase domain-containing protein 14B-like	100621118	0.25	0.00410	0.43	0.05	-0.03	0.01	0.71	0.38	0.15	0.07	0.44	0.34
LOC100512279	BTB/POZ domain-containing protein KCTD17-like	100512279	0.25	0.00396	0.35	0.17	0.42	0.08	0.48	0.31	0.51	-0.12	0.09	0.25
ARHGEF19	Rho guanine nucleotide exchange factor (GEF) 19	100521628	0.25	0.00808	0.13	0.43	-0.11	0.18	0.63	-0.03	0.17	0.23	0.29	0.61
LOC100623005	uncharacterized protein C14orf43-like	100623005	0.25	0.00812	0.81	0.48	0.00	0.23	0.66	0.25	0.18	0.00	0.23	-0.09
LOC100151957	par-6 partitioning defective 6 homolog beta	100151957	0.25	0.00833	0.57	0.52	-0.01	0.31	-0.03	0.41	0.06	0.61	0.24	-0.15
TDRD7	tudor domain containing 7	100152652	0.25	0.00982	0.48	0.13	0.06	-0.19	0.49	0.44	0.04	0.06	0.65	0.18
LOC100518983	rap guanine nucleotide exchange factor 5-like	100518983	0.25	0.00464	0.43	0.27	-0.15	0.10	0.11	0.72	0.15	0.25	0.46	0.21
MAST2	microtubule associated serine/threonine kinase 2	100512112	0.25	0.00403	0.31	0.71	-0.10	0.02	0.38	0.35	0.25	0.36	0.24	0.00

LOC100518575	coiled-coil domain-containing protein 56-like	100518575	0.25	0.00063	0.21	0.17	-0.06	0.30	0.27	0.57	0.14	0.27	0.33	0.31
LOC100517809	uncharacterized LOC100517809	100517809	0.25	0.00945	0.31	0.08	0.13	0.48	0.68	0.19	0.48	-0.22	0.25	0.13
LUZP1	leucine zipper protein 1	100620499	0.25	0.00273	0.50	0.22	0.09	0.12	0.60	0.39	0.20	-0.15	0.20	0.33
LOC100524975	olfactory receptor 10H1-like	100524975	0.25	0.00524	0.22	0.29	-0.15	0.56	0.28	0.01	0.41	0.29	0.43	0.14
CPAMD8	C3 and PZP-like, alpha-2-macroglobulin domain containing 8	100515128	0.25	0.00747	0.39	0.27	-0.20	0.39	0.49	0.10	0.02	0.34	0.36	0.33
LNX2	ligand of numb-protein X 2	100510925	0.25	0.00018	0.46	0.23	0.16	0.12	0.99	0.33	0.26	0.08	0.35	0.18
DGAT	diacylglycerol acyltransferase	397118	0.25	0.00743	0.33	-0.06	0.12	0.13	0.62	0.55	0.25	-0.23	0.33	0.43
LOC100625977	dynein intermediate chain 1, axonemal-like	100625977	0.25	0.00462	0.23	0.31	0.29	0.53	0.42	-0.22	0.25	0.09	0.32	0.25
LRRFIP2	leucine rich repeat (in FLII) interacting protein 2	100626059	0.24	0.00200	0.17	-0.04	0.01	0.33	0.77	0.45	0.11	0.09	0.44	0.33
MGLL	monoglyceride lipase	100233193	0.24	0.00252	0.29	-0.10	0.37	0.27	0.21	0.49	0.16	0.05	0.16	0.53
GJD3	gap junction protein, delta 3, 31.9kDa	100048959	0.24	0.00507	0.39	0.23	0.33	0.17	0.68	0.18	0.12	-0.04	-0.05	0.42
MYO1E	myosin IE	100154204	0.24	0.00053	0.51	0.07	0.07	0.31	0.30	0.16	0.27	0.09	0.31	0.33
MICAL2	microtubule associated monoxygenase, calponin and LIM domain containing 2	100519075	0.24	0.00316	0.50	0.24	0.01	0.11	0.68	0.30	0.23	-0.08	0.19	0.25
ANKRD46	ankyrin repeat domain 46	100152758	0.24	0.00359	0.28	0.39	-0.04	0.23	0.32	0.66	0.00	0.10	0.38	0.09
LOC100518928	putative cleavage and polyadenylation specificity factor subunit 4-like protein-like	100518928	0.24	0.00954	0.12	0.54	0.15	0.67	0.14	0.05	0.27	0.04	0.37	0.07
FOXO3	forkhead box O3	733621	0.24	0.00075	0.26	0.09	0.03	0.23	0.79	0.48	0.12	0.32	0.25	0.09
CYP17A1	cytochrome P450 17A1	403330	0.24	0.00954	0.25	0.08	0.04	0.22	0.25	0.39	-0.12	0.00	0.54	0.46
LOC100625066	zinc finger protein 500-like	100625066	0.24	0.00905	0.11	0.11	0.53	0.77	0.11	-0.23	0.23	0.37	0.11	0.30
LOC100154044	bcl-2-like protein 2-like	100154044	0.24	0.00899	0.17	0.41	0.39	0.11	0.57	0.39	0.15	-0.18	0.21	0.20
SMAD6	SMAD family member 6	100152069	0.24	0.00372	0.66	0.24	0.19	0.06	0.34	0.26	0.26	0.11	0.11	0.17
ZNF605	zinc finger protein 605	100155634	0.24	0.00576	0.23	0.28	0.30	-0.05	0.34	0.06	0.41	0.03	0.57	0.22
LOC100156267	CLRN3 protein	100156267	0.24	0.00522	0.02	0.15	-0.20	0.30	0.36	0.45	0.20	0.21	0.67	0.23
LOC100156669	protein FAM89A-like	100156669	0.24	0.00192	0.35	0.50	0.16	-0.02	0.24	0.14	0.25	0.28	0.33	0.17
LOC100152820	UPF0235 protein C15orf40 homolog	100152820	0.24	0.00516	0.39	0.41	0.24	-0.15	0.34	0.41	0.11	0.12	0.15	0.26
LOC100516293	phosphoinositide 3-kinase regulatory subunit 4-like	100516293	0.24	0.00162	0.40	-0.02	0.17	0.04	0.47	0.06	0.25	0.18	0.40	0.42
AKAP12	A kinase (PRKA) anchor protein 12	100152595	0.24	0.00531	0.30	0.12	-0.09	-0.03	0.29	0.70	0.36	0.12	0.33	0.27
RMND5A	required for meiotic nuclear division 5 homolog A (S. cerevisiae)	100516072	0.24	0.00529	0.44	0.14	0.17	-0.10	0.21	0.67	0.12	0.21	0.48	0.01
LOC100525411	nesprin-3-like	411	0.23	0.00252	0.33	0.02	0.26	0.42	0.21	0.10	0.25	-0.01	0.32	0.25
ACAP2	ArfGAP with coiled-coil, ankyrin repeat and PH domains 2	100627914	0.23	0.00206	0.34	0.12	0.11	0.44	0.22	0.62	0.07	0.38	0.25	0.10
LOC100621028	RING finger protein 223-like	100621028	0.23	0.00487	0.53	0.20	0.06	0.58	0.44	-0.03	0.07	0.10	0.22	0.16
LOC100153977	Glutamate-cysteine ligase, modifier subunit	100153977	0.23	0.00543	0.26	0.11	0.64	0.32	-0.18	0.05	0.28	0.21	0.40	0.25
LOC100516614	plasmolipin-like	100516614	0.23	0.00454	0.57	0.26	0.14	0.13	0.27	-0.15	0.31	0.00	0.35	0.46
LOC100512419	lipid phosphate phosphohydrolase 3-like	100512419	0.23	0.00696	0.64	0.17	0.50	-0.22	0.22	0.32	0.38	0.05	0.05	0.21
ZSWIM6	zinc finger, SWIM-type containing 6	100620165	0.23	0.00142	0.40	0.14	0.04	0.66	0.99	0.66	0.24	0.09	0.28	0.30

LOC100526142	uncharacterized LOC100526142	100526142	0.23	0.00590	0.29	0.09	-0.07	0.18	0.29	0.18	0.43	0.07	0.39	0.45
KREMEN1	kringle containing transmembrane protein 1	100154052	0.23	0.00987	0.64	0.07	0.24	0.33	0.33	-0.07	0.10	-0.14	0.45	0.15
GLTP	glycolipid transfer protein	396765	0.23	0.00180	0.20	0.37	0.16	0.13	0.50	0.50	-0.09	0.16	0.19	0.17
KCNH6	potassium voltage-gated channel, subfamily H (eag-related), member 6	100514537	0.23	0.00778	0.10	-0.05	0.43	0.32	0.28	-0.07	0.25	0.35	0.31	0.40
LOC100522542	uncharacterized LOC100522542	100522542	0.23	0.00600	-0.03	0.41	-0.04	0.39	0.60	0.05	0.10	0.29	0.23	0.29
ASXL3	additional sex combs like 3 (Drosophila)	100518495	0.23	0.00869	0.24	0.39	0.29	0.57	0.47	0.04	-0.02	0.14	0.25	-0.10
LOC100517478	putative hydroxypyruvate isomerase-like	100517478	0.23	0.00788	0.29	-0.12	0.32	0.30	0.68	0.31	0.04	0.07	0.18	0.20
LMNA	lamin A/C	100126859	0.23	0.00600	0.52	-0.01	0.22	0.39	0.56	0.16	-0.09	-0.05	0.22	0.35
TMEM171	transmembrane protein 171	100514277	0.23	0.00660	0.11	0.43	0.00	0.35	0.28	0.70	-0.17	0.20	0.23	0.14
SERINC2	serine incorporator 2	100626004	0.23	0.00148	0.33	0.08	0.27	0.18	0.24	0.25	0.31	-0.16	0.39	0.37
LOC100523918	LIM domain only protein 7-like	100523918	0.22	0.00979	0.12	0.05	0.22	0.54	-0.01	-0.17	0.23	0.56	0.62	0.18
MBOAT1	membrane bound O-acyltransferase domain containing 1	100152017	0.22	0.00020	0.11	0.08	0.15	0.25	0.32	0.21	0.19	0.26	0.31	0.36
LOC100525150	CD44 antigen-like	100525150	0.22	0.00143	0.24	0.22	0.21	-0.08	0.33	0.54	0.34	0.20	0.06	0.17
HSD17B8	hydroxysteroid (17-beta) dehydrogenase 8	100154372	0.22	0.00671	0.05	-0.14	0.25	0.06	0.66	0.55	0.44	0.30	0.28	0.27
LOC100621404	protein unc-13 homolog B-like	100621404	0.22	0.00508	-0.07	0.03	0.24	0.09	0.44	0.33	0.01	0.26	0.66	0.33
LOC100515636	2-oxoglutarate dehydrogenase, mitochondrial-like	100515636	0.22	0.00636	0.49	-0.06	-0.07	0.04	0.45	0.18	0.33	0.03	0.34	0.48
COX10	COX10 homolog, cytochrome c oxidase assembly protein, heme A: farnesyltransferase (yeast)	100521863	0.22	0.00063	0.20	0.08	0.15	0.24	0.29	0.21	0.20	0.43	0.15	0.27
LOC100737883	UPF0366 protein C11orf67 homolog	100737883	0.22	0.00561	0.38	0.15	0.19	0.21	0.20	0.46	-0.01	0.07	0.13	0.44
FAM107A	family with sequence similarity 107, member A	100516512	0.22	0.00237	0.41	0.21	0.33	0.27	0.10	0.33	0.17	0.15	0.03	0.23
ZNF410	zinc finger protein 410	100512059	0.22	0.00205	0.26	0.32	0.07	0.38	0.27	0.39	0.16	0.15	0.35	0.06
LOC100739329	uncharacterized LOC100739329	100739329	0.22	0.00959	0.59	0.21	0.18	0.11	0.32	0.47	0.17	-0.28	0.04	0.18
LOC100625487	lon protease homolog 2, peroxisomal-like	100625487	0.22	0.00136	0.43	0.28	0.20	0.00	0.00	0.30	0.15	0.27	0.40	0.08
BCL2L13	BCL2-like 13 (apoptosis facilitator)	100517587	0.22	0.00847	0.32	0.34	0.19	-0.13	0.15	0.73	0.01	0.19	0.25	0.13
OSBPL10	oxysterol binding protein-like 10	100526184	0.22	0.00396	0.11	0.03	0.18	0.11	0.45	0.10	0.47	0.08	0.16	0.39
TXNRD1	thioredoxin reductase 1	396681	0.22	0.00020	0.27	0.18	0.29	0.37	0.07	0.18	0.19	0.30	0.20	0.13
LOC100156717	heparan-sulfate 6-O-sulfotransferase 1-like	100156717	0.22	0.00889	0.20	0.58	0.25	0.24	0.57	0.00	0.16	0.11	-0.06	0.13
B4GALT5	UDP-Gal:betaGlcNAc beta 1,4-galactosyltransferase, polypeptide 5	100522653	0.22	0.00070	0.35	0.17	0.15	0.07	0.50	0.02	0.21	0.16	0.28	0.27
DOK4	docking protein 4	100620637	0.22	0.00970	0.58	0.44	-0.15	0.26	0.05	0.29	0.14	0.04	0.16	0.34
LOC100517911	ankyrin repeat and SOCS box protein 13-like	100517911	0.22	0.00277	0.38	0.14	0.34	0.10	0.39	0.35	0.12	-0.12	0.28	0.16
LOC100158185	adenine nucleotide translocator 2	100158185	0.21	0.00183	0.28	0.11	-0.19	0.27	0.16	0.40	0.19	0.03	0.40	0.22
LOC100736777	E3 SUMO-protein ligase NSE2-like	100736777	0.21	0.00725	0.08	0.36	0.23	0.55	0.22	0.43	-0.15	0.07	0.36	0.28
PTPRS	protein tyrosine phosphatase, receptor type, S	100515359	0.21	0.00697	0.45	0.12	0.18	0.19	0.59	0.39	0.08	-0.02	-0.03	0.18
SBF2	SET binding factor 2	100521123	0.21	0.00728	0.40	-0.09	0.26	-0.11	0.23	0.59	0.26	0.16	0.24	0.19
NECD	necdin	100144475	0.21	0.00652	0.15	0.20	0.04	0.11	0.19	0.18	0.25	0.41	0.02	0.37

LOC100622361	uncharacterized protein C7orf36-like	100622361	0.21	0.00997	0.56	0.01	0.29	0.22	0.48	0.11	0.03	-0.10	0.30	0.22
LOC100523432	protein deltex-2-like	100523432	0.21	0.00837	-0.13	0.27	0.29	0.06	0.35	0.08	0.12	0.45	0.19	0.44
CAPN5	calpain 5	100524513	0.21	0.00407	0.55	0.12	0.19	0.22	0.41	0.02	0.11	-0.09	0.34	0.25
LOC100520165	transmembrane protein 164-like	100520165	0.21	0.00324	0.43	0.28	0.24	0.44	0.77	0.22	0.99	0.33	0.99	0.31
SGK1	serum/glucocorticoid regulated kinase 1	100625739	0.21	0.00716	-0.074	0.04	0.88	0.73	0.53	0.16	0.26	-0.601	0.01	0.32
TET3	tet methylcytosine dioxygenase 3	100520484	0.21	0.00640	0.61	0.38	0.16	0.09	0.19	0.22	0.00	0.22	0.21	0.00
LOC100516943	SET-binding protein-like	100516943	0.21	0.00933	0.15	0.05	0.57	0.11	0.09	0.11	0.99	0.22	0.11	0.09
NHSL1	NHS-like 1	100156257	0.21	0.00437	0.41	0.35	0.00	0.16	0.31	0.04	0.27	-0.704	0.26	0.33
LOC100627727	UDP-glucose 6-dehydrogenase-like	100627727	0.21	0.00414	0.36	-0.608	0.22	0.11	0.03	0.51	0.06	0.45	0.20	0.19
LOC10052525860	clathrin light chain B-like	100525860	0.21	0.00516	0.34	0.34	0.10	0.30	0.00	0.14	-0.104	0.24	0.57	0.14
LOC100627418	cyclin-dependent kinase 18-like	100627418	0.21	0.00403	0.35	0.07	0.33	-0.1007	0.47	0.17	0.23	0.14	0.08	0.32
LOC10052424137	ankyrin repeat domain-containing protein 43-like	100524137	0.20	0.00131	0.24	0.03	0.28	0.19	0.29	0.29	0.21	0.04	0.44	0.33
NET1	neuroepithelial cell transforming 1	100516825	0.20	0.00459	0.35	0.08	-0.04	-0.02	0.41	0.43	0.10	0.06	0.35	0.29
LOC10051010946	band 4.1-like protein 4B-like	100510946	0.20	0.00492	0.37	0.19	-0.01	-0.01	0.20	0.55	0.08	0.12	0.42	0.13
LOC10052222629	oligoribonuclease, mitochondrial-like	100522629	0.20	0.00666	0.46	0.02	0.13	0.22	0.38	0.43	0.26	-0.607	0.15	0.04
LOC10073739472	transmembrane BAX inhibitor motif-containing protein 1-like	100739472	0.20	0.00145	0.35	0.15	0.11	0.42	0.37	0.06	0.08	0.14	0.14	0.18
LRP6	low density lipoprotein receptor-related protein 6	100515777	0.20	0.00582	0.49	0.11	0.15	-0.07	0.19	0.11	0.14	0.07	0.22	0.18
SOCS6	suppressor of cytokine signaling 6	100155619	0.20	0.00841	0.27	0.46	-0.14	0.44	-0.02	0.43	0.17	0.36	0.18	0.05
ACOT7	acyl-CoA thioesterase 7	100514773	0.20	0.00904	0.13	0.54	0.10	0.18	-0.08	0.41	0.14	0.45	0.02	0.09
LOC10073737982	protein FAM59A-like	100737982	0.20	0.00921	0.40	-0.100	0.00	0.38	0.24	0.32	0.12	0.08	0.55	0.06
MYH14	myosin, heavy chain 14, non-muscle	100525110	0.20	0.00818	0.60	0.08	0.02	-0.06	0.17	0.12	0.29	0.19	0.43	0.12
LOC10052323232	olfactory receptor 2L2-like	100523232	0.19	0.00650	0.02	0.34	0.11	-0.16	0.19	0.24	0.15	0.36	0.25	0.45
AGAP1	ArfGAP with GTPase domain, ankyrin repeat and PH domain 1	100620812	0.19	0.00888	0.43	0.23	0.17	0.20	0.53	0.21	-0.021	0.06	0.10	0.04
TIPARP	TCDD-inducible poly(ADP-ribose) polymerase	100626111	0.19	0.00972	0.06	0.48	0.01	0.18	0.18	0.63	0.08	0.16	0.13	0.04
BMP4	bone morphogenetic protein 4	100113425	0.19	0.00198	0.38	0.23	0.24	0.11	0.12	0.30	0.19	-0.109	0.24	0.23
DST	dystonin	100152205	0.19	0.00464	0.34	0.23	0.17	-0.01	0.01	0.36	0.06	0.27	0.40	0.10
LRRC1	leucine rich repeat containing 1	100154780	0.19	0.00203	0.11	0.12	0.18	0.05	0.03	0.22	0.12	0.27	0.49	0.26
MPP5	membrane protein, palmitoylated 5 (MAGUK p55 subfamily member 5)	100152635	0.19	0.00656	0.22	0.33	0.12	0.15	-0.02	0.51	-0.061	0.30	0.02	0.13
LOC100155640	trace amine-associated receptor 3-like	100155640	0.19	0.00848	0.26	-0.046	0.25	0.31	0.24	0.11	0.15	0.13	0.51	-0.021
LOC10052323552	F-actin-capping protein subunit alpha-3-like	100523552	0.19	0.00454	0.28	0.02	0.04	0.33	0.40	0.21	0.18	0.06	0.35	0.05
LOC10062020929	anamorsin-like	100620929	0.19	0.00534	0.36	0.17	0.25	0.14	0.28	0.22	0.66	-0.105	0.09	0.19
ALDH18A1	aldehyde dehydrogenase 18 family, member A1	100151977	0.19	0.00449	0.31	0.09	0.00	0.09	0.49	0.26	0.18	-0.068	0.27	0.28
COX7A2	COX7A2 protein	100038000	0.19	0.00669	0.19	0.32	0.48	-0.013	0.03	0.43	0.10	0.17	0.05	0.12
CTR1	high-affinity copper uptake protein	397186	0.19	0.00317	0.32	0.10	0.22	-0.036	0.21	0.58	0.21	0.18	0.05	0.07
LOC10052424666	abhydrolase domain-containing protein FAM108A-like	100524666	0.19	0.00291	0.23	0.14	0.09	0.44	0.36	0.01	0.15	0.05	0.15	0.36

		100516		0.00	-0.	0.2	0.2	0.3	0.2	0.3	0.1	0.0	0.2	0.1
WDR20	WD repeat domain 20	626	0.19	821	11	9	3	2	9	2	0	8	0	6
LOC1006	inactive phospholipase	100622		0.00	0.2	0.0	0.2	0.2	0.3	0.1	0.0	0.1	0.0	0.2
22421	D5-like	421	0.19	625	7	4	8	9	0	3	5	9	3	9
LOC1005	gamma-glutamylaminecyc	100524		0.00	0.4	0.1	0.0	0.3	0.3	0.1	0.1	0.0	0.1	0.0
24456	lotransferase-like	456	0.19	606	7	3	8	1	6	6	3	0	8	7
	makorin ring finger protein	100514		0.00	0.2	0.0	-0.	0.1	0.1	0.2	0.2	0.2	0.3	0.2
MKRN1	1	261	0.18	216	3	1	03	2	5	8	6	0	5	6
		100192		0.00	0.2	0.1	0.3	0.1	0.1	0.0	0.1	-0.	0.2	0.2
NCK2	NCK adaptor protein 2	439	0.18	346	5	1	6	8	6	5	7	01	7	8
	FAT tumor suppressor	100154		0.00	0.3	0.2	0.0	0.2	0.3	0.0	0.1	0.1	0.0	0.2
FAT1	homolog 1 (Drosophila)	043	0.18	207	4	3	4	2	5	5	1	8	5	4
LOC1006	lon protease homolog 2,	100625		0.00	0.2	0.0	0.2	0.1	0.0	0.4	0.2	0.0	0.0	0.3
25597	peroxisomal-like	597	0.18	317	7	5	0	2	4	1	3	8	8	2
LOC1007	uncharacterized	100736		0.00	0.3	0.1	0.1	0.1	0.2	0.3	0.0	-0.	0.3	0.2
36865	LOC100736865	865	0.18	769	2	4	5	1	5	0	6	18	7	8
LOC1005		100517		0.00	0.4	0.0	0.2	0.2	0.2	0.3	0.0	0.0	0.1	0.0
17160	proline-rich protein 13	160	0.18	713	7	1	0	1	8	5	1	5	9	3
LOC1005	protein SCO1 homolog,	100517		0.00	0.2	0.2	0.0	0.0	0.1	0.2	0.1	0.1	0.4	-0.
17855	mitochondrial-like	855	0.18	568	5	2	4	6	8	9	8	2	6	01
		100157		0.00	0.2	0.1	0.1	0.0	0.0	0.3	0.1	0.1	0.4	0.0
CAB39	calcium binding protein 39	453	0.18	404	8	4	2	4	6	4	1	7	4	6
LOC1005	sphingosine-1-phosphate	100524		0.00	0.3	0.1	0.2	0.0	0.0	0.2	0.1	0.1	0.3	0.0
24832	phosphatase 2-like	832	0.18	385	5	9	0	1	6	0	6	5	8	5
LOC1001		100153		0.00	0.2	0.0	-0.	0.2	0.4	0.1	0.0	0.1	0.3	0.1
53898	ezrin-like	898	0.17	549	0	5	05	4	6	4	1	7	9	4
	coiled-coil													
LOC1005	domain-containing protein	100518		0.00	0.2	0.0	0.3	0.2	0.0	0.3	0.3	0.0	0.0	0.1
18956	124-like	956	0.17	614	4	2	6	0	7	4	1	1	9	0
LOC1005	uncharacterized	100517		0.00	0.2	0.0	0.0	0.1	0.1	0.3	0.0	0.2	0.1	0.1
17953	LOC100517953	953	0.17	678	9	6	7	6	6	8	6	4	7	1
LOC1001		100137		0.00	0.4	-0.	0.1	0.1	0.1	0.1	0.0	-0.	0.3	0.3
37084	EPLIN-b	084	0.17	849	0	04	9	3	8	3	9	07	5	2
LOC1006		100623		0.00	0.1	0.0	0.0	0.1	0.1	0.1	0.2	0.2	0.3	0.1
23017	olfactory receptor 5B2-like	017	0.17	317	2	2	8	6	7	5	9	2	0	6
LOC1005		100522		0.00	0.2	0.0	0.1	0.2	0.1	-0.	0.1	0.1	0.4	0.1
22603	olfactory receptor 8K3-like	603	0.17	675	1	6	0	8	5	02	5	6	0	7
LOC1005	uncharacterized	100521		0.00	-0.	0.2	0.1	0.1	0.0	0.2	0.1	0.1	0.2	0.3
21654	LOC100521654	654	0.17	907	11	3	8	9	1	7	8	0	9	4
	zinc finger, AN1-type	100155		0.00	0.0	0.1	-0.	0.2	0.1	0.4	0.1	0.1	0.2	0.1
ZFAND6	domain 6	656	0.16	712	4	0	01	2	5	3	8	3	6	1
LOC1006	lon protease homolog 2,	100625		0.00	0.2	0.1	0.0	0.1	0.1	0.4	0.1	0.0	0.1	0.1
25283	peroxisomal-like	283	0.16	595	6	0	6	6	1	4	7	1	3	5
		100156		0.00	0.2	-0.	0.2	0.0	0.1	0.2	0.0	0.1	0.2	0.2
SLK	STE20-like kinase	642	0.15	780	6	13	4	8	9	6	7	1	4	2
	jumonji, AT rich	100524		0.00	0.2	0.2	0.2	0.0	-0.	-0.	0.2	0.1	0.2	0.2
JARID2	interactive domain 2	569	0.15	931	1	2	6	5	03	03	8	6	2	0
LOC1000		100037		0.00	0.0	0.2	0.0	0.0	0.2	0.2	0.2	0.0	0.1	0.1
37981	HC12887	981	0.15	429	7	5	5	5	2	0	6	6	6	4
	lectin, galactoside-binding,	100038		0.00	0.1	0.0	0.0	0.1	0.0	0.1	0.0	0.1	0.1	0.1
LGALS3	soluble, 3	033	0.10	934	7	3	9	1	2	8	5	5	0	5
LOC1005	UPF0458 protein	100516		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
16361	C7orf42-like	361	-0.14	708	31	23	20	03	12	16	07	13	06	07
	mediator complex subunit	100157		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
MED12	12	450	-0.14	662	19	15	04	19	14	29	01	13	13	17
LOC1001	5-formyltetrahydrofolate	100152		0.00	-0.	-0.	-0.	0.0	-0.	0.0	-0.	-0.	-0.	-0.
52422	cyclo-ligase-like	422	-0.16	791	21	33	19	0	18	8	20	15	24	17
		100157		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
TLN1	talin 1	220	-0.16	965	19	12	06	15	02	44	17	3	27	19
	outer dense fiber of sperm	100156		0.00	-0.	0.0	0.0	-0.	-0.	-0.	-0.	-0.	-0.	-0.
ODF2	tails 2	735	-0.16	739	25	1	7	17	25	28	14	18	22	18
		100511		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
TCF4	transcription factor 4	671	-0.16	991	29	20	06	18	20	19	04	2	48	01
	solute carrier family 30													
	(zinc transporter), member	100156		0.00	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.	-0.	-0.
SLC30A7	7	498	-0.17	721	39	24	19	11	07	1	01	23	35	07
LOC1005	uncharacterized	100521		0.00	-0.	-0.	0.0	-0.	-0.	-0.	-0.	-0.	-0.	-0.
21760	LOC100521760	760	-0.17	298	15	29	1	15	19	19	13	16	37	08
	lysine (K)-specific	100517		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
KDM3A	demethylase 3A	522	-0.17	542	08	24	14	01	16	16	40	05	17	29
	RUN and FYVE domain	100519		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0
RUFY1	containing 1	354	-0.17	947	32	47	11	16	07	21	24	09	03	0
	high density lipoprotein	100233		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
HDLBP	binding protein	187	-0.17	503	11	02	04	02	21	41	17	22	37	15
	claudin domain containing	100154		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
CLDND1	1	619	-0.17	403	43	29	19	06	08	24	04	4	23	21

CDKAL1	CDK5 regulatory subunit associated protein 1-like 1	100512355	-0.18	0.00472	-0.32	-0.30	-0.28	-0.18	-0.30	0.00	-0.09	-0.14	0.05	-0.24
NXF1	nuclear RNA export factor 1	100628213	-0.19	0.00541	-0.13	-0.06	-0.25	-0.46	-0.42	-0.05	-0.09	-0.08	-0.28	-0.06
LOC100624587	non-histone chromosomal protein HMG-14-like	100624587	-0.19	0.00615	-0.39	-0.30	0.01	-0.13	-0.41	0.04	-0.16	-0.14	-0.20	-0.20
LOC100738762	WW domain-binding protein 11-like	100738762	-0.19	0.00869	-0.39	-0.23	-0.11	-0.12	-0.53	0.04	-0.12	-0.20	-0.25	0.01
RPN1	ribophorin I	397606	-0.19	0.00930	-0.31	0.01	0.04	-0.15	-0.47	-0.09	-0.08	-0.10	-0.56	-0.20
GRN	granulin	733645	-0.19	0.00827	-0.60	-0.20	-0.02	-0.16	-0.06	-0.32	-0.31	-0.15	-0.23	-0.09
ABCE1	ATP-binding cassette, sub-family E (OABP), member 1	100524881	-0.19	0.00970	-0.39	-0.47	-0.05	-0.05	-0.33	-0.24	-0.09	-0.18	-0.32	-0.19
LOC100738870	alpha-enolase-like	870	-0.20	0.00255	-0.17	-0.21	-0.09	-0.07	-0.29	-0.22	-0.28	-0.12	-0.50	-0.21
LOC100524280	guanine nucleotide-binding protein subunit beta-4-like	280	-0.20	0.00498	-0.50	-0.15	-0.14	-0.08	-0.28	-0.25	-0.08	-0.26	-0.31	-0.14
LOC100513892	dolichyl-diphosphooligosaccharide--protein glycosyltransferase subunit 2-like	100513892	-0.20	0.00412	-0.38	-0.04	-0.03	-0.11	-0.38	-0.16	-0.12	-0.25	-0.55	-0.10
LOC100154836	deoxycytidylate deaminase-like	836	-0.21	0.00635	-0.09	-0.02	-0.28	-0.03	-0.51	-0.26	-0.31	-0.27	-0.15	-0.38
LOC100737879	thyroid adenoma-associated protein homolog	879	-0.21	0.00862	-0.31	-0.52	-0.25	-0.33	-0.32	-0.28	-0.07	-0.06	-0.19	-0.04
LOC100512373	transmembrane protein 163-like	373	-0.21	0.00819	-0.21	-0.28	-0.20	-0.21	-0.03	-0.28	-0.08	-0.46	-0.40	-0.06
ANAPC16	anaphase promoting complex subunit 16	100155019	-0.21	0.00502	-0.34	-0.49	-0.31	-0.23	-0.21	-0.09	-0.32	-0.25	-0.13	-0.02
LOC100737320	olfactomedin-4-like	320	-0.21	0.00723	-0.20	-0.01	-0.09	-0.13	-0.79	-0.21	-0.08	-0.21	-0.32	-0.19
ARPC1B	actin related protein 2/3 complex, subunit 1B, 41kDa	100174964	-0.21	0.00657	-0.39	-0.11	-0.08	-0.00	-0.32	-0.37	-0.07	-0.02	-0.58	-0.30
LOC100738598	disintegrin and metalloproteinase domain-containing protein 17-like	598	-0.22	0.00968	-0.31	-0.00	-0.00	-0.26	-0.28	-0.69	-0.17	-0.07	-0.29	-0.33
HIVEP2	human immunodeficiency virus type I enhancer binding protein 2	100525369	-0.22	0.00897	-0.51	-0.01	-0.04	-0.33	-0.56	-0.14	-0.19	-0.09	-0.22	-0.29
LOC100737847	pleckstrin homology domain-containing family A member 2-like	847	-0.22	0.00793	-0.58	-0.03	-0.13	-0.62	-0.20	-0.13	-0.01	-0.03	-0.20	-0.25
DNAJC10	DnaJ (Hsp40) homolog, subfamily C, member 10	100523288	-0.22	0.00256	-0.08	-0.32	-0.01	-0.12	-0.50	-0.22	-0.40	-0.32	-0.06	-0.26
RARS	arginyl-tRNA synthetase	414410	-0.22	0.00959	-0.16	-0.19	-0.02	-0.12	-0.21	-0.65	-0.01	-0.27	-0.42	-0.38
SRSF6	serine/arginine-rich splicing factor 6	768102	-0.22	0.00436	-0.52	-0.06	-0.07	-0.04	-0.30	-0.34	-0.14	-0.25	-0.47	-0.25
SH3KBP1	SH3-domain kinase binding protein 1	100522530	-0.22	0.00834	-0.29	-0.23	-0.08	-0.04	-0.35	-0.43	-0.03	-0.01	-0.72	-0.11
HCFC1	host cell factor C1 (VP16-accessory protein)	100523834	-0.22	0.00178	-0.14	-0.25	-0.00	-0.12	-0.26	-0.47	-0.20	-0.12	-0.44	-0.24
RAMP2	receptor (G protein-coupled) activity modifying protein 2	397155	-0.23	0.00684	-0.64	-0.36	-0.08	-0.15	-0.41	-0.09	-0.06	-0.17	-0.41	-0.05
ADAM19	ADAM metallopeptidase domain 19	100521528	-0.23	0.00861	-0.36	-0.17	-0.01	-0.02	-0.05	-0.74	-0.37	-0.17	-0.36	-0.17
LOC100620483	mis18-binding protein 1-like	483	-0.23	0.00728	-0.49	-0.11	-0.02	-0.20	-0.62	-0.20	-0.21	-0.01	-0.21	-0.27
TGIF1	TGFB-induced factor homeobox 1	100625732	-0.23	0.00161	-0.04	-0.12	-0.09	-0.16	-0.27	-0.49	-0.33	-0.22	-0.14	-0.43
LYST	lysosomal trafficking regulator	100049676	-0.23	0.00323	-0.38	-0.05	-0.24	-0.43	-0.15	-0.45	-0.04	-0.10	-0.45	-0.08
LOC100522211	E3 ubiquitin-protein ligase RNF135-like	211	-0.23	0.00810	-0.63	-0.42	-0.05	-0.17	-0.33	-0.19	-0.01	-0.34	-0.20	-0.18
MIC-2	MHC class I related antigen 2	100135034	-0.23	0.00711	-0.07	-0.29	-0.61	-0.04	-0.42	-0.14	-0.14	-0.23	-0.29	-0.07
ERCC4	excision repair cross-complementing rodent repair deficiency, complementation group 4	100516427	-0.23	0.00094	-0.34	-0.15	-0.26	-0.24	-0.27	-0.17	-0.36	-0.21	-0.22	-0.10

LOC100525611	protein O-glucosyltransferase 1-like	100525611	-0.23	0.00332	-0.25	-0.10	-0.17	-0.35	-0.43	-0.62	-0.04	-0.23	-0.18	0.01
TGFBR1	transforming growth factor, beta receptor 1	396665	-0.24	0.00505	-0.51	-0.52	-0.06	-0.07	-0.22	-0.27	-0.28	0.09	-0.39	-0.22
LOC100621521	ABI gene family member 3-like	100621521	-0.24	0.00773	-0.05	-0.49	-0.10	0.02	-0.36	-0.46	-0.15	0.06	-0.42	-0.40
GPX1	glutathione peroxidase 1	397403	-0.24	0.00145	-0.56	-0.14	-0.01	-0.12	-0.36	-0.32	-0.23	-0.20	-0.40	-0.13
LOC100517301	dTDP-D-glucose 4,6-dehydratase-like	100517301	-0.24	0.00139	-0.17	-0.08	-0.25	-0.14	-0.51	-0.35	-0.28	-0.14	-0.09	-0.36
FLVCR2	feline leukemia virus subgroup C cellular receptor family, member 2	100217391	-0.24	0.00694	-0.17	-0.08	-0.16	-0.00	-0.56	-0.39	-0.41	-0.18	-0.40	-0.28
TLR2	toll-like receptor 2	396623	-0.24	0.00971	-0.31	-0.03	-0.13	-0.02	-0.65	-0.40	-0.38	-0.01	-0.32	-0.42
LOC100153642	ankyrin repeat domain-containing protein 37-like	100153642	-0.24	0.00334	-0.35	-0.13	-0.35	-0.43	-0.09	-0.38	-0.37	-0.07	-0.13	-0.14
ELOVL6	ELOVL fatty acid elongase 6	100312970	-0.24	0.00848	-0.37	-0.25	-0.03	-0.05	-0.66	-0.26	-0.28	-0.44	-0.31	-0.25
LRP11	low density lipoprotein receptor-related protein 11	100153355	-0.25	0.00690	-0.22	-0.13	-0.06	-0.04	-0.52	-0.38	-0.42	-0.16	-0.06	-0.55
FUT8	fucosyltransferase 8 (alpha (1,6) fucosyltransferase)	396933	-0.25	0.00610	-0.39	-0.22	-0.00	-0.30	-0.66	-0.26	-0.01	-0.01	-0.59	-0.16
ARRDC3	arrestin domain containing 3	100520233	-0.25	0.00365	-0.45	-0.18	-0.09	-0.20	-0.34	-0.05	-0.72	-0.13	-0.13	-0.36
SCLT1	sodium channel and clathrin linker 1	100518025	-0.25	0.00362	-0.32	-0.37	-0.22	-0.25	-0.22	-0.36	-0.69	-0.00	-0.07	-0.15
B3GALNT1	beta-1,3-N-acetylgalactosaminyltransferase 1 (globoside blood group)	397634	-0.25	0.00277	-0.22	-0.36	-0.08	-0.06	-0.51	-0.17	-0.33	-0.36	-0.32	-0.25
MAP3K1	mitogen-activated protein kinase kinase kinase 1, E3 ubiquitin protein ligase	396617	-0.26	0.00053	-0.45	-0.05	-0.17	-0.41	-0.49	-0.30	-0.08	-0.05	-0.31	-0.29
LOC100155363	UPF0536 protein C12orf66-like	100155363	-0.27	0.00175	-0.30	-0.04	-0.30	-0.23	-0.29	-0.44	-0.45	-0.33	-0.05	-0.30
LOC100157723	purinergic receptor P2X4	723	-0.27	0.00544	-0.29	-0.73	-0.41	-0.19	-0.09	-0.40	-0.45	-0.31	-0.05	-0.25
LOC100515590	coiled-coil domain-containing protein 69-like	590	-0.27	0.00715	-0.55	-0.07	-0.29	-0.29	-0.30	-0.76	-0.00	-0.04	-0.23	-0.31
LOC100152536	multidrug resistance-associated protein 4-like	536	-0.27	0.00461	-0.07	-0.20	-0.30	-0.02	-0.09	-0.68	-0.41	-0.10	-0.58	-0.47
CXCL14	chemokine (C-X-C motif) ligand 14	494467	-0.27	0.00160	-0.08	-0.45	-0.06	-0.08	-0.01	-0.47	-0.54	-0.43	-0.50	-0.27
WIPI1	WD repeat domain, phosphoinositide interacting 1	100462758	-0.27	0.00631	-0.29	-0.87	-0.00	-0.18	-0.30	-0.25	-0.15	-0.50	-0.33	-0.03
LOC100623014	transient receptor potential cation channel subfamily M member 2-like	100623014	-0.28	0.00120	-0.26	-0.04	-0.29	-0.46	-0.52	-0.31	-0.11	-0.21	-0.34	-0.21
GALE	UDP-galactose-4-epimerase	100621392	-0.28	0.00669	-0.31	-0.17	-0.05	-0.01	-0.21	-0.80	-0.44	-0.59	-0.38	-0.01
LOC100154996	glycoprotein endo-alpha-1,2-mannosidase-like	100154996	-0.28	0.00664	-0.64	-0.35	-0.21	-0.18	-0.76	-0.03	-0.13	-0.03	-0.49	-0.25
NCALD	neurocalcin delta	100156785	-0.28	0.00658	-0.92	-0.39	-0.18	-0.14	-0.11	-0.19	-0.16	-0.40	-0.04	-0.40
GBA	glucosidase, beta, acid	449572	-0.28	0.00237	-0.57	-0.09	-0.06	-0.12	-0.67	-0.40	-0.19	-0.19	-0.44	-0.25
LOC100517795	AP-1 complex subunit sigma-2-like	100517795	-0.28	0.00115	-0.57	-0.27	-0.35	-0.10	-0.58	-0.06	-0.06	-0.29	-0.22	-0.39
NIN	ninein (GSK3B interacting protein)	100157402	-0.28	0.00736	-0.38	-0.02	-0.03	-0.32	-0.37	-0.55	-0.17	-0.04	-0.89	-0.18
TANK	TRAF family member-associated NFKB activator	100156366	-0.28	0.00953	-0.62	-0.03	-0.03	-0.22	-0.60	-0.68	-0.05	-0.04	-0.57	-0.27
DEGS1	delta(4)-desaturase, sphingolipid 1	100135677	-0.28	0.00307	-0.63	-0.00	-0.04	-0.09	-0.19	-0.44	-0.35	-0.34	-0.67	-0.23
VRK2	vaccinia related kinase 2	100511127	-0.28	0.00345	-0.34	-0.30	-0.14	-0.43	-0.78	-0.02	-0.15	-0.08	-0.19	-0.41
LOC100516193	uncharacterized LOC100516193	100516193	-0.28	0.00339	-0.33	-0.16	-0.09	-0.37	-0.92	-0.25	-0.13	-0.39	-0.12	-0.22
LOC100624193	lysosomal-associated transmembrane protein 5-like	100624193	-0.28	0.00607	-0.72	-0.34	-0.05	-0.37	-0.56	-0.58	-0.06	-0.08	-0.25	-0.24

		100153		0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.1	-0.	-0.	-0.
STK38	serine/threonine kinase 38	410	-0.28	810	19	11	8	65	83	44	2	01	42	41
	structural maintenance of	100152		0.00	-0.	-0.	0.1	-0.	-0.	0.1	-0.	-0.	-0.	-0.
SMC4	chromosomes 4	361	-0.28	835	70	28	4	46	77	8	19	08	51	17
	baculoviral IAP repeat	100622		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
BIRC2	containing 2	859	-0.29	762	35	07	21	06	70	89	19	4	22	21
	hypoxia inducible factor 1, alpha subunit (basic helix-loop-helix			0.00	-0.	-0.	-0.	0.0	-0.	-0.	-0.	-0.	-1.	-0.
HIF1A	transcription factor)	396696	-0.29	897	21	03	02	1	48	56	22	24	08	05
LOC1005	39S ribosomal protein	100513		0.00	-0.	0.0	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
13036	L51, mitochondrial-like	036	-0.29	432	20	6	39	41	76	17	23	5	44	40
LOC1005	uncharacterized	100511		0.00	-0.	0.4	-0.	-0.	0.1	-0.	-0.	-0.	-0.	-0.
11129	LOC100511129	129	-0.29	909	64	3	42	24	1	36	66	56	26	31
ARHGAP	Rho GTPase activating	100523		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.	-0.
4	protein 4	659	-0.30	346	69	32	05	24	23	79	1	21	29	24
	tyrosylprotein	100154		0.00	-0.	-0.	0.0	0.0	-0.	-0.	-0.	-0.	-0.	-0.
TPST2	sulfotransferase 2	448	-0.30	655	88	09	2	9	50	52	07	19	69	16
		100628		0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.0	-0.	-0.	-0.
SOAT1	sterol O-acyltransferase 1	166	-0.30	120	35	26	5	35	61	61	5	31	30	32
ARHGAP	Rho GTPase activating	100514		0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.2	-0.	-0.	-0.
25	protein 25	401	-0.30	937	59	19	1	29	86	67	8	02	31	36
	phosphatidylinositol-5-phosphate 4-kinase, type II,	100520		0.00	-0.	-0.	0.0	-0.	-0.	-0.	-0.	0.2	-0.	-0.
PIP4K2A	alpha	446	-0.30	720	69	36	8	44	73	33	05	9	29	49
LOC1006		100620		0.00	-0.	-0.	-0.	0.2	-0.	-0.	-0.	-0.	-0.	-0.
20551	latexin-like	551	-0.30	179	60	34	18	5	54	50	39	24	13	33
				0.00	-0.	-0.	0.2	-0.	-0.	-0.	-0.	-0.	-0.	0.0
FADS1	fatty acid desaturase 1	444995	-0.30	385	80	28	1	46	29	15	26	35	66	2
LOC1005		100522		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.	0.0
22138	protein FAM65A-like	138	-0.30	336	21	53	05	62	37	60	3	11	55	0
		100156		0.00	-0.	-0.	0.0	-0.	-0.	-0.	-0.	-0.	-0.	-0.
PHF19	PHD finger protein 19	643	-0.30	101	36	38	2	19	12	60	54	33	29	22
LOC1005	phosphofurin acidic cluster	100521		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.3	-0.	-0.	-0.
21836	sorting protein 1-like	836	-0.31	445	61	53	13	61	32	54	6	07	35	30
LOC1007	integral membrane protein	100738		0.00	-0.	-0.	-0.	0.2	-0.	-0.	-0.	-0.	-0.	-0.
38945	GPR137B-like	945	-0.31	843	72	33	06	6	69	28	80	05	27	15
	folylpolyglutamate													
LOC1007	synthase,	100736		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.
36908	mitochondrial-like	908	-0.31	306	48	15	46	08	33	76	28	62	9	06
				0.00	-0.	-0.	0.2	-0.	-0.	-0.	-0.	-0.	-0.	-0.
FADS2	fatty acid desaturase 2	444997	-0.31	385	58	05	5	06	49	29	37	43	79	32
LOC1005	uncharacterized	100525		0.00	-0.	0.0	-0.	-0.	-0.	-0.	0.1	-0.	-0.	-0.
25431	LOC100525431	431	-0.31	627	74	1	28	64	55	19	6	30	04	57
		100153		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.
ZNF318	zinc finger protein 318	069	-0.31	192	07	65	46	65	22	28	16	36	4	43
	alpha-N-acetylneuraminide													
LOC1006	alpha-2,8-sialyltransferase	100621		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	0.0
21251	-like	251	-0.31	296	48	19	54	60	37	22	26	0	63	3
LOC1005	cysteine-rich hydrophobic	100526		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	0.0	-0.
26199	domain 1 protein-like	199	-0.32	402	68	38	46	48	71	02	34	6	6	19
	sec1 family domain	100738		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
SCFD2	containing 2	927	-0.32	117	53	11	10	27	66	06	35	3	69	39
LOC1005	scm-like with four MBT	100515		0.00	-0.	0.0	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.
15572	domains protein 2-like	572	-0.32	604	79	2	41	67	74	22	07	7	23	22
LOC1005	PQ-loop repeat-containing	100525		0.00	-0.	-0.	0.0	0.0	-0.	-0.	-0.	-0.	-0.	-0.
25694	protein 3-like	694	-0.32	236	77	51	0	8	44	38	09	23	68	16
LOC1006	FCH and double SH3	100622		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.
22758	domains protein 2-like	758	-0.32	079	37	23	28	47	47	77	06	1	27	36
	ADAM metallopeptidase			0.00	-0.	0.2	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.
ADAM17	domain 17	397343	-0.32	401	47	7	20	18	59	74	37	0	58	42
LOC1005	KDEL motif-containing	100511		0.00	-0.	0.0	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
11896	protein 1-like	896	-0.32	313	16	9	15	67	18	28	35	18	76	55
LOC1001	carbohydrate	100157		0.00	-0.	-0.	-0.	0.0	-0.	-0.	0.0	-0.	-0.	-0.
57186	sulfotransferase 11-like	186	-0.32	424	60	28	23	8	29	85	2	08	71	26
	diacylglycerol kinase,			0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.2	-0.	-0.	-0.
DGKA	alpha 80kDa	397097	-0.32	464	50	66	5	80	28	41	0	23	52	06
	CD40 molecule, TNF receptor superfamily			0.00	-0.	-0.	-0.	-0.	-1.	-0.	0.0	0.1	-0.	-0.
CD40	member 5	397395	-0.32	946	91	14	16	29	00	36	5	2	19	33
LOC1006	ena/VASP-like	100625		0.00	-0.	-0.	0.0	-0.	-0.	-0.	-0.	0.0	-0.	-0.
25637	protein-like	637	-0.32	650	82	26	8	74	76	27	11	7	08	34
	phosphatidylinositol transfer protein,	100233		0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.3	-0.	-0.	-0.
PITPNC1	cytoplasmic 1	199	-0.32	977	21	32	9	21	73	90	2	11	49	66

LOC1001	WAS/WASL-interacting protein family member	100154		0.00	-1.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.
54822	1-like	822	-0.32	314	00	37	08	54	41	42	12	1	30	10
	bactericidal/permeability-i	100286		0.00	-0.	-0.	0.0	0.0	-0.	-0.	-0.	-0.	-0.	-0.
BPI	ncreasing protein	800	-0.33	077	50	26	4	9	59	66	37	59	14	29
LOC1005		100525		0.00	-0.	-0.	0.2	-0.	-0.	-0.	-0.	-0.	-0.	-0.
25217	tribbles homolog 2-like	217	-0.33	559	39	17	3	64	59	12	14	31	90	21
LOC1005		100515		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.	-0.
15873	septin-6-like	873	-0.33	221	95	42	02	36	60	33	7	04	25	37
LOC1007	uncharacterized	100739		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.3	-0.	-0.
39736	LOC100739736	736	-0.33	584	51	08	54	14	81	69	14	5	58	11
	mucin 20, cell surface	100134		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.3	-0.
MUC20	associated	954	-0.33	634	61	41	73	40	26	69	01	17	7	37
	RAS guanyl releasing													
RASGRP	protein 2 (calcium and	100626		0.00	-0.	-0.	-0.	-0.	0.0	-0.	0.2	-0.	-0.	-0.
2	DAG-regulated)	093	-0.33	763	49	20	27	96	8	39	3	19	63	46
	pigeon homolog	100512		0.00	-0.	-0.	0.0	-0.	-0.	-0.	-0.	0.2	-0.	-0.
PION	(Drosophila)	622	-0.33	275	82	63	5	42	38	48	21	1	33	29
LOC1001	Fc receptor-like protein	100152		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.
52824	1-like	824	-0.33	161	50	59	31	77	28	25	12	19	9	39
LOC1007		100739		0.00	-0.	-0.	-0.	-0.	0.2	-0.	-0.	-0.	-1.	-0.
39768	beta-parvin-like	768	-0.33	746	13	42	13	17	8	80	31	36	00	27
	v-rel reticuloendotheliosis													
	viral oncogene homolog	100525		0.00	-0.	-0.	0.1	-0.	-0.	-0.	0.0	0.0	-0.	-0.
REL	(avian)	104	-0.33	591	85	33	2	48	92	51	4	9	20	30
				0.00	-0.	-0.	-0.	-0.	-0.	0.0	0.2	-0.	-0.	-0.
C3	complement component 3	397072	-0.33	509	64	19	39	65	97	1	9	25	48	08
	beta-galactoside													
LOC1005	alpha-2,6-sialyltransferase	100512		0.00	0.1	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.4	-0.
12775	2-like	775	-0.34	739	3	28	43	32	61	81	62	32	8	59
	large neutral amino acids													
LOC1006	transporter small subunit	100623		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
23009	4-like	009	-0.34	206	69	24	16	12	16	97	38	09	54	03
		100512		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.1	0.2	-0.	-0.
LIMD2	LIM domain containing 2	735	-0.34	602	75	46	37	41	01	83	3	4	61	36
LOC1005	non-histone chromosomal	100525		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.2	0.0	-0.	-0.
25406	protein HMG-14-like	406	-0.34	407	54	46	63	12	85	23	1	0	34	44
	nucleic acid binding	100155		0.00	-0.	0.0	-0.	-0.	-0.	-0.	0.0	0.1	-0.	-0.
OBFC2A	protein 1	066	-0.34	967	25	9	14	31	83	91	8	8	89	45
	serine peptidase inhibitor,			0.00	-0.	-0.	0.0	0.0	-0.	-0.	-0.	-0.	-0.	-0.
SPINK4	Kazal type 4	396872	-0.34	095	18	42	4	4	47	88	50	58	09	39
	interleukin 2 receptor,			0.00	-0.	-0.	-0.	0.0	-0.	-0.	-0.	-0.	-0.	-0.
IL2RA	alpha	396814	-0.35	041	48	29	40	6	46	09	46	30	51	50
	ER degradation-enhancing													
LOC1007	alpha-mannosidase-like	100738		0.00	-0.	-0.	-0.	0.0	-0.	-0.	-0.	0.0	-0.	-0.
38285	1-like	285	-0.35	026	48	40	36	3	58	67	26	1	51	27
	interleukin 10 receptor,	100525		0.00	-0.	-0.	0.1	-0.	-0.	-1.	-0.	0.0	-0.	-0.
IL10RA	alpha	654	-0.35	698	96	34	7	11	55	02	32	7	38	07
	AT-rich interactive													
LOC1005	domain-containing protein	100524		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
24615	5A-like	615	-0.35	420	86	15	01	25	83	49	08	01	74	10
		100518		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
PHF11	PHD finger protein 11	446	-0.35	011	56	20	15	30	49	66	22	03	58	34
	fibroblast activation	100736		0.00	-0.	-0.	-0.	0.3	-0.	-0.	-0.	-0.	-0.	-0.
FAP	protein, alpha	572	-0.35	249	07	42	35	5	30	84	78	40	34	35
		100155		0.00	-0.	0.0	-0.	0.1	-0.	-1.	-0.	-0.	-0.	-0.
ARG2	arginase, type II	893	-0.35	982	01	2	46	0	60	16	21	02	78	42
				0.00	-0.	-0.	0.0	0.3	-0.	-1.	-0.	-0.	-0.	-0.
SRM	spermidine synthase	414385	-0.35	990	09	26	1	9	18	18	48	58	74	44
LOC1006	uncharacterized	100622		0.00	-0.	-0.	-0.	-0.	-0.	0.0	0.0	-0.	-0.	-0.
22759	LOC100622759	759	-0.36	258	62	19	19	89	88	7	0	10	38	38
	serum/glucocorticoid													
	regulated kinase family,	100521		0.00	-0.	-0.	0.1	-0.	-1.	-0.	-0.	0.0	-0.	-0.
SGK3	member 3	447	-0.36	567	50	08	6	22	03	59	02	1	82	52
	Abelson helper integration	100514		0.00	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.	-0.
AHI1	site 1	806	-0.36	138	84	31	30	81	39	21	2	14	25	50
	family with sequence	100157		0.00	-0.	-0.	-0.	0.0	-0.	-0.	0.1	-0.	-0.	-0.
FAM49B	similarity 49, member B	574	-0.36	399	72	32	11	0	92	84	8	01	47	42
ARHGDI	Rho GDP dissociation	100155		0.00	-1.	-0.	0.0	-0.	-0.	-0.	0.2	0.1	-0.	-0.
B	inhibitor (GDI) beta	466	-0.36	921	13	66	3	39	80	44	6	2	56	06
		100512		0.00	-0.	-0.	0.0	-0.	-0.	0.1	-0.	-0.	-1.	-0.
ITGA8	integrin, alpha 8	676	-0.37	897	26	24	7	55	29	0	64	39	39	08
	signaling lymphocytic													
	activation molecule family	100511		0.00	-1.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
SLAMF1	member 1	325	-0.37	376	00	65	17	20	19	79	01	04	52	11
	sphingomyelin													
SMPDL3	phosphodiesterase,	100153		0.00	-0.	-0.	-0.	0.0	-0.	-0.	-0.	-0.	-0.	-0.
A	acid-like 3A	442	-0.37	067	88	47	37	6	17	55	61	09	52	09

LOC100738208	solute carrier organic anion transporter family member 5A1-like	100738208	-0.37	0.00935	-0.54	-0.16	-0.45	-0.79	-0.47	-0.07	0.06	0.39	-0.64	-1.02
LOC100524363	proline-rich protein 16-like	100524363	-0.37	0.00243	0.00	-0.37	0.00	-0.68	-0.30	-0.26	-0.23	-0.30	-0.06	-0.53
Sep-01	septin 1	100514708	-0.37	0.00285	-0.87	-0.78	-0.15	-0.67	-0.24	-0.67	0.17	-0.01	-0.31	-0.19
LOC100518524	GTPase IMAP family member 6-like	100518524	-0.37	0.00454	-1.08	-0.40	-0.04	-0.15	-0.94	-0.40	-0.16	0.22	-0.49	-0.33
LOC100511860	protein tyrosine phosphatase receptor type C-associated protein-like	100511860	-0.38	0.00231	-1.06	-0.39	-0.31	-0.48	-0.41	-0.79	0.13	0.01	-0.30	-0.28
SPI1	spleen focus forming virus (SFFV) proviral integration oncogene spi1	414912	-0.38	0.00795	-0.84	-0.12	0.02	0.02	-0.81	-0.02	-0.02	-0.04	-0.88	-0.17
LOC100518506	protein FAM177B-like	100518506	-0.38	0.00094	-0.28	-0.51	-0.33	0.37	-0.50	-0.68	-0.65	-0.67	-0.28	-0.23
LOC100620522	transcription regulator protein BACH2-like	100620522	-0.38	0.00386	-0.33	-0.11	-0.41	-1.16	-0.29	-0.45	0.27	-0.30	-0.46	-0.53
DOCK8	dedicator of cytokinesis 8	100153522	-0.38	0.00186	-0.94	-0.34	-0.07	-0.44	-0.57	-0.92	-0.08	0.10	-0.19	-0.34
ABAT	4-aminobutyrate aminotransferase	397500	-0.38	0.00946	-0.57	-0.32	-0.05	0.15	-0.21	-0.03	-0.63	-0.07	-0.33	0.16
LOC100520583	C4b-binding protein beta chain-like	100520583	-0.38	0.00103	-0.42	-0.42	-0.21	0.06	-0.86	-0.55	-0.65	-0.45	0.07	-0.37
MAP4K2	mitogen-activated protein kinase kinase kinase kinase 2	100523716	-0.38	0.00685	-0.65	-0.32	-0.18	-0.75	-0.32	-0.21	0.16	-0.32	-0.25	-0.16
LOC100514466	protein THEMIS2-like	100514466	-0.38	0.00414	-0.19	-0.15	-0.47	-0.07	-0.27	-0.39	-0.09	-0.34	-0.57	-0.30
LOC100737965	runt-related transcription factor 2-like	100737965	-0.38	0.00326	-0.83	-0.39	-0.21	-0.72	-0.50	-0.42	-0.02	-0.09	-0.94	-0.09
LOC100518347	GTPase IMAP family member 4-like	100518347	-0.38	0.00770	-1.11	-0.45	-0.04	-0.27	-1.05	-0.60	-0.09	-0.03	-0.56	-0.04
MST4	serine/threonine protein kinase MST4	100233205	-0.38	0.00376	-0.93	-0.29	-0.02	-0.59	-0.86	-0.54	-0.00	-0.01	-0.41	-0.51
ST8SIA4	ST8 alpha-N-acetyl-neuraminide alpha-2,8-sialyltransferase 4	641356	-0.38	0.00243	-0.74	-0.39	-0.06	-0.16	-0.66	-0.65	-0.24	-0.02	-0.99	-0.16
LOC100512951	uncharacterized LOC100512951	100512951	-0.38	0.00214	-0.97	-0.11	-0.04	-0.70	-0.56	-0.64	-0.01	-0.28	-0.34	-0.42
CIITA	class II, major histocompatibility complex, transactivator	100736732	-0.39	0.00948	-1.33	-0.07	-0.11	-0.43	-0.03	-0.42	-0.12	-0.01	-0.29	-0.26
GCET2	germinal center-associated, signaling and motility	100626350	-0.39	0.00881	-0.80	-0.84	-0.25	-0.70	-0.63	-0.14	-0.16	-0.03	-0.08	-0.86
LOC100520025	cysteine-rich with EGF-like domain protein 2-like	100520025	-0.39	0.00346	-0.49	-0.13	-0.03	-0.14	-0.04	-0.60	-0.51	-0.04	-0.84	-0.47
APOBEC3F	apolipoprotein B mRNA editing enzyme, catalytic polypeptide-like 3F	100037939	-0.39	0.00238	-1.00	-0.67	-0.06	-0.38	-0.48	-0.69	-0.31	-0.01	-0.14	-0.39
LOC100521239	coatomer subunit epsilon-like	100521239	-0.39	0.00873	-0.12	-0.34	-0.02	-0.02	-0.60	-0.41	-0.36	-0.12	-0.24	-0.10
LOC100512932	acid ceramidase-like	100512932	-0.39	0.00369	-0.87	-0.33	-0.11	-0.00	-0.23	-0.64	-0.34	-0.13	-0.21	-0.16
LOC100625333	high mobility group protein B2-like	100625333	-0.39	0.00246	-0.78	-0.16	-0.01	-0.46	-0.05	-0.40	-0.22	-0.10	-0.59	-0.48
PARP8	poly (ADP-ribose) polymerase family, member 8	100511108	-0.39	0.00281	-0.83	-0.63	-0.03	-0.41	-0.88	-0.53	-0.09	-0.08	-0.70	-0.27
LOC100152849	EMI domain-containing protein 1-like	100152849	-0.40	0.00295	-0.23	-0.73	-0.04	-0.09	-0.49	-0.21	-0.01	-0.48	-0.50	-0.46
BCL6	B-cell CLL/lymphoma 6	100156549	-0.40	0.00070	-0.59	-0.01	-0.33	-0.46	-0.63	-0.62	-0.00	-0.05	-0.85	-0.56
LOC100525029	protein FAM111A-like	100525029	-0.40	0.00086	-0.50	-0.13	-0.53	-0.08	-0.06	-0.49	-0.29	-0.03	-0.68	-0.27
LOC100625253	DNA-binding protein Ikaros-like	100625253	-0.40	0.00064	-0.69	-0.66	-0.18	-0.36	-0.69	-0.68	-0.09	-0.04	-0.53	-0.40
LOC100524418	prostaglandin D2 receptor-like	100524418	-0.40	0.00193	-0.74	-0.02	-0.05	-0.00	-0.21	-0.63	-0.11	-0.36	-0.70	-0.27
LOC100518723	interleukin-21 receptor-like	100518723	-0.41	0.00890	-0.12	-0.21	-0.31	-0.91	-0.57	-0.22	-0.00	-0.08	-0.73	-0.58
LOC100625140	protein CASC4-like	100625140	-0.41	0.00159	-0.24	-0.10	-0.17	-0.08	-0.32	-0.20	-0.80	-0.42	-0.28	-0.63

BIN2	bridging integrator 2	100519772	-0.41	0.00821	-1.30	-0.70	0.25	-0.40	-0.45	-0.83	-0.12	0.02	-0.66	0.07
APOD	apolipoprotein D	100157318	-0.41	0.00105	-0.27	-0.62	-0.07	-0.59	-0.28	-0.49	-0.10	-1.13	-0.32	-0.23
FCRL3	Fc receptor-like 3	100158069	-0.41	0.00520	-0.56	-0.22	-0.21	-0.58	-1.20	-0.73	0.51	-0.31	-0.39	-0.41
DOCK2	dedicator of cytokinesis 2	100512021	-0.41	0.00470	-1.05	-0.60	-0.31	-0.57	-0.89	-0.59	-0.05	0.46	-0.46	-0.05
LOC100152206	C2 calcium-dependent domain-containing protein 4A-like	100152206	-0.41	0.00922	-0.26	0.05	-0.64	0.57	-0.41	-1.22	-0.43	-0.46	-0.41	-0.91
LOC100739094	phospholipase B-like 1-like	100739094	-0.41	0.00333	0.32	-0.64	0.13	-0.40	-0.56	-0.32	-0.49	-0.33	-0.99	-0.86
DUOX2	dual oxidase 2	397060	-0.42	0.00305	-0.16	0.05	-0.47	-0.14	-0.38	-0.36	-1.38	-0.01	-0.85	-0.47
LOC100518944	dedicator of cytokinesis protein 11-like	100518944	-0.42	0.00355	-1.15	-0.62	-0.12	-0.74	-0.81	-0.65	0.01	0.19	-0.15	-0.16
RHOH	ras homolog family member H	100513215	-0.42	0.00685	-1.23	-0.35	0.18	-0.33	-0.85	-0.87	0.02	-0.72	-0.72	-0.27
LOC100154617	histidine ammonia-lyase-like	100154617	-0.42	0.00499	-0.43	-0.69	-0.27	0.23	-0.82	-0.15	-0.83	-0.06	0.08	-0.31
LOC100525094	SAM and SH3 domain-containing protein 3-like	100525094	-0.43	0.00064	-1.03	-0.44	-0.27	-0.29	-0.49	-0.88	-0.05	-0.41	-0.50	
LOC100738167	mitogen-activated protein kinase kinase kinase kinase 1-like	100738167	-0.43	0.00284	-0.94	-0.51	-0.02	-0.51	-0.82	-0.92	-0.75	-0.52	-0.46	
AKNA	AT-hook transcription factor	100152364	-0.43	0.00011	-0.69	-0.43	-0.24	0.01	-0.41	-0.84	-0.25	-0.78	-0.45	-0.21
CHIA	chitinase, acidic	100155231	-0.43	0.00063	-0.61	-0.93	-0.61	-0.14	-0.74	-0.19	-0.21	-0.67	-0.15	-0.04
LOC100620588	synaptosomal-associated protein 25-like	100620588	-0.43	0.00056	-0.41	-0.20	-0.07	-0.30	-0.55	-0.60	-0.39	-0.25	-0.16	-0.39
LOC100516246	EGF-like module-containing mucin-like hormone receptor-like 3-like	100516246	-0.43	0.00426	-0.26	-0.27	-0.00	-0.75	-0.44	-0.12	-0.08	-0.16	-0.61	-0.37
RASSF2	Ras association (RalGDS/AF-6) domain family member 2	100157105	-0.43	0.00083	-0.64	-0.02	-0.24	-0.07	-0.68	-0.13	-0.28	-0.06	-0.71	-0.50
BHLHE40	basic helix-loop-helix family, member e40	100514873	-0.43	0.00625	-0.63	-0.09	-0.57	-0.08	-0.26	-0.39	-0.56	-0.11	-0.92	-0.38
ELK3	ELK3, ETS-domain protein (SRF accessory protein 2)	100622445	-0.44	0.00722	-0.61	-0.36	-0.05	-0.29	-0.31	-0.77	-0.20	-0.21	-0.68	-0.03
LOC100523976	uncharacterized LOC100523976	100523976	-0.44	0.00524	-1.34	-0.43	-0.30	-0.80	-0.59	-0.79	-0.04	-0.05	-0.41	
ACAP1	ArfGAP with coiled-coil, ankyrin repeat and PH domains 1	100623422	-0.44	0.00103	-1.04	-0.66	-0.05	-0.35	-0.57	-0.86	-0.05	-0.20	-0.55	-0.27
LOC100157293	Transmembrane protein 71	100157293	-0.44	0.00943	-0.78	-0.36	-0.02	-0.52	-0.29	-0.61	-0.43	-0.22	-0.63	-0.32
PDE3B	phosphodiesterase 3B, cGMP-inhibited	100516060	-0.44	0.00740	-1.35	-0.64	-0.07	-0.45	-0.89	-0.89	-0.03	-0.03	-0.40	-0.27
LOC100521317	myeloid cell nuclear differentiation antigen-like	100521317	-0.44	0.00080	-0.35	-0.14	-0.15	-0.29	-0.31	-0.73	-0.33	-0.29	-0.58	-0.27
SUSD1	sushi domain containing 1	100516174	-0.44	0.00191	-0.54	-0.47	-0.21	-0.02	-0.83	-0.37	-0.15	-0.24	-0.48	-0.17
KLHL6	kelch-like 6 (Drosophila)	100625137	-0.44	0.00025	-0.86	-0.01	-0.59	-0.68	-0.45	-0.28	-0.00	-0.62	-0.33	-0.65
CCR7	chemokine (C-C motif) receptor 7	396663	-0.45	0.00305	-1.09	-0.18	-0.33	-0.53	-0.42	-0.13	-0.30	-0.01	-0.53	-0.57
TTC39C	tetratricopeptide repeat domain 39C	100511088	-0.45	0.00151	-1.25	-0.29	-0.03	-0.90	-0.76	-0.37	-0.29	-0.15	-0.08	-0.38
LOC100520045	fas apoptotic inhibitory molecule 3-like	100520045	-0.45	0.00408	-1.03	-0.45	-0.38	-0.37	-0.41	-0.65	-0.27	-0.06	-0.18	-0.25
C2	complement component 2	448981	-0.45	0.00476	-1.10	-0.03	-0.14	-0.57	-0.52	-0.56	-0.16	-0.24	-0.03	-0.26
SLC5A8	solute carrier family 5 (iodide transporter), member 8	100524807	-0.46	0.00065	-0.40	-0.55	-0.40	-0.29	-0.88	-0.01	-0.48	-0.44	-0.27	-0.37
LOC100624581	lysosome-associated membrane glycoprotein 3-like	100624581	-0.46	0.00982	-1.06	-0.18	-0.06	-0.28	-0.41	-0.21	-0.24	-0.14	-0.44	-0.41
LOC100526183	SLAIN motif-containing protein 1-like	100526183	-0.46	0.00787	-1.12	-0.33	-0.78	-0.04	-0.89	-0.41	-0.38	-0.35	-0.60	-0.15

LOC1005	alkaline phosphatase,	100523		0.00	-0.	-1.	-1.	-0.	-0.	-0.	-0.	0.4	-0.	-0.
23216	placental type-like	216	-0.46	387	15	28	02	32	60	56	57	6	36	20
				0.00	-0.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.	-0.
CFH	complement factor H	397525	-0.46	564	91	17	10	39	80	03	51	19	33	21
	IKAROS family zinc	100510		0.00	-1.	-0.	-0.	-0.	-1.	-0.	-0.	0.4	0.0	-0.
IKZF2	finger 2 (Helios)	935	-0.47	578	37	78	33	40	04	41	19	5	7	68
LOC1005	peptidyl-prolyl cis-trans	100524		0.00	-0.	-0.	0.1	-0.	-0.	-0.	-0.	-0.	-1.	-0.
24935	isomerase FKBP11-like	935	-0.47	018	58	60	3	17	53	63	44	61	01	25
	WD repeat and FYVE	100157		0.00	-0.	0.0	-0.	-0.	-0.	-1.	0.0	-0.	-0.	-0.
WDFY3	domain containing 3	904	-0.47	130	70	1	17	84	58	23	5	15	60	46
	hydrogen voltage-gated	100152		0.00	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.	-0.	-0.
HVCN1	channel 1	115	-0.47	041	73	51	46	13	35	51	03	10	81	07
LOC3967				0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
57	trappin 8	396757	-0.47	000	36	21	43	28	98	67	53	48	41	37
LOC1006	rho GTPase-activating	100624		0.00	-1.	-0.	-0.	-0.	-0.	-0.	-0.	0.0	-0.	-0.
24751	protein 30-like	751	-0.47	100	21	20	23	64	91	87	16	6	37	20
LOC1005	protein kinase C beta	100522		0.00	-1.	-0.	-0.	-0.	-0.	-0.	0.1	-0.	-0.	-0.
22792	type-like	792	-0.48	156	11	40	03	98	98	45	8	03	57	37
	purinergic receptor P2Y,	100157		0.00	-1.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	0.1	-0.
P2RY10	G-protein coupled, 10	702	-0.48	688	64	56	07	99	49	24	42	5	6	69
LOC1005	SHC SH2 domain-binding	100522		0.00	-1.	-0.	0.0	-0.	-0.	-0.	-0.	0.0	-1.	-0.
22493	protein 1-like	493	-0.48	926	46	09	7	60	59	03	19	4	48	49
LOC1005	calpain small subunit	100518		0.00	-0.	-0.	-0.	0.4	-0.	-1.	-0.	-1.	0.2	-0.
18072	2-like	072	-0.48	902	16	39	72	5	30	39	68	27	2	59
LOC1007		100736		0.00	-0.	0.1	0.0	-0.	-1.	-0.	0.1	0.0	-1.	-0.
36918	protein EURL homolog	918	-0.48	900	83	6	7	67	51	61	8	4	25	42
	CD79a molecule, immunoglobulin-associate	100190		0.00	-1.	-0.	-0.	-1.	-0.	-0.	0.5	-0.	-0.	-0.
CD79A	d alpha	992	-0.49	456	36	32	51	05	38	14	3	19	93	52
	lymphocyte cytosolic	100156		0.00	-1.	-0.	0.0	-0.	-0.	-1.	-0.	0.2	-0.	-0.
LCP1	protein 1 (L-plastin)	254	-0.49	346	14	51	2	29	91	38	01	2	55	35
	CD79b molecule, immunoglobulin-associate	100511		0.00	-0.	-0.	-0.	-1.	-0.	-0.	0.0	-0.	-0.	-0.
CD79B	d beta	898	-0.49	088	54	38	49	36	52	03	8	54	84	30
	chemokine (C-C motif)			0.00	-0.	-0.	-1.	-0.	-1.	-0.	-0.	0.0	0.0	-0.
CCL28	ligand 28	554303	-0.49	119	39	69	08	20	21	16	73	8	3	60
	phosphoinositide-3-kinase	100155		0.00	-0.	-0.	-0.	-0.	-0.	-1.	0.3	-0.	-0.	-0.
PIK3AP1	adaptor protein 1	165	-0.50	657	49	08	26	36	77	86	3	23	82	44
LOC1005	uncharacterized protein	100513		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.
13515	C14orf105-like	515	-0.50	000	37	49	33	43	36	50	86	66	49	48
LOC1005		100525		0.00	-1.	-0.	0.2	-0.	-0.	-1.	0.1	-0.	-0.	-0.
25302	protein EVI2A-like	302	-0.50	512	16	34	6	63	99	36	6	38	37	17
LOC1001	leukocyte differentiation	100152		0.00	-1.	-0.	0.1	-0.	-0.	-1.	-0.	0.1	-0.	-0.
52851	antigen CD84	851	-0.50	253	02	55	4	58	88	29	04	7	57	37
	regulator of G-protein			0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	0.1	-1.	-0.
RGS16	signaling 16	397544	-0.50	102	78	02	72	53	21	92	24	6	14	61
				0.00	-0.	0.0	-0.	-0.	-1.	-1.	0.0	0.3	-1.	-0.
CD69	CD69 molecule	397165	-0.50	961	99	2	10	40	22	26	9	6	27	26
	kynurenine 3-monooxygenase (kynurenine			0.00	-1.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	0.2	-0.
KMO	3-hydroxylase)	397148	-0.50	141	25	48	14	33	21	77	28	41	8	45
LOC1007		100737		0.00	-0.	-0.	-0.	-0.	0.0	-0.	-2.	-0.	0.1	-0.
37508	cytochrome P450 2G1-like	508	-0.50	992	39	36	01	61	2	32	02	90	2	56
	putative sodium-coupled													
LOC1007	neutral amino acid	100737		0.00	0.1	-0.	-0.	0.4	-0.	-1.	-1.	-0.	-0.	-0.
37086	transporter 11-like	086	-0.51	681	9	40	33	8	73	18	20	81	08	98
	receptor-type													
LOC1007	tyrosine-protein	100738		0.00	-1.	-0.	0.0	-0.	-1.	-0.	0.1	0.2	-0.	-0.
38552	phosphatase C-like	552	-0.51	271	09	52	8	66	21	99	6	2	59	46
LOC1006	mitogen-activated protein	100622		0.00	-0.	-0.	-0.	0.0	-0.	-1.	-0.	0.0	-0.	-0.
22217	kinase kinase kinase 8-like	217	-0.51	515	73	78	13	3	93	80	29	0	23	22
		100520		0.00	-1.	-0.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.
PARVG	parvin, gamma	379	-0.51	052	20	56	14	34	62	15	39	20	43	05
	AF4/FMR2 family,	100512		0.00	-0.	-0.	0.0	-0.	-0.	-0.	0.0	-0.	-0.	-1.
AFF2	member 2	515	-0.52	041	74	32	0	66	75	51	1	22	91	09
LOC1006	protein kinase C theta	100621		0.00	-1.	-0.	0.0	-1.	-1.	-0.	0.3	0.3	-0.	-0.
21326	type-like	326	-0.52	846	56	59	5	08	02	84	2	2	22	59
LOC1005	interleukin-22 receptor	100513		0.00	-0.	-0.	-0.	-0.	-0.	-1.	0.0	-0.	-0.	-0.
13324	subunit alpha-2-like	324	-0.52	191	83	27	33	14	20	64	5	53	52	82
LOC1006	cytohesin-interacting	100627		0.00	-1.	-0.	0.0	-0.	-0.	-1.	0.2	0.2	-1.	-0.
27882	protein-like	882	-0.52	991	57	37	0	20	78	44	6	4	08	29
	signal transducer and			0.00	-1.	-0.	-0.	-0.	-1.	-0.	-0.	0.2	-0.	-0.
STAT4	activator of transcription 4	397261	-0.53	248	46	52	12	59	22	67	18	6	46	29
	protein tyrosine phosphatase, receptor type,	100522		0.00	-1.	-0.	-0.	-0.	-1.	-1.	0.1	0.2	-0.	-0.
PTPRC	C	631	-0.53	194	01	69	01	59	21	08	6	5	58	49

LOC1005	G-protein coupled receptor	100522		0.00	-1.	-0.	0.4	-0.	-1.	-0.	-0.	0.0	-0.	-0.
22635	183-like	635	-0.53	896	74	15	2	30	30	85	13	4	83	43
		100607		0.00	-0.	-0.	-0.	-0.	-1.	-1.	-0.	-0.	-0.	0.2
WAP-3	elafin family member	966	-0.53	665	28	16	61	71	59	58	31	24	02	1
LOC1001		100156		0.00	-0.	-0.	-0.	-1.	-0.	-1.	0.1	-0.	-0.	-0.
56280	gremlin-1-like	280	-0.54	283	04	47	13	69	21	03	7	91	36	69
LOC1001		100157		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-1.
57947	GTPase SLIP-GC-like	947	-0.54	083	38	08	69	96	98	48	04	32	14	34
		100153		0.00	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-0.	-2.	-0.
LCN2	lipocalin 2	501	-0.54	390	35	05	25	03	72	52	79	36	00	33
		100125		0.00	-0.	-0.	0.3	-0.	-1.	-1.	-0.	-0.	-0.	0.0
LY96	lymphocyte antigen 96	555	-0.54	351	55	78	1	65	45	32	18	27	59	5
	chromogranin B			0.00	-0.	-0.	-0.	0.4	-0.	-1.	-1.	-0.	0.3	-0.
CHGB	(secretogranin 1)	397154	-0.55	687	05	84	46	9	64	41	36	85	7	73
LOC1007	SLAIN motif-containing	100739		0.00	-1.	-0.	-0.	-0.	-0.	-0.	0.4	-0.	-1.	-0.
39847	protein 1-like	847	-0.55	338	52	71	05	48	73	40	0	05	17	81
				0.00	-0.	-0.	-0.	-2.	-0.	-0.	0.6	-0.	-0.	-0.
CD19	CD19 molecule	397669	-0.55	928	34	30	45	08	81	21	3	86	45	66
LOC1000	chitinase 3-like 2 isoform	100037		0.00	-0.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.	-0.
37944	A	944	-0.56	018	74	17	40	29	47	52	29	37	64	68
		100511		0.00	-1.	-0.	-0.	0.0	-0.	-1.	0.2	0.2	-1.	-0.
CD48	CD48 molecule	130	-0.57	808	42	38	17	5	99	53	8	0	34	35
				0.00	-1.	-0.	0.0	-0.	-0.	-1.	0.0	0.0	-1.	-0.
IL6R	interleukin 6 receptor	399522	-0.57	334	47	06	1	66	68	36	6	9	16	46
LOC1005		100524		0.00	-1.	-0.	0.1	-0.	-1.	-1.	-0.	0.0	-0.	-0.
24827	protein EVI2B-like	827	-0.57	385	34	44	7	32	05	65	07	2	91	14
				0.00	-0.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.	-0.
C4	complement C4	445467	-0.58	039	93	21	24	83	54	94	28	39	05	40
	cytidine monophosphate-N-acetyln euraminic acid			0.00	-0.	-0.	-0.	-0.	-1.	-1.	0.2	-0.	0.1	-0.
CMAH	hydroxylase	396918	-0.58	245	80	52	44	24	10	78	5	81	5	52
LOC1005	lymphoid-restricted	100518		0.00	-0.	-0.	-0.	-1.	-1.	-0.	0.1	-0.	-0.	-0.
18428	membrane protein-like	428	-0.59	061	96	49	07	19	30	85	2	11	47	61
		100156		0.00	-0.	-0.	-0.	0.0	-0.	-2.	0.1	-0.	-0.	-0.
BCL2A1	BCL2-related protein A1	860	-0.60	820	70	09	31	2	89	43	4	41	74	60
	serpin peptidase inhibitor, clade A (alpha-1													
SERPINA	antiproteinase,			0.00	0.0	0.4	0.0	-1.	-0.	-1.	-1.	-0.	-0.	-0.
1	antitrypsin), member 1	397688	-0.60	933	0	2	4	36	16	93	25	82	16	79
LOC1001	A-kinase anchor protein	100153		0.00	-1.	-0.	-0.	-0.	-1.	-0.	-0.	-0.	-0.	0.0
53460	5-like	460	-0.60	441	99	85	11	95	21	60	15	14	03	2
	interleukin 2 receptor,			0.00	-1.	-0.	-0.	-0.	-1.	-1.	0.0	-0.	-0.	-0.
IL2RG	gamma	397156	-0.60	076	46	50	10	50	00	40	8	20	58	38
		100038		0.00	-1.	-0.	-0.	0.0	-1.	-0.	-0.	-1.	0.0	-0.
PCD1B	CD1B antigen	007	-0.62	066	08	31	41	2	49	55	57	16	3	63
				0.00	-0.	0.0	-0.	-1.	-0.	-0.	0.1	-0.	-1.	-0.
CLU	clusterin	397025	-0.63	158	76	4	26	94	50	64	1	53	27	52
	acyloxyacyl hydrolase	100522		0.00	-1.	-0.	-0.	-0.	-1.	-0.	0.3	-0.	-1.	-0.
AOAH	(neutrophil)	290	-0.64	528	88	43	31	15	24	97	4	02	49	22
LOC1005		100520		0.00	-1.	0.4	0.0	-0.	-2.	-1.	0.1	-0.	-1.	-0.
20876	sorting nexin-10-like	876	-0.66	926	41	6	1	23	03	53	2	19	16	64
		100156		0.00	-1.	-0.	-0.	-0.	-1.	-1.	-0.	0.0	-0.	-0.
SLAMF6	SLAM family member 6	912	-0.67	020	57	70	34	76	17	10	13	2	44	56
	membrane-spanning 4-domains, subfamily A,	100627		0.00	-1.	0.3	-0.	-2.	-0.	-0.	0.4	-0.	-0.	-0.
MS4A1	member 1	952	-0.68	603	08	6	90	35	77	37	8	78	43	93
LOC1005	transmembrane protein	100525		0.00	-1.	-0.	-0.	-0.	-1.	-0.	-0.	0.0	-0.	-0.
25349	156-like	349	-0.69	007	17	27	60	74	46	79	63	8	67	69
	lymphoid	100170		0.00	-1.	0.1	-0.	-1.	-0.	-0.	0.0	-0.	-1.	-0.
LEF1	enhancer-binding factor 1	126	-0.70	123	10	4	38	81	91	71	8	30	55	51
LOC1007	monocarboxylate	100739		0.00	-0.	-0.	-0.	0.0	-0.	-2.	-0.	-0.	-1.	-0.
39042	transporter 7-like	042	-0.71	146	85	34	83	0	25	18	33	70	38	21
ARHGAP	Rho GTPase activating	100520		0.00	-1.	-0.	-0.	-0.	-1.	-1.	0.4	-0.	-1.	-0.
15	protein 15	808	-0.74	209	40	47	11	81	34	81	8	06	48	41
LOC1005		100512		0.00	-0.	-0.	-0.	-0.	-2.	-0.	-0.	-0.	-1.	-0.
12873	antileukoproteinase-like	873	-0.74	065	73	25	94	09	23	58	88	35	22	15
LOC1005	placenta-specific gene 8	100525		0.00	-1.	-0.	0.1	-1.	-2.	-0.	-0.	-0.	-0.	-0.
25175	protein-like	175	-0.78	046	23	08	2	11	17	89	48	65	66	67
LOC1001	SLAM family member	100154		0.00	-1.	-0.	-0.	-0.	-1.	-2.	0.0	0.0	-1.	-0.
54053	7-like	053	-0.78	584	04	32	10	43	34	92	0	4	40	30
C4H1orf1	chromosome 1 open	100627		0.00	0.2	-0.	-0.	-1.	-0.	-2.	0.8	-0.	-2.	-1.
62	reading frame 162 ortholog	962	-0.78	624	2	24	75	15	68	16	2	84	07	00
		100511		0.00	-0.	-0.	-0.	0.4	-1.	-1.	-1.	-1.	-0.	-0.
TPH1	tryptophan hydroxylase 1	002	-0.80	065	29	73	48	5	10	88	59	04	36	95
	BPI fold containing family	100113		0.00	-0.	-0.	-0.	-0.	-1.	-2.	-0.	-0.	-0.	-0.
BPIFB2	B, member 2	424	-0.84	003	62	69	90	59	24	01	57	85	13	80

LOC1005		100524		0.00	-1.	0.0	-0.	0.2	-1.	-2.	0.0	-0.	-2.	-0.
24766	P2Y purinoceptor 13-like	766	-0.86	809	72	3	26	5	94	62	0	18	01	19
	chemokine (C-X-C motif)			0.00	-1.	-0.	-0.	-0.	-0.	-1.	-0.	-0.	-1.	-1.
CXCR4	receptor 4	396659	-0.89	005	42	21	50	37	76	62	54	49	85	16
	immunoresponsive 1	100524		0.00	-1.	0.3	-0.	0.2	-2.	-2.	-0.	-0.	-2.	-1.
IRG1	homolog (mouse)	951	-0.93	558	17	4	21	4	07	59	24	15	28	17
	C-type lectin domain	100038		0.00	-1.	-0.	0.0	-0.	-2.	-2.	0.0	-0.	-1.	-0.
CLEC7A	family 7, member A	025	-0.94	372	29	66	8	16	36	98	7	49	14	47
	secretory leukocyte			0.00	-1.	-0.	-0.	-0.	-1.	-1.	-1.	-1.	-1.	-0.
SLPI	peptidase inhibitor	396886	-1.13	003	07	08	20	39	92	47	83	91	48	93
LOC1005	C4b-binding protein alpha	100520		0.00	-1.	-0.	-0.	-0.	-2.	-3.	-0.	-1.	-4.	-0.
20761	chain-like	761	-1.42	190	41	65	27	08	39	61	45	12	00	25
	chitinase 3-like 1 (cartilage			0.00	-2.	0.3	-0.	-1.	-2.	-3.	-0.	-2.	-2.	-1.
CHI3L1	glycoprotein-39)	396865	-1.88	008	96	2	66	84	84	82	53	38	89	24

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Jing

张京

About the author

Curriculum vitae

Jing Zhang was born on February 24th, 1983, in Fushun, China. After completing high school in 2002, she started her BSc studies in Animal Science at the Nanjing Agricultural University during the period from September 2002 to June 2006. She continued with the study "Gastrointestinal microbiology" at Nanjing Agricultural University from September 2006 to June 2009 to obtain her MSc. The MSc thesis, titled "Isolation and identification of amino acid utilizing bacteria from pig small intestine" was defended under the supervision of Prof. Dr. Weiyun Zhu in June 2009. In the same year, she was granted a fellowship from the China Scholarship Council to obtain the doctoral degree at Laboratory of Microbiology at Wageningen University under the supervision of Prof. Dr. Hauke Smidt and Prof. Dr. Willem de Vos from February 2010 till now. She was involved in several pigs' and chicken projects, including: 1) the EU-FP7 funded project entitled "Interplay of microbiota and gut function in the developing pig – Innovative avenues towards sustainable animal production", and 2) Wageningen University Strategic Research Agenda IP/OP funded program entitled "Satiety & Satisfaction". Her main role in these two projects was to analyze the microbial diversity and composition in the pig's gut, which are the main findings of this thesis.

List of publications

1. Jansman AJ, **Zhang J**, Koopmans SJ, Dekker RA, Smidt H. Effects of a simple or a complex starter microbiota on intestinal microbiota composition in caesarean derived piglets. Journal of Animal Science, 2012. 90(Supplement 4): p. 433-435.
2. Haenen D*, **Zhang J***, Souza da Silva C, Bosch G, van der Meer IM, van Arkel J, van den Borne JJ, Pérez Gutiérrez O, Smidt H, Kemp B, Müller M, Hooiveld GJ. A diet high in resistant starch modulates microbiota composition, SCFA concentrations, and gene expression in pig intestine. The Journal of Nutrition, 2013. 143(3): p. 274-283.
3. Haenen D, Souza da Silva C*, **Zhang J***, Koopmans SJ, Bosch G, Vervoort J, Gerrits WJ, Kemp B, Smidt H, Müller M, Hooiveld GJ. Resistant starch induces catabolic but suppresses immune and cell division pathways and changes the microbiome in the proximal colon of male pigs. The Journal of Nutrition, 2013. 143(12): p. 1889-1898.
4. Arnal ME, **Zhang J**, Messori S, Bosi P, Smidt H, Lallès JP. Early changes in microbial colonization selectively modulate intestinal enzymes, but not inducible heat shock proteins in young adult swine. PLoS One, 2014. 9(2): p. e87967.
5. Schokker D*, **Zhang J***, Zhang L, Vastenhouw SA, Heilig HGHJ, Smidt H, Rebel JMJ, Smits MA. Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. PLoS One, 2014. 9(6): p. e100040
6. Hugenholtz F*, **Zhang J***, O'Toole PW, Smidt H. Studying the mammalian intestinal microbiome using animal models. Accepted for publication.
7. Arnal ME, **Zhang J**, Erridge C, Smidt H, Lallès JP. Early changes in microbial colonization selectively modulate various aspects of colonic physiology in young and adult swine offspring. Re-submitted for publication after revision.
8. **Zhang J**, Jansman AJM*, El Aidy S*, Schokker D*, Perez O, Feng Y, Koopmans SJ, Dekker RA, Smidt H. Post-natal microbial association has impact on gut microbial colonization and intestinal gene expression in caesarean derived piglets. Manuscript in preparation.
9. **Zhang J**, Rwibasira P, Arnal ME, Perez-Gutteriez O, Lallès JP, Smidt H. Influence of maternal antibiotic treatment on intestinal microbiota in piglets. Manuscript in preparation.
10. **Zhang J**, Hornung B, Hugenholtz F, Ramiro Garcia, J, Souza da Silva C, Bosch G, Schaap P, Smidt H. Metatranscriptomic analysis of microbial composition and function in pig gut. Manuscript in preparation.

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Overview of completed training activities

Discipline specific activities

Genetics and physiology of food-associated micro-organisms, Wageningen, the Netherlands, 2010

Gut day 2010, Ghent, Belgium, 2010

System biology course Statistics of ~omics data analysis, Wageningen, the Netherlands, 2010

Functional Metagenomics of the Intestinal Tract and Food-Related Microbes, Helsinki, Finland, 2011

Gut day 2011, Wageningen, the Netherlands, 2011

^{12}th International Symposium on Digestive Physiology in Pigs, Keystone, USA, 2012

^{14}th International Symposium on Microbial Ecology, Copenhagen, Denmark, 2012

^{4}th International Human Microbiome Congress, Hangzhou, China, 2013

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