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TITOLO TESI

ANALYSIS OF FACTORS AFFECTING THE BACTERIAL COMMUNITY VARIATIONS IN THE GASTROINTESTINAL TRACT OF PIGS

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Abstract

The gastrointestinal tract (GIT) hosts a dense microbial population that establishes a complex network of symbiotic relationships with the host, which determine the homeostasis of the host-microbiota system.

Over the recent years, an ever-increasing number of conditions have been associated with different configurations of the microbial communities and it has been seen that by acting on the structure of the microbiota it is possible to affect the health status, the welfare and the performances of the host.

However, the knowledge we have about the variables involved in determining the structure of the microbial communities are still limited and the identification of the key factors in the microbiota shaping represents a very active line of research that aims at the development of tools and strategies for modulating the microbiota as a way to improve the welfare and the health status of the host, the food efficiency, and as an alternative way to the reduce the use of antibiotics in livestock production.

The aim of this work was to test the effect of some factors that could play a role in shaping the bacterial communities of the porcine gastrointestinal tract.

In the first study, the microbiota profile from oxyntic mucosa, pyloric mucosa, gastric groove and luminal content of the stomach was analysed in weaned pigs, testing the hypothesis of the existence of multiple microbial niches in the gastric environment. A different pattern between mucosal and luminal bacterial communities was reported.

In the second study, the effect of a long-term formic acid administration was tested on growth performances, on the H^+/K^+-ATPase presence in the oxyntic mucosa, on the expression of gene markers for inflammatory response in jejunal mucosa, and on jejunal bacterial community structure, in weaners piglets. The overall results suggested an adaptive response to the long-term administration of formic acid, and the bacterial community showed a reduction in lactic and butyric acid producing bacteria.

In the third study, the piglet's faecal microbiota rearrangement from the lactation period to the post-weaning phase was analysed taking into account the potential impact of the host A0 blood group. The

weaning shift in bacterial community suggested a role of milk-derived lipids in microbiota shaping, while no effects related to the blood group were reported.

These studies contributed to the knowledge on bacterial community shaping in young pigs, focusing the attention on aspects not yet well explored for the porcine GIT microbiota, such as the role of the gastric environment and of the blood group, and showing the effects of potential adaptation to treatments currently used in the swine industry such as the administration of the organic acids.

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Overview and Aims

The mammalian gastrointestinal tract (GIT) is a ‘lotic’ ecosystem characterized by a succession of stratified environments, colonized by microbial communities. The term ‘Microbiota’ refers to these communities, which are composed of Bacteria, Archaea, Viruses and Eukaryota. Nevertheless, the term ‘Microbiota’ is commonly used in literature as a synonym for ‘bacterial community’ that is currently the most studied portion of the GIT microbial ecosystem. The interplay between microbial communities and their habitat (the host) originates a complex network of symbiotic interactions that determine the homeostasis of the host-microbial system. The microbiota plays key roles in the barrier effect against pathogens and in the availability of dietary components otherwise unusable for the host, which are of fundamental importance for the efficiency of the gastrointestinal tract and for the state of health of the host. The microbiota is also essential for the development, education and functionality of the immune system and is involved in the development and regulation of the "gut-brain axis" and the endocrine system.

The gastrointestinal ecosystem is highly dynamic, and the microbiota is susceptible to rapid changes in response to various factors such as age, health status and genetic makeup of the host, diet, administration of drugs and many others, which can therefore, influence the structure and metabolic potential of the microbial community.

In last years the accessibility to high-throughput sequencing technologies has allowed the bloom of the bacterial communities profiling studies based on the sequencing of the 16S rRNA gene hypervariable regions, however, despite the large amount of data produced, we are still at the dawn of understanding of the complex network of variables involved in the shaping of the GIT microbiota. The identification of the factors involved in the shaping of the bacterial communities is a fundamental prerequisite for setting tools and strategies aimed at the manipulation and modulation of these communities in order to improve the health and welfare of the host, the efficiency in the use of diet-derived nutrients and in perspective of the reduction of the antibiotics use.

The aim of the present work was to assess the effect of different factors which may impact on the structure of the gastrointestinal bacterial community in pigs.

The specific aims of the three studies presented in this thesis were:

1) In the first work, the bacterial community of the stomach was studied. Was testing the hypothesis that different areas (for functions and characteristics) of the gastric mucosa could correspond to different microbial niches. The V6 region of the 16S rRNA gene was used for the microbiota profiling of three different areas of the gastric mucosa (fundus, pylorus and gastric groove) and of gastric content from weaned piglets.

Study published in PLoS One. doi:10.1371/journal.pone.0173029

2) The aim of the second study was to evaluate the impact of two doses of formic acid supplementation for six weeks to weaner pigs. The long-term effects of formic acid were tested for growth performance, microbiota composition in the jejunum using the V3-V4 region of the 16S rRNA gene, expression of marker genes for inflammation in the jejunum, and the quantification of acid-secreting cells in the gastric oxyntic mucosa using immunohistochemistry.

Study published in Animal Feed Science and Technology. doi:10.1016/j.anifeedsci.2017.06.015

3) The aims of the third study were to test the hypothesis that the genotypes for A0 blood groups impact on piglets' fecal bacterial community long some crucial moments such as suckling and weaning, and contribute to enriching the knowledge about the development of the early microbiota in piglets, identifying potential key points in its shaping. The region V3-V4 of the 16S rRNA gene was used for the profiling of the fecal bacterial community of the suckling and weaned piglets, and the bacterial metabolic potential was also evaluated by metagenomic predictions.

Literature review

1 The gut Colonization

The settlement of GIT microbiota in pigs, as in other mammals, is a complex succession of events in which we can detect a longitudinal progression, in terms of time (birth→adulthood) and in terms of space (proximal→distal). Although recent studies advanced the "in utero colonization hypothesis", the current scientific evidence does not support this hypothesis (Perez-Muñoz et al., 2017), thus, more than a century later his statement, the "sterile womb paradigm" remains the central dogma, and any intra-uterine bacterial presence is assumed to be dangerous for the foetus (Funkhouser and Bordenstein, 2013).

The GIT can be compared to an open tube constantly subjected to new microbial inputs via oral route (Isaacson and Kim, 2012), immediately after birth the early microbial colonizers invade the intestine of the new-borns, triggering an ecological succession primarily driven by changes in intestine from aerobic to anaerobic environment and by changes in diet composition (Savage, 1977). The succession leads to the alternation of different predominant bacterial groups until the establishment of a climax community, characterized by a certain dynamic equilibrium, which is affected by several changes in response to the continuous external microbial inputs, the environmental changes determined by the host's physiological state, and the dietary changes (Isaacson and Kim, 2012).

As the GIT is an open ecosystem, a critical point in defining gut microbiota colonization is the distinction between autochthonous and allochthonous component of the communities, this distinction is difficult especially in new-borns, in whom the bacteria acquired transiently in peripartum phase and then from environmental inputs, represent a fundamental prerequisite (inoculum) for the development of the autochthonous microbiota. This implies that only those bacteria able to multiply in a specific niche with a rate that can equal or exceed the shedding rate and/or able to adhere permanently to the intestinal surface will form the autochthonous communities, whereas, the

allochthonous components will not be able to colonize the same niche except under aberrant conditions (Mackie et al., 1999).

The microbiota vary qualitatively and quantitatively accordingly with the chemical and nutrient gradients, and with the physiological and immune compartmentalisation, from the proximal to the distal part of the GIT, establishing the densest communities in caecum and colon, in addition, the differences over the intestinal cross-section axis determine compartments between mucosal folds and also between lumen and intestinal wall, which can represent microhabitats with peculiar microbial communities. Several other factors, of both bacterial and host origin, influence the gut colonization, the mains one are: the immune reactivity of the host, the presence of mucosal receptors, the nutrient composition and availability, the intestinal content flow, the pH and the O_2 concentration, and other exogenous factors such as the use of antimicrobials (Figure 1) (Donaldson et al., 2015).

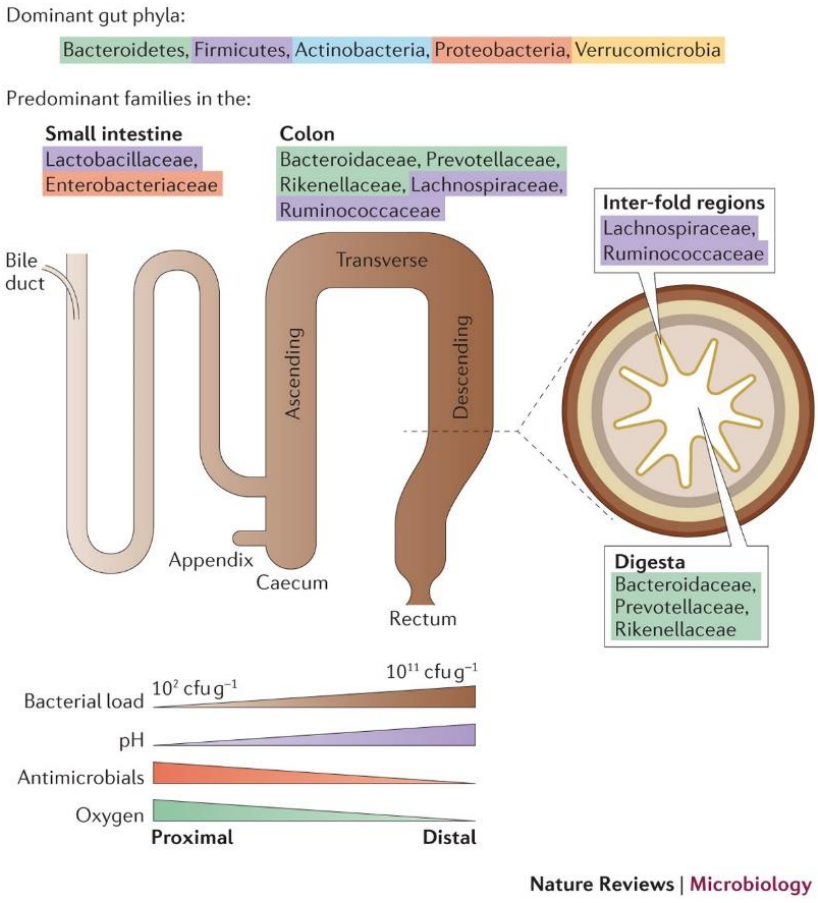

Figure 1: Microbial habitats in gastrointestinal tract (human)
Reprinted by permission from Macmillan Publishers Ltd: [REVIEWS IN MICROBIOLOGY] Donaldson et Al. "Gut biogeography of the bacterial microbiota" Reviews Microbiology 14, 20–32 copyright 2015. Nature Publishing Group and Copyright Clearance Center, Licence number 4241930175915.

1.1 Early colonizers in piglets and milk oriented microbiota

From the moment of partum, the sterile GIT of the piglets is suddenly exposed to a plethora of microbes through contact with the vagina, stools and mother's skin, and through the surrounding environment. During the first 48 hours after birth the gastric pH decreases to 2.0 inhibiting the proliferation of most bacteria except for Lactobacilli which become the predominant genus in the stomach, whereas *Escherichia coli* finds its niche preferential in the small and large intestine.

The maternal microbiota represents the first inoculum and it can be detected in early stage, in fact, the analysis of bacterial metabolic fingerprint in faeces of the new-borns shows high similarity with that of their mothers, however, despite the close contact that piglets still have with their dams, these similarities disappear already seven days after birth when the sows-derived 'birth microbiota' shift to a 'milk oriented microbiota' during the suckling period (Frese et al., 2015; Katouli et al., 1997).

Swords and colleagues (1993) followed the pig colonic microbiota evolution starting from the birth to four months of life and showed the extremely fast shift during the early colonization. In the first hours after birth the aerobes and facultative anaerobes bacteria derived from sows and environment represent the predominant group, comprising 80% of the entire bacterial community, however, the metabolic activities of the pioneer colonizers modify the GIT environment and the situation already changes 24 hours after birth. In fact, the rate of oxygen utilization by facultative organisms is faster than the rate of oxygen solution, as consequence, the environment in the lower gut became highly reducing, the oxygen tension is lower, resulting in more favourable conditions for the anaerobes, which two days after birth represent 90% of colonic bacterial community in piglets (Figure 2) (Swords et al., 1993).

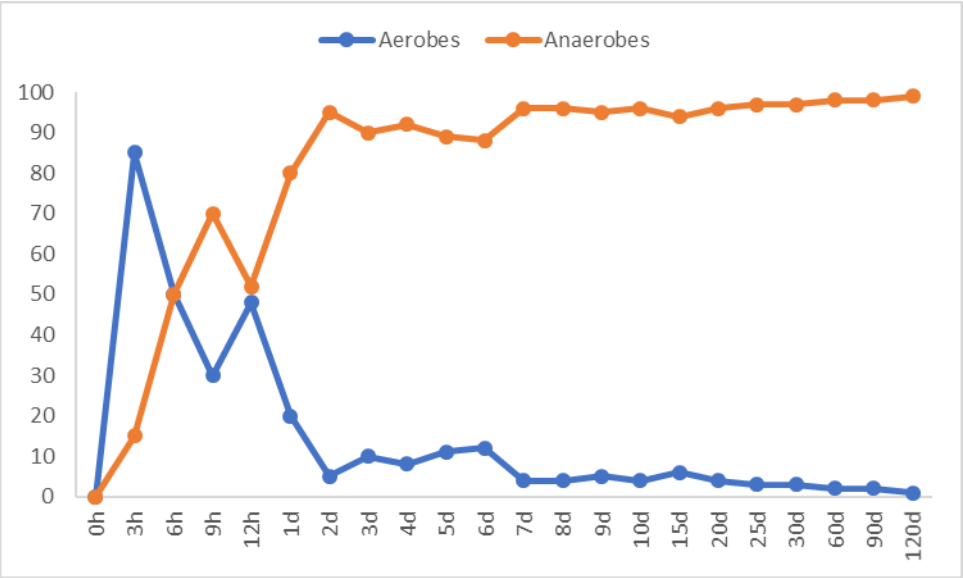

Figure 2 Changes in densities (%) of aerobic and anaerobic bacteria in distal colon of pigs from birth to 120 days of life.

Adapted from Swords et al. (1993) "Postnatal Changes in Selected Bacterial Groups of the Pig Colonic Microflora" Biol Neonate. 1993;63(3):191-200. Copyright © 1993 Karger Publishers, Basel, Switzerland. Karger Publishers and Copyright Clearance Center, Licence number 4242651087941.

It is also important to report the colostrum intake as another key factor for piglet's development and for the microbiota shaping in the first few days of life. Indeed, in addition to being rich in immunoglobulins, the colostrum also contains a set of others factors impacting the immune system and antimicrobial factors, that include: antibacterial enzyme lactoperoxidase, antibacterial and lytic enzyme lysozyme, defensins, iron-binding antimicrobial protein lactoferrin, soluble CD14 and oligosaccharides that act as equivalent of mucosal microbial ligands (Hurley and Theil, 2011). All these factors may play a role in modulating, directly (antimicrobials) and/or indirectly (immune activation), the early microbial colonization of the GIT.

During the following suckling period, the microbial biodiversity increases and became quite stable in its taxonomic composition. The milk-based diet shapes the composition of the bacterial community towards a milk oriented microbiota (MOM), which at this stage is dominated by bacteria capable of metabolizing milk glycans not digested by the host in the upper part of the intestine, and is dominated by Enterobacteriaceae and Bacteroidaceae (Pajarillo et al. 2014; Mach et al. 2015; Frese et al. 2015). It is also significant to note that pathobiont taxa, such as *Escherichia spp.*, are part of the bacterial

community in the lactation phase, awaiting potential stresses that can lead them to became pathogenic (Chen et al., 2017; Kim et al., 2011; Mach et al., 2015).

1.2 Weaning transition and dysbiosis

Current practices in pig industry include an early weaning of piglets (3-4 weeks of life), this event represent a very stressful phase for piglets which concern social, environmental and nutritional changes (Montagne et al., 2007). The weaning leads to a period of post-weaning anorexia, which causes growth stasis of piglets, that is accompanied by adverse changes in intestinal morphology, determined by a series of events reviewed by Lalles et al. (2004), among which are reported: a reduction in villous length, an alteration in electrolyte and fluid balance and an augmented mucosal permeability, decreased enzymatic activities are also reported, an enrichment in expression of genes for proinflammatory cytokines as well as the levels of mucins and goblet cell density are reduced.

Montagne and colleagues (2007) define this period of gut integrity rearrangement as *Post-weaning acute phase* and attribute it to a duration of about five days in the immediate post-weaning. The time span ranging from day 5 to 15 after weaning is defined, in the same study, as *Adaptation to the weaning diet*. The re-feeding and the resulting arrival of nutrients in the intestine represent the driving factor in this phase. The main changes concern the partial restoration of the length of the villi, hence an increased mass of the jejunum, and an increased activity of maltase, amylase and pancreatic trypsin and a low lipase activity. These enzymatic changes can be related with the shift from the milk-based diet, rich in lipids, to weaner diets and the presence of starch in these, as well as the development and maturation of the distal intestine represent another marker of this phase.

These drastic changes in the intestinal environment are reflected on the bacterial community that becomes particularly unstable in the immediate post-weaning, with a decrease in biodiversity (Hu et al., 2016) which is then restored between two and three weeks after weaning (Inoue et al. 2005; Pajarillo et al. 2014). The main changes during the weaning transition concern the increase in bacteria

capable of using the complex plant-derived carbohydrates (weaners diet) in distal intestine, as Prevotellaceae and Ruminococcaceae. However, there are differences in the variations found in the different studies for the non-dominant taxa and that can be related to the different techniques used, the different experimental conditions and of course, to the different intestinal tract analysed. In a recent review, Gresse and co-workers (2017) summarized the results of the principal studies which analysed the microbiota shift during the weaning transition (table1).

Table1 Influence of weaning transition on pig GIT microbiota

Modified from Gresse et Al. 2017 "Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health" Trends in microbiology Volume 25, Issue 10, p851–873, October 2017. Elsevier and Copyright Clearance Center, Licence number 4242630646251.

Age of piglets	**Diet and breeding**	**Origin of samples**	**Method of microbiota analysis**	**Qualitative modifications of gut microbiota**	**Quantitative composition of gut microbiota**		**Refs**
					Before weaning	**After weaning**	
Healthy piglets							
19 days	32 piglets weaned with a cereal and protein-based diet	Ileum, Colon	PCR-DGGE, qPCR, 16S amplicon sequencing	Decrease in *Lactobacillus* in the ileum	No clones of *E. coli* in the ileum and colon $7.1 \pm 1.3 \times 10^7$ of total Lactobacillus in ileal samples of unweaned piglets	30% of clones highly similar to *E. coli* and *Shigella flexneri;4.4 ± 0.5 × 10^7 of total Lactobacillus in ileal samples 2 days postweaning*	(Konstantinov et al., 2006)
7 to 35 days	~30 piglets from 3 different litters, fed with cereal-based diet and no antibiotics	Stomach, Jejunum, Ileum	PCR-DGGE, qPCR, 16S amplicon sequencing	Decrease in *Lactobacillus* spp. relative abundance in the stomach, jejunum and ileum	*Streptococcus suis* not detected	10^7 copies/g of potentially harmful *Streptococcus suis*	(Su et al., 2008)
4 to 6 weeks	15 piglets weaned at 28 days and housed in controlled environmental conditions	Feces	16S amplicon sequencing (454-pyrosequencing)	Increase in *Prevotella* relative abundance, Increase in Shannon-Weaver diversity index, Shift from Firmicutes to Bacteroidetes, Shift from Firmicutes to Bacteroidetes, New species of Clostridium detected	54.0% of Firmicutes; 38.7% of Bacteroidetes; 4.2% of Proteobacteria; 0.7% of Spirochates	35.8% of Firmicutes; 59.6% of Bacteroidetes; 1.0% of Proteobacteria; 2.0% of Spirochates	(Alain B Pajarillo et al., 2014)
7 to 32 days	Piglets weaned at 25 days, randomly divided into 4 litters	Stomach Ileum Colon	PCR DGGE, qPCR	Decrease in diversity in the ileum and colon, No change in the stomach	ND	ND	(Tao et al., 2015)

14 to 70 days	31 piglets weaned at 28 days fed with carbohydrate-based concentrate	Feces	16S amplicon sequencing (454-pyrosequencing)	Increase in *Acetivibrio*, *Dialister*, *Oribacerium* and *Prevotella*	At 14 days: ~9% of Bacteroides; ~2%of Escherichia/Shigella; ~3%of Lactobacillus; ~0.5% of Prevotella	At 36 days: ~0% of Bacteroides; ~0%of Escherichia/Shigella; ~0.5% of Lactobacillus; ~28% of Prevotella	(Mach et al., 2015)
21 days to 3 weeks	36 crossbred castrated male pigs weaned at 21 days and fed with mash diet	Feces	qPCR, 16S amplicon sequencing (Illumina MiSeq)	Increase in Proteobacteria relative abundance at 1 week postweaning and decrease at 3 weeks postweaning, Increase in anaerobic fiber-fermenting Firmicutes	ND	40.8–45.8% of Bacteroidetes; 35.8–45.1% of Firmicutes; 0.9–12.9% of Proteobacteria; 0.7–5.2% of Tenericutes; 1.4–3.1% of Spirochaetes; 0–1.3% of Planctomycetes	(Yang et al., 2016)
0 to 7 weeks	40 Meishan and Yorkshire piglets weaned at 28 days and separated in mixed groups at birth	Feces	16S amplicon sequencing (454-pyrosequencing)	Disappearance of *Fusobacterium; Lower relative abundance of Lactobacillus; Prevotella, Ruminococcaceae, Spirochaetaceaemore abundant;*	~45–70% of Firmicutes; ~20–40% of Bacteroidetes; ~3–20% of Fusobacterium	~55–70% of Firmicutes; ~20–30% of Bacteroidetes; ~0–10% of Fusobacterium	(Bian et al., 2016)
35 days	120 piglets weaned at 2 days and fed with basal diet for 14 days	Jejunum	qPCR	Decrease in *Lactobacillus; Increase in Enterococcus and Escherichia coli*	ND	ND	(Wei et al., 2017)

The drastic rearrangement of the GIT ecosystem after weaning can lead to a condition defined as 'dysbiosis'. This condition has a crucial role as a predisposing factor in gastrointestinal disorders like post-weaning diarrhoea in piglets, which represent a critical point for the pig industry. In fact, the F4+ and F18+ *E. coli* - the main infectious agents of post-weaning diarrhoea in piglets - causes significant impacts in terms of growth retardation, mortality and high use of antimicrobials (Nguyen et al., 2017). The characteristics of the dysbiosis are still not well defined, however, the literature available in this regard describes an imbalance of the microbial community characterized by a decline in strictly anaerobic bacteria belonging to Clostridia and Bacteroidia classes which correspond to an increase in facultative anaerobes including Enterobacteriaceae species (Winter et al., 2013).

The links between dysbiosis and post-weaning gastrointestinal disorders are not completely clarified, however, Gresse et al. (2017) summarized the mechanisms involved as follow:

The decreased microbial diversity makes glycan derived from mucus layer more available for use by pathogenic bacteria residing in intestine, e.g. invitro studies revealed that some *E. coli* strains are able to use the fucose produced by commensal *Bacteroides* to activate the type III secretion system (T3SS) which is used to detect and adhere to the intestinal absorptive cells of the host. The reduction in biodiversity is also associated to a greater intestinal permeability which can favour the transit of toxins and pathogens through the epithelium. Moreover, the inflammatory host-response to the weaning stress can lead to a nitrate-rich environment that could represent an advantage for *E. coli* which have nitrate reductase genes. In addition, the intestinal inflammation calls for increased blood flow with a consequent increase in oxygen supply, this can result in a reduction in the relative abundance of obligate anaerobes and further promote Enterobacteriaceae and other facultative anaerobes. In this context, the administration of antibiotics can feed this vicious circle by promoting the inflammatory response and by further reducing the variability of the bacterial community (Figure 3).

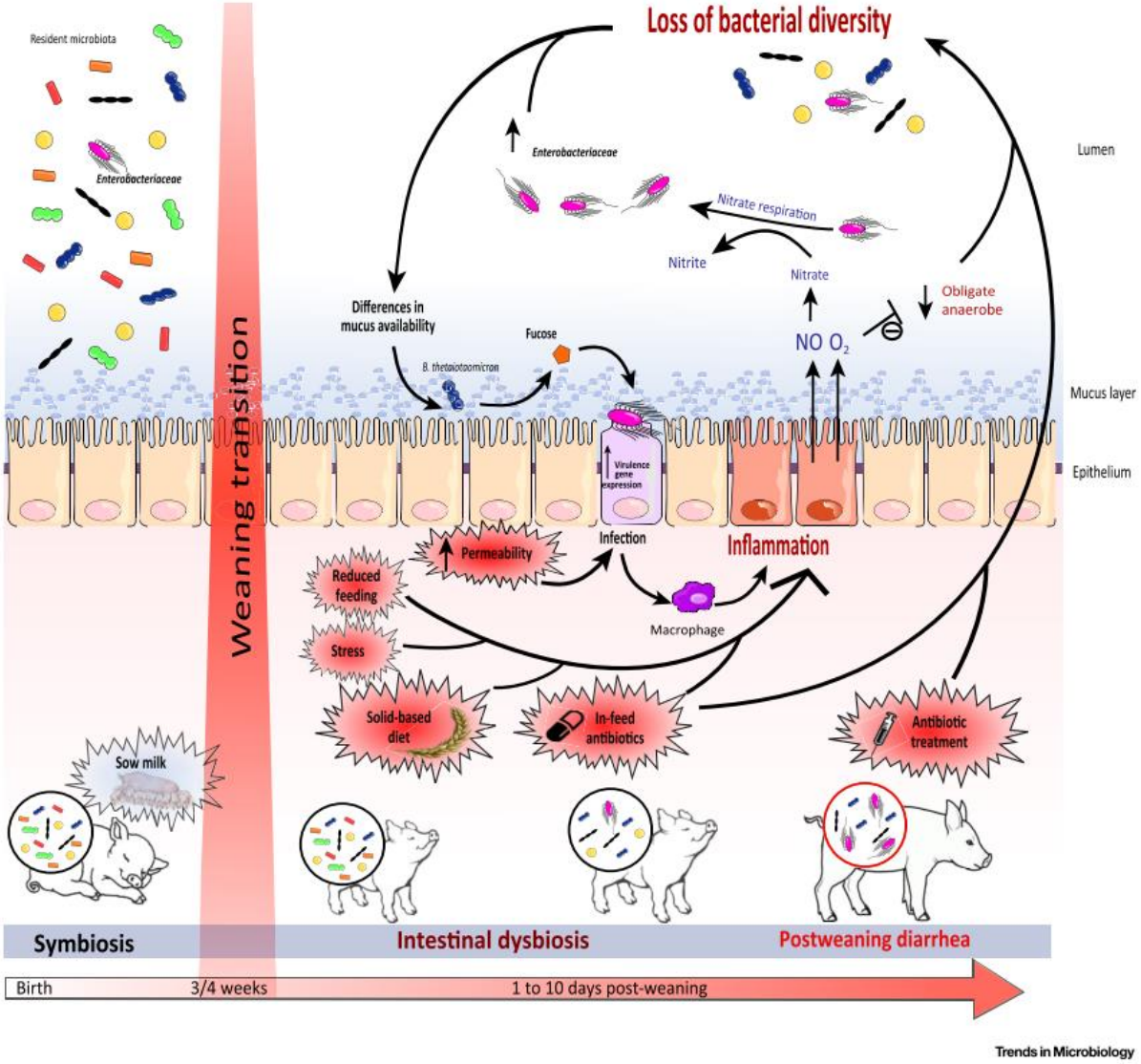

Figure 3: Impact of Weaning Transition on Piglet Gut Microbiota and Expansion of Infectious Agents.
From Gresse et Al. 2017 "Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health" Trends in microbiology Volume 25, Issue 10, p851–873, October 2017. Elsevier and Copyright Clearance Center, Licence number 4242630646251.

Furthermore, the weaning is characterized by different kind of stressors for piglets, such as the mixing with non-littermates, which lead to; hypothalamic pituitary adrenal axis (HPA) activation, augmented circulation of stress-related mediators (cortisol, corticotropin releasing factor), changes in intestinal transepithelial permeability and a higher susceptibility to pathogens (Jones et al., 2001; Moeser et al., 2017). Even if the mechanisms involved are still to be elucidated, the microbiota seems to play a central role in the development of the gut-brain axis, and a part of the research in this area aims at the development of "psychobiotic-based" and diet-based interventions for the treatment of stress-related disorders (Foster et al., 2017).

1.3 The mature microbiota

After weaning, the microbiota of the healthy animal becomes quite stable and it is possible to better define a proximal→distal gradient along the GIT.

Pigs are omnivores with a simple gastrointestinal system without a functional caecum. Contrary to ruminants, in pigs, most of the feed is digested by endogenous enzymes in the proximal GIT, where take places the degradation of proteins, carbohydrates and fats into amino acids, monosaccharides, monoglycerides, glycerol and free fatty acids and the subsequent absorption for systemic distribution. The proximal GIT of pigs contains a relatively low number of autochthonous bacteria whereas the highest bacterial concentration is confined to the distal intestine, where the short-chain fatty acids derived by bacterial fermentations can provide about 10-30 % of the energy requirements (Brunnberg et al., 2011).

Because of the low pH, the peristaltic movements and the rapid transit of the bolus, the stomach and the proximal part of the small intestine are considered an unfavourable habitat for a stable bacterial colonization. The discovery of *Helicobacter pylori* weakened the dogma for which 'the stomach is a sterile organ', in fact, some recent studies in humans suggest that the mucosal surface of the healthy stomach can represent a colonization site for a resident gastric microbiota in which the presence of genera such as *Prevotella*, *Streptococcus*, *Veillonella*, *Rothia*, and *Haemophilus* were reported (Nardone and Compare, 2015). Furthermore, the gastric environment represents a strong selection factor for bacteria and its role of ecological filter is fundamental in shaping the structure of the whole gut microbial community (Beasley et al., 2015). In pigs very few studies are focused on the proximal GIT microbiota and the Lactobacilli and Streptococci are reported as dominant genera in the stomach (Brunnberg et al., 2011; Mann et al., 2014; Mu et al., 2017).

The distal part of the small intestine is considered a transition region leading to the large intestine (Jensen and Jørgensen, 1994). The environmental conditions gradually change along the small intestine and the slower flow of digesta and the higher pH determine an increased bacterial density

and diversity. *Lactobacillus, Streptococcus, Clostridium, Escherichia, Helicobacter, Anaerobacter, Turicibacter, Acinetobacter* are the principal genera reported for the Ileum and Jejunum, with quite variable proportions in different studies (Looft et al., 2014; Mann et al., 2014; Zhao et al., 2015).

The cecum and colon are the major sites for bacterial fermentations in the pig gut, characterized by a highly diverse population. The pH close to neutrality, the reducing environment, the slowest transit and therefore the greater substrate availability constitutes an ideal environment for the development of a diverse and stable microbiota (Fonty and Gouet, 1989). The fiber fermenters bacteria such as *Prevotella*, Ruminococcaceae, and Clostridiales are the dominant taxa reported for the large intestine in pigs (Looft et al., 2014; Mann et al., 2014; Zhao et al., 2015).

Although the bacterial community is more stable after weaning, longitudinal studies showed that the microbiota of pigs continues to change, becoming more uniform among subject as pigs aged. The main changes reported regarding the decrease in Proteobacteria and Bacteroidetes and an increase in Firmicutes and Spirochaetes in the faecal bacterial community (Kim et al., 2011; Zhao et al., 2015).

A wide range of variability can be still determined by different factors like environment and host genetic, as well as, the different methods and techniques used in various studies on microbiota profiling can represent a confounding factor, and the definition of a 'core microbiota' can be difficult. In last years a high number of studies have used high-throughput sequencing of the 16S rRNA gene (hypervariable regions) to define the structure and composition of the gut microbiota, in a recent work Holman and colleagues (2017) performed a Meta-analysis using 20 of these data sets publicly available deriving from studies on swine gut microbiota. They concluded that the most significant factors in defining the microbial profile of the pig are the localization along the gastrointestinal tract and the "study effect". This, on the one hand, highlights that technical limitations which make it difficult to study such a complex ecosystem still exist, but on the other hand, it confirms the peculiarity of bacterial communities in relation to well-defined ecological conditions (figure 4).

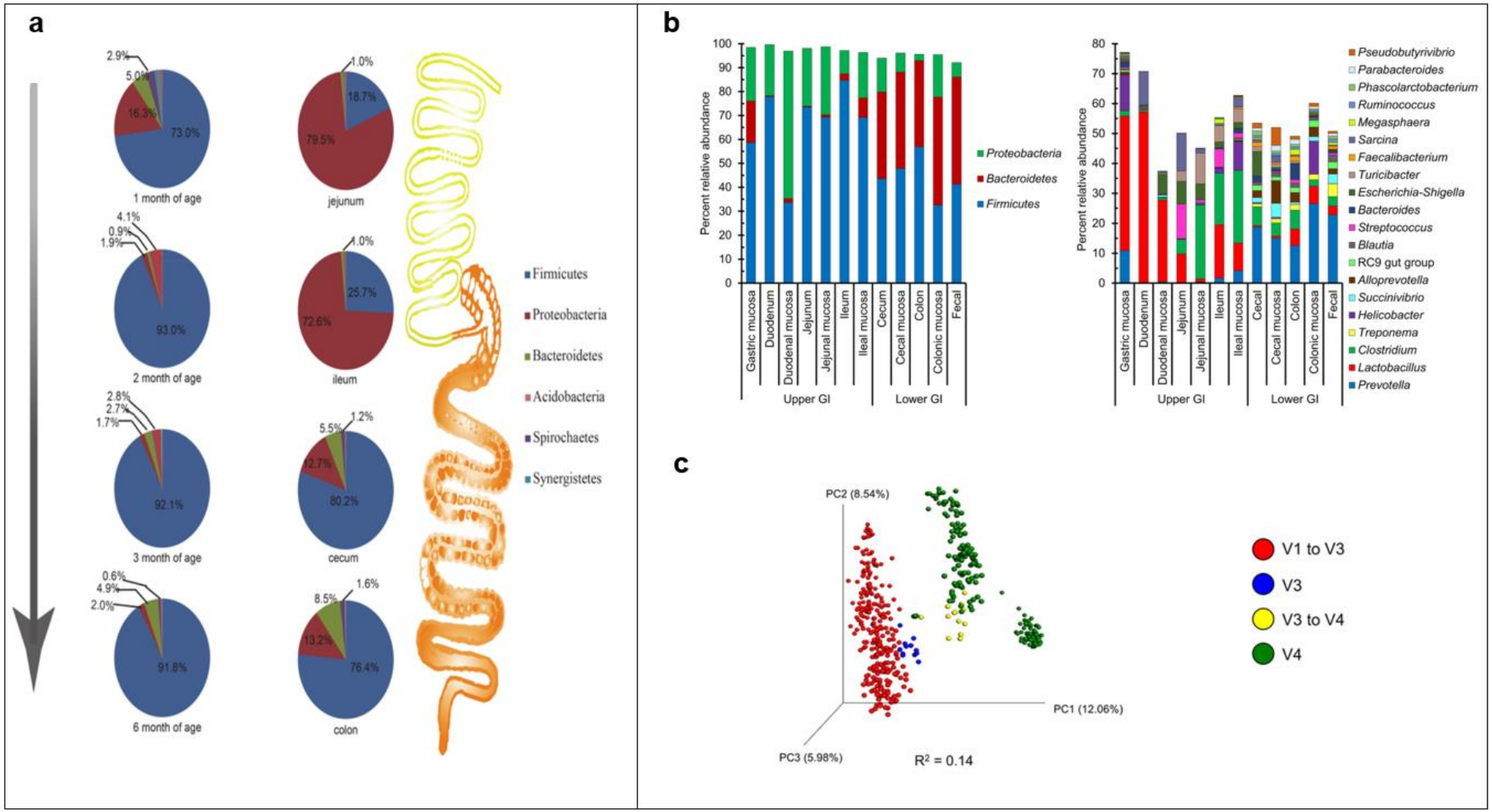

Figure 4 microbiota composition in GIT of pigs. a) Bacterial phyla in faeces at different age and in different intestinal tracts (age 6 month) b) Bacterial phyla and genera in different section of the GIT in pigs (meta-analysis from different studies) c) "study effect" on porcine faecal microbiota, impact of the different hypervariable regions sequenced on the beta diversity.

a) from Zhao et Al. (2015) https://doi.org/10.1371/journal.pone.0117441; b) c) from Holman et Al (2017) https://doi.org/10.1128/mSystems.00004-17.

2 The diet as gut microbiota modulator

As already reported in the previous sections, the main factors determining the structure of the gut microbiota are the different location along the gastrointestinal tract and the composition of the diet. The gut microbiota plays an important role in the degradation of various dietary compounds which are indigestible by the host's enzymes and the large intestine is the anaerobic compartment mainly involved in bacterial fermentation along the gastrointestinal tract of pigs. The variations among the distinct intestinal tracts therefore imply differences in the use of nutrients by the microbial community, indeed, if the competition for nutrients in the proximal intestine can be more detrimental than beneficial to the host, bacterial fermentations occurring in the large intestine make available to the host otherwise unusable energy sources.

More generally, the effect of the diet on shaping the microbiota is clearly described by studies comparing the faecal bacterial communities of carnivorous, omnivorous and herbivorous mammals, in which, apart from differences in diet, differences in the digestive physiology of the host (e.g. hind-gut, fore-gut fermenters) were also highlighted (Ley et al., 2008; Muegge et al., 2011). The results from these studies showed less variability in bacterial communities from carnivorous and high variability in the herbivorous gut microbiota, furthermore, the microbiota genetic potential analysed through whole metagenome sequencing highlighted an enrichment in amino acid biosynthetic pathways for herbivorous microbiota, whereas, amino acid degradation pathways were mostly enriched in bacterial communities from carnivorous.

The idea of manipulating the intestinal microbiota through diet has a quite long history (Torrey, 1919) and has been widely discussed and explored in many studies, however the diet-mediated modulation acts and is influenced at different levels (metagenomics, transcriptomics, proteomics, metabolomics) and although some variations can be empirically manifested, the causal links are often complex and not entirely clear (de Toro-Martín et al., 2017; Sonnenburg and Bäckhed, 2016). However, if we are

still far from precision nutrition that can associate a specific diet to a precise taxonomic and metabolic pattern of the microbiota, we have various knowledge about bacterial metabolisms in the intestine.

2.1 Dietary fiber and gut microbiota

As mentioned in the first chapter, one of the major changes during microbial colonization of the gastrointestinal tract occurs at weaning time, this drastic shift in the composition of the microbial community is mainly due to the different source of dietary glycans. Frese and colleagues (2015) showed that in suckling piglets the highly sialylated milk glycans play a pivotal role in shaping the milk-oriented faecal microbiota dominated by Bacteroidaceae and Enterobacteriaceae, while the transition to a vegetal-based diet after weaning leads to the dominance of Prevotellaceae and Ruminococcaceae and to the consequent enrichment in enzymes for the degradation of vegetal complex carbohydrates.

Because of the inability of mammals to degrade dietary fiber by endogenous enzymes, the carbohydrates represent the main energy source for the gut microbiota, for this reason, the carbohydrates are the most studied dietary component interacting with the intestinal bacterial community (Englyst, 1989; Flint, 2004; Trowell et al., 1976). Whereas the rumen microbiota of the fore-gut fermenters can access to all glycans provided by diet, the microbial communities in the large intestine of monogastric can use only the carbohydrates which escape the host's digestion in small intestine, these 'non-digestible' carbohydrates include the plant polysaccharides such as cellulose, xylan, pectin a variety of polysaccharide and oligosaccharides and feed additives, and also a portion of dietary starch (resistant starch). The digestion of these compounds depends completely on the activity of different bacteria that express saccharolytic enzymes, cellulases, hemicellulases, pectinases and xylanases (Salyers et al., 1977; Varel and Yen, 1997).

Swine microbiota contains cellulolytic and hemicellulolytic bacterial taxa also reported for rumen, such as *Fibrobacter*, *Ruminococcus*, *Butyrivibrio* and *Prevotella* (Varel and Yen, 1997), others

carbohydrates like B-glucans and pectins are preferentially fermented by lactobacilli (Graham et al., 1986; Hill et al., 2005).

Although the fiber fermentations mainly happen in the large intestine, a certain amount of dietary fiber, in particular the soluble fraction, can be fermented in the small intestine of pigs (Jørgensen et al. 1996; Jha et al. 2010; Jha and Leterme 2012), for example, it was reported that more than 55% of dietary inulin was digested in the small intestine (Bohmer et al., 2005), and it has also been seen that microbial fermentations in the small intestine can contribute with a small but significant contribution to the supply of amino acids to the host (Torrallardona et al., 2003).

The fermentations in large intestine of pigs can provide up to 30% energy supply and the Short-chain fatty acids produced, in particular, butyric acid, represent the major energy sources for the colonic epithelium (Csordas, 1996), conversely, lactic acid is the main organic acid in the stomach and small intestine (Bach Knudsen et al., 1991), their production rates and concentrations depend on the type and quantity of carbohydrate available (Topping and Clifton 2001).

The degradation of carbohydrates in large intestine involves different taxa in a cross-feeding system, these processes are better described for rumen, nevertheless can be representative also for part of the interactions happening in the large intestine of other mammals (Flint, 2004).

The primary degraders of vegetal cell wall comprise a small number of species, however, many others benefit of breakdown products. For instance, bacteria such as *Fibrobacter succinogenes* and *Ruminococcus flavefaciens*, release xylo-oligosaccharides that they cannot further metabolize, so these "intermediate" products can be efficiently utilized by non-cellulolytic bacteria such as *Prevotella spp*., in turn, also the cellulolytic bacteria depend on other members of the gut microbial community for vitamins and precursors for amino acid synthesis (Scott and Dehority 1965; Hungate and Stack 1982). In addition to these symbiotic relationships between bacteria, antagonistic mechanisms must be highlighted, these are often mediated by the production of antibacterial compounds such as bacteriocin which can represent important factors in "interspecific" competition dynamics (Kalmokoff and Teather, 1997; Riboulet-Bisson et al., 2012; Walsh et al., 2014).

Although the variations in different types and amount of fiber in pig diet can be related to potential benefits in microbiota manipulation, contrasting results are reported in literature regarding the inclusion of fiber from different sources and the association with dysbiotic events related to the major gastrointestinal disorders affecting the swine, such as post-weaning diarrhoea (PWD) due to E. coli and swine dysentery caused by *Brachyspira hyodysenteriae.*

Some studies in the past suggested that the administration of oats, wheat, and barley fiber and insoluble fiber may play a role in containment of pathogenic *E. coli* proliferation (Bertschinger et al. 1979; Thomlinson and Lawrence 1981). However, more recent studies have suggested that weaner diets rich in high fermentable carbohydrate sources, can be detrimental in terms of growth performances and also favouring the proliferation of pathogenic *E. coli* (McDonald et al., 2001, 1999). Kim and colleagues (2008) have shown instead that more than the fiber content per se, the balance between fermentable carbohydrates and proteins could affect the clinical manifestation of post-weaning diarrhoea.

Similarly, the data regarding the association between dietary fiber and swine dysentery also are controversial. In some studies, a low level of dietary fiber and resistant starch is reported as preventive against *Brachispira hyodisenteriae* infection (Durmic et al., 1998; Pluske et al., 1996), however, more recent works showed that the levels of inclusion of dietary fiber have no effect on swine dysentery (Kirkwood et al., 2000; Lindecrona et al., 2003); Bauman and Bilkei (2002) concluded instead, that high levels of highly fermentable fiber in diet may increase health and performance in pigs infected with *Brachyspira hyodysenteriae* (Baumann and Bilkei, 2002).

Regardless the unclear effects of fiber in gastrointestinal disorders, a major concern regarding this dietary component in mono-gastric animals is that high dietary fiber content is negatively correlated with nutrient utilization and net energy values, however, a certain level of fiber is important for maintaining normal physiological function in the digestive tract and as prebiotic factor (Lindberg, 2014). A better understanding of the effects of different fiber types on the microbial community and

the development of techniques that can improve its nutritional values may provide fundamental tools in managing the administration of this dietary component.

2.2 Dietary protein and gut microbiota

The dietary proteins that escape digestion in the small intestine, and some proteins of endogenous origin, represent another source available for bacterial fermentations. The end products of protein fermentations are different from those of fiber fermentations and mainly consist in branched chain fatty acid (BCFA) such as iso-butyrate, valerate and iso-valerate - in variable proportions depending on the substrate - derived by the metabolism of branched chain amino acids such as valine, leucine and isoleucine (Macfarlane et al., 1992). In addition, some potentially toxic metabolites such as ammonia, amines phenols, indols and sulphurous compounds are also produced; these compounds are mainly transferred to the liver where they are detoxified to glucuronides and then excreted in urine, instead a small part is directly excreted in faeces (Le et al., 2007).

Various bacterial species such as *Escherichia coli, Klebsiella* spp., *Campylobacter* spp., *Streptococcus* spp., *Clostridium perfringens*, *Clostridium difficile* and *Bacteroides fragilis* have been reported as major protein fermenters. The bacterial protein degradation is normally scarce in small intestine, even if some amino acid bacterial degradation was suggested also for the upper gastrointestinal tract (Davila et al., 2013). Conversely, the large intestine represents the main "bioreactor" also for protein fermentations and this bacterial proteolytic activity results increased when carbohydrate sources were depleted (Piva et al. 1996; Jha and Berrocoso 2016).

Although the amino acid degradation by bacteria contribute to the formation of SCFA in GIT, the above mentioned toxic product of protein fermentations like ammonia can inhibit the oxidative metabolism of SCFA in colonocytes, compromising the energy supply of these cells (Blachier et al., 2007) and can also readily pass the gut wall reach other tissues adversely affecting the host's health (Bikker et al., 2006; Cone et al., 2005). Furthermore the amino acids decarboxylation results in the

production of biogenic amines like histamine that can lead to chloride secretion in the colon of pigs and watery faeces (Kröger et al., 2013). High-protein diet in pigs is also reported as predisposing factors to the colonization of small intestine by enterotoxigenic *E. coli* and consequent post-weaning diarrhoea (Heo et al., 2009).

Because of this, the excess of protein in the diet is considered detrimental for the balance of the microbial community and for the general homeostasis of the gastrointestinal tract. However, protein level balance with adequate amounts of dietary fiber seems to be a viable food strategy to contain the production of harmful metabolites and to counteract dysbiotic events linked to protein fermentations in pigs intestine (Jha and Berrocoso 2016).

2.3 Dietary lipids and gut microbiota

The roles of lipids in shaping the gut microbiota received less attention and little is known about the degradation of dietary lipids by bacterial metabolisms along the GIT. Major knowledge about the pathways for extracellular fatty acid utilization are reported in model prokaryotes such as *Escherichia coli* (Gram-) and *Bacillus subtilis* (Gram+*)* (Fujita et al., 2007).

In *E. coli* the exogenous fatty acids can be converted to acyl-CoA thioester by an acyl-CoA synthetase (FadD) and then the acyltransferases (PlsB and PlsC) use the acyl-CoAs as substrates for membrane phospholipid biosynthesis. The second fate for acyl-CoA is the utilization as a carbon and energy source in beta-oxidation pathway. A key point in *E. coli* (and probably in the other Gram-) is that the extracellular fatty acid cannot be used for lipopolysaccharide (LPS) biosynthesis (Yao and Rock, 2015). The high-fat diets were associated with gut microbiota modification leading to higher LPS levels in the circulatory system, however, these can be due to an excessive chylomicron formation incorporating LPS, which lead to an extra-hepatic exposure to LPS (Laugerette et al., 2014; Moreira et al., 2012).

In *B. subtilis* the straight-chain fatty acids added to the culture medium showed no degradation, suggesting insignificant levels of beta-oxidation for these bacteria. However, a considerable number of genes involved in the b-oxidation of fatty acids were revealed in *B. subtilis*, this may be related to the activation of beta-oxidation only in certain physiological condition such as sporulation (Fujita et al., 2007).

Furthermore, the microbiota plays a role in cholesterol reduction to coprostanol and coprostanone. Different bacteria belonging to the *Eubacterium, Bacteroides*, *Bifidobacterium* and *Clostridium* genera are able to metabolize cholesterol to coprostanol, and it is reported that germ free rats excrete unmodified cholesterol whereas conventional rats excrete coprostanol and coprostanone up to 55% of the total faecal sterols (McNamara et al. 1981; Baron and Hylemon 1997).

Another role of the gut microbiota linked to lipid dietary content regards the metabolism of the bile. The main function of bile is to act as an emulsifier and to solubilize fats, this also confers antimicrobial properties on bile and gives it an important role in the host's physicochemical defences. Gram+ bacteria seem to be more sensitive to the bile than Gram-, however bile tolerance is extremely variable among different strains and cannot be generalized at taxonomic level of species or genera, furthermore the results from tolerance studies conducted in bile broth systems can be highly different between them and may not truly reflect the in vivo situation. Apart from these limitations that still exist, bile could represent another host-derived factor that acts in shaping the gut microbial community (Begley et al., 2005).

In addition, the composition and size of the bile acids (BA) can be altered by intestinal microbiota via various metabolic transformation such as deconjugation, dehydrogenation, epimerization and the secondary BAs bacteria-derived can regulate the activation of nuclear farnesoid X receptor (FXR) modulating the BA synthesis and the lipid metabolism of the host. Furthermore the activation of the host’s BA secretion may represent a mechanism of cross-talk used by some bacteria for the interspecific competition (Nie et al., 2015).

Starting from the first work on human enterotypes (Arumugam et al., 2011), a growing number of studies continued to associate the "Bacteroides enterotype" with the western diet, richer in fat and protein. Studies expressly focused on the interaction between dietary fat and microbiota in pigs are almost absent, however, in the lactation phase the intestinal microbiota of piglets could be influenced by the high fat levels in sow milk and the *Bacteroides* genus seems to be positively correlated with these (Bian et al., 2016).

2.4 Organic acids and gut microbiota

The introduction of antibiotics as feed additives in 1940's revolutionized the livestock production, the commercial diets were regularly supplemented with antibiotics in subtherapeutic doses for gastrointestinal disorders prevention and in order to improve feed efficiency and growth performances. In the same years, the phenomenon of antibiotic resistance was already known and became better documented since the 1960s (Davies and Davies 2010). In the following years, the accumulation of evidence on the spread of antibiotic resistance and on the high risks for human health led, from 2006, to the ban of the use of antibiotics as growth promoters for livestock production in EU. As a consequence, the search for alternatives to antibiotic use has become of primary interest, in particular in order to contrast the lower post-weaning daily weight gain and a high prevalence of post weaning diarrhoea.

Currently it is commonly recognized that the efficacy of antibiotics is mainly due to the microbiota modulation, however, antibiotic specificities are variable also depending on microbiota composition, and their effects on distinct microbial communities and the exact mode of action in promoting growth performances are not completely defined (Gaskins et al., 2002).

Among the various feed additives such as copper sulphate, zinc oxide, probiotics, prebiotics and herbs studied in newly weaned piglets several organic acids and their salts are currently used as modulators of the gut ecosystem in pigs. Some of the most used are: formic, acetic, propionic, butyric, lactic, fumaric, Ca-formate, Ca-propionate, K-diformate, and Na-benzoate (Mroz, 2005).

The exact mechanism of action of organic acid and their salt is still unclear, although two main hypotheses about the modes of action in contrasting dysbiosis have been proposed.

The first one regards the reduction of pH.

The protein digestion begins in the stomach through the action of pepsin, which is secreted as pepsinogen precursor, both the conversion of pepsinogen and the action of pepsin work better in an acidic environment (pH 2.0 to 3.5). At weaning, the lower acid secretion of piglet's stomach, the lack of lactose substrate for the lactate producing bacteria, and the buffer effect of meals can result in elevated pH, often to over 5.0. This poorly acidic environment in the stomach can lead to a reduction in proteolytic activity and to a reduced bacterial selectivity mediated by gastric acute acidity, as a result, bacteria such as Enterobacteriaceae can settle into the small intestine and proliferate by fermenting the undigested proteins leading to a dysbiotic intestinal ecosystem (Suiryanrayna and Ramana 2015).

The second mechanism regards the bactericidal action.

The organic acids are lipophilic in their undissociated form, because of this they are able to penetrate the cell membrane of Gram- bacteria. The higher cytosolic pH of the bacteria cells induces the dissociation of the acid with consequent accumulation of hydrogen ions and reduction of the intracellular pH, this causes increased energy requirement to expel protons via the H+ATPase, reduction of bacterial metabolic activities and of macromolecules synthesis, leading to the destruction of bacterial cells (Lückstädt and Mellor 2011).

In addition, organic acids may represent an energetic substrate and/or modulator for mucosal development and epithelial cell growth increasing absorptive capacity, and can be involved also as precursors for the synthesis of non-essential amino acids, DNA and on lipids required for intestinal growth (Mroz, 2005).

The results regarding microbial community modulation by organic acid are contrasting and some authors did not find changes after the administration of different acidifiers in pig diet (Risley et al., 1992; Zentek et al., 2013).

Moreover, an excessive acidification of stomach via organic acid may inhibit the secretion of gastrin hormone and consequently reduce the hydrochloric acid secretion resulting in a loss of effectiveness against harmful bacteria, however, the use of protection can represent a tool to promote the slower release of organic acid and direct their action in the distal small intestine (Bosi et al., 1999).

3 Host-derived factors

One of the main questions in the study of the microbiota is: Does the host influence the microbiota or the microbiota influence the host?

Recently Rosenberg and Rosenberg (2016), starting from the experimental data and the theoretical elements concerning the host-microbiota interaction, formalized the concepts of hologenome and holobiont as follows:

"*The hologenome concept of evolution asserts that the holobiont with its hologenome, acts as a unique biological entity and therefore also as a level of selection in evolution. […] The major arguments for considering the holobiont as a level of selection are the following.*

i. *All multicellular organisms contain abundant and diverse microbiota. Often, the number of microbial cells and the sum of their genetic information are above that of their host.*

ii. *Not only the host genome but also the microbiome can be transmitted between generations with reasonable fidelity and thus maintains the unique properties of the holobiont.*

iii. *Microbiotas and their hosts interact in a manner that affects the fitness of the holobiont in many ways, including its morphology, development, behavior, physiology, and resistance to disease. Taken together, these interactions characterize the holobiont as a single and unique biological entity."*

(Rosenberg and Zilber-Rosenberg 2016)

Despite the huge amount of data produced by microbiota studies, the information on how host genetic makeup can affect the gut bacterial community structure is still scarce. This is also due to technical and methodological difficulties, such as the need for a large number of samples to carry out genome wide association (GWA) studies or the difficulty in characterizing and correctly evaluating the smaller bacterial groups, which although little abundant could have a major impact on the host (Fu et al. 2016; Dąbrowska and Witkiewicz 2016).

Widespread studies on the association between the microbiota and the host genetic background have been conducted in murine models, these studies reported correlations between specific bacterial taxa and some QTLs related to the host metabolism (weight, fat content) and immune response (Benson et al., 2010; Leamy et al., 2014). However, in another study Carmody and co-workers used mice knockout for genes linked to host-microbial interactions [MyD88(-/-), NOD2(-/-), ob/ob, and Rag1(-/-)], in order to test the diet and genotype effects on microbiota structure. The results showed a predominant role of the diet on the host genotype in shaping the bacterial community. In fact, the faecal microbiota was modified by the diet administration similarly in both wild-type and knockout mice, furthermore, the shifts in microbial composition observed after dietary changes were rapid, reproducible, and reversible confirming the dominance of the diet factor (Carmody et al., 2015). In addition, another study which used a cross-fostering model between two different mouse strains showed that the nursing mother was the principal factor in determining bacterial colonization, instead of the birth mother (Daft et al., 2015).

For pigs, extensive study to connect the host genome and microbiota composition are less frequent and more difficult respect to use murine models. However, a certain level of heritability of the microbiota and a correlation with the host's immune traits was described in some recent studies which suggest the hypothesis that the microbiota may be considered as a phenotypic trait (Camarinha-Silva et al., 2017; Estellé et al., 2014). In addition, other studies have reported some differences in faecal bacterial communities in pig of different breeds, indicating that the artificial selection can also act at

the microbial level (Pajarillo et al., 2014; Xiao et al., 2017; Yang et al., 2014). Conversely, two studies that used the cross-fostering pattern among different breeds and the introduction of solid feed during the lactation period showed that although it is possible to detect a weak effect of both the nursing-mother factor and the breed factor, the diet factor prevails in modifying the bacterial community of piglets (Bian et al., 2016; Xian et al., 2014).

3.1 Bacterial adhesion and host-derived factors

Understanding the complex associations involving the host genome and the microbial metagenome is one of the major scientific challenges for the coming years, in which the first steps are moving. However, it is possible to focus the attention on specific mechanisms, and their genetic background, which may explain how the host selects certain bacteria and distances others in the different stages of the gut maturation and in different areas along the gastrointestinal tract, starting from "model events" such as bacterial infections.

In the mucosa colonization process an essential step is the recognition of a tissue, and hence, of a specific site on the host's intestinal surface by the microorganism. This allows the bacteria to have an optimal position for access to nutrients and for the release of any toxins in the target tissue. This tropism is mediated by proteins called adesine expressed on the microbial surface, the adesins are lectins capable of recognizing specific carbohydrates in receptors expressed on the host cell membrane (Ofek and Sharon 1990).

In fimbriated *E. coli* these proteins have a typical polymeric structure called fimbriae or pili. The specific receptors are differentially expressed along the gastrointestinal tract of the host and this results in the localization of the different bacteria in specific niches. For example, in the case of *E. coli* F4+, the presence of specific receptors varies along the gastro-intestinal tract of the pig with a higher expression of these receptors (F4R) in the mid-small intestine (Cox and Houvenaghel 1993). Conversely, these receptors are absent in the cecum and in large intestines (Chandler et al., 1994).

Likewise, *E. coli* F18+ showed a stronger tropism for mid jejunum and ileum (Bertschinger and Pohlenz 1983). Host susceptibility to these pathogens is therefore associated with the expression of specific receptors (F4R and F18R). Pigs resistant to *E. coli* F4+ and *E. coli* F18+ infections exist in fact, and this is due to the lack of expression of these receptors at the level of the intestinal mucosa.

The genetic basis of this mechanisms is still not well known, even so, it has been associated with the mucin 4 gene (*MUC4*) on chromosome 13 which has been proposed as a candidate gene for the F4R expression, and a XbaI polymorphism in intron 7 of *MUC4* has been identified to discriminate F4R positive and F4R negative pigs (Joergensen et al., 2003). This polymorphism was used to screen the animals in studies with experimental infection and the F4R+ genotype pigs showed a significantly higher occurrence of diarrhoea compared to pigs with the F4R- genotype (Jensen et al., 2006; Trevisi et al., 2012). However, even if MUC4 genotype is considered as a marker, a direct causality relationship between MUC4 and susceptibility to *E. coli* F4 has not been confirmed, e.g., Rasschaert and colleagues did not find a complete association between genotype and in vitro adhesion testing, suggesting that there may be at least one other receptor involved in susceptibility to *E. coli* F4 strains (Rasschaert et al., 2007).

The receptor for *E. coli* F18 is still not described in pigs, however the alpha (1,2)-fucosyltransferase (*FUT1*) gene on chromosome 6 has been proposed as a candidate gene (Meijerink et al., 1997) and a polymorphism at nucleotide 307 was reported as discriminating between resistant and susceptible pigs (Meijerink et al., 2000), furthermore, the efficacy of *FUT1* as a marker for susceptibility to *E. coli* F18 was confirmed by adhesion tests that showed an almost complete association in particular for the F18ab phenotype of *E. coli* (Luo et al., 2010).

In addition to the spatial differences in the expression of these receptors (different intestinal tracts) there are also chronological differences (different maturation stages). For example, the receptor for *E. coli* F5+ (NeuGc-GM3) is expressed exclusively in the intestine of the new-born piglets and this explains the incidence of diarrhoea by coli F5+ in neonates but not in adults (Kyogashima et al., 1989; Teneberg et al., 1990).

The mechanisms for adhesion of pathogenic bacteria to the intestinal epithelium and their genetic bases could represent an important factor in explaining the causal relationship between host genetics and commensal microbiota.

McLoughlin and colleagues (2016) proposed a series of models based on host-provided adhesiveness as a mechanism in microbiota colonization. They predicted, using an in-silico approach, scenarios in which the host can use adhesion to favour the settlement and competitiveness of beneficial commensals. The environmental fluctuation due to diet changes can have a high impact on gut bacterial community structure. The models proposed show that the host-provided factors able to select particular bacteria and/or bacterial genotype may limit the effect of these fluctuations providing specific ecological refugia-niche for the resident bacteria during the perturbation events affecting the gastrointestinal ecosystem.

A factor under host control which may play an important role in gut microbiota colonization is the mucus. The mucus secretion in GIT is extremely variable, particularly during infection or dysbiotic conditions, indeed, a mucus hyperproduction by goblet cells is reported during inflammatory events (Boshuizen et al., 2005; Guilmeau et al., 2008). The autochthonous bacteria often reside in mucus layer covering the epithelia and the mucus flow rate can be decisive in the adhesion process, by approaching or bypassing the different bacterial cells on the epithelial surface (McLoughlin et al., 2016). Furthermore, the mucins have a high degree of glycosylation, and many bacteria are able to attach these glycans, both to use them as an energy source (Koropatkin et al., 2012; Sonnenburg et al., 2005) but also as an anchorage (Naughton et al., 2013; Derrien et al., 2010; Huang et al., 2011).

The fucosylation controlled by Fucosyltransferase2 (FUT2) gene can represent an example of this mechanism.

The *FUT2* gene encodes a galactoside 2-α-l-fucosyltransferase 2, this enzyme adds an L-fucose residue in α1–2 linkage to the terminal β-d-galactose residue of gut mucus glycans, and the L-fucose attached to glycoproteins and glycolipids is available for bacteria as energetic substratum and as a target for adhesion (Bry et al., 1996). About 20% of humans lack functional copies of this gene, these

subjects are called “nonsecretors” and, because of that, the mucins in their distal gut are devoid in terminal fucose residues (Kelly et al., 1995).

It has been shown, in fact, that the *FUT2* genotype (*FUT2*$^{+}$ vs *FUT2*$^{-}$) affects the gut microbiota colonization in normal condition and during dysbiotic events and that the genotype effect can be outclassed by varying the glycans content in the diet (Kashyap et al., 2013; Pickard et al., 2014).

Another potential candidate for the modulation of the microbial adhesion are the immunoglobulins A (IgAs).

IgAs are normally produced by the host at the level of the gut epithelium in response to the resident microbiota, coating most of bacteria (D’Auria et al., 2013; Palm et al., 2014; van der Waaij et al., 2004). Indeed, these immunoglobulins are able to bind specific microbial epitopes by a hypervariable region but also a wide range of bacteria by a non-specific binding region (Mathias and Corthésy 2011; Nowrouzian et al. 2013), furthermore, the non-specific region is also used to bind the mucins which constitute the mucus matrix a (Biesbrock et al. 1991; Phalipon et al. 2002; Bergstrom and Xia 2013). These evidences have led McLoughlin and colleagues (2016) to hypothesize that specific IgA could be used by the host to select specific bacteria to approach the epithelial surface under normal conditions, and conversely, in infection conditions, the specific adhesion capabilities of the immunoglobulins and the greater mucus secretion could be a strategy to remove pathogens from the epithelial surface.

Mice studies indicate that IgAs can modulate microbial composition by selecting specific taxa in a site-specific manner, showing that polyclonal IgAs from different intestinal tissues express microbiota-reactive and polyreactive specificities (Bunker et al., 2017; Sait et al., 2003). Although the IgAs interaction mechanisms are still not well known, Fransen and colleagues (2015) showed that mouse strains with distinct genetic background have a different arrangement in microbiota selection and that this is correlated with the amount and diversity assortment of innate (genetically determined) IgAs and with milk-derived IgAs after birth. They also showed that, when the mice were microbiota depleted by antibiotic treatment and then inoculated with a new microbiota by other mice strains, the

recipients did not show a microbiota similar to that of donors, but was more similar to their original microbiota, suggesting that this can be due to the already-defined IgAs pattern that drives bacterial colonization and that could be a limitation in faecal transplant procedures (Fransen et al., 2015).

It should also not be overlooked that the recognition/adhesion is not a one-way mechanism. Indeed, the expression of specific glycoconjugates by the host also occurs in response to the presence of bacteria (Lu and Walker, 2001) in a "cross-talk" system. The intestinal microbiota appears to play a crucial role in stimulating the initial expression of cellular glycoconjugates required by certain bacteria to occupy a certain niche, and in influencing the glycosylation patterns, both quantitatively and qualitatively (glycan motif) (Freitas et al., 2002; Umesaki et al., 1995), thus modifying the adhesion sites.

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Experimental studies

Exploring gastric bacterial community in young pigs

Abstract

Microbiota plays an important role in the homeostasis of the gastrointestinal tract. Understanding the variations of the commensal microbiota composition is crucial for a more efficient control of enteric infectious diseases and for the reduction of the use of antibiotics in animal production, which are the main points of interest for improved animal healthcare and welfare and for consumer health protection.

Even though the intestinal microbiota has been extensively studied, little is known about the gastric microbiota. This pilot study was aimed at a descriptive analysis of the gastric microbiota in healthy pigs and at the identification of any differences among four potentially distinct microbial niches in the stomach.

Gastric mucosal samples from the oxyntic area, the pylorus and the gastric groove, and a sample of gastric contents were collected from four healthy weaned pigs. Bacterial DNA was isolated and extracted from each sample and amplicons from the V6 region of the 16S rRNA gene were sequenced using Ion Torrent PGM. The data were analysed by an "unsupervised" and a "supervised" approach in the Ribosomal Database Project (RDP) pipeline. Proteobacteria was the dominant phylum in all the samples.

Differences in bacterial community composition were found between mucosal and content samples (one-way ANOSIM pairwise post hoc test, $p < 0.05$); instead, the different mucosal regions did not show differences between them.

The mucosal samples were characterised by *Herbiconiux* and *Brevundimonas*, two genera which include cellulolytic and xylanolytic strains. Nevertheless, additional larger trials are needed to support the data presented in this pilot study and to increase the knowledge regarding the resident microbiota of the stomach.

Introduction

The importance of the microbiota to the health status of the gastrointestinal tract is widely recognised. Over the years, the Microbial Ecology of the GI tract has been extensively explored (Zoetendal et al., 2004) but the stomach ecosystem has received less attention; this was due to the technical limitations and to the fact that the gastric environment was considered too inhospitable. The potentiality of the gastric environment as a microbial niche was reconsidered after the discovery of *Helicobacter pylori* and thanks to successive technological advances (Walker and Talley, 2014). In recent years, one of the most used methods for exploring the microbial diversity of an environment is 16S rRNA profiling conducted using Next Generation Sequencing (NGS) approaches; however, the studies which use these techniques to analyses the stomach microbiota of monogastric animals (non-human) are still infrequent (Mann et al., 2014; Perkins et al., 2012). In particular, as regards the pig, only the work of Mann et al. (2014) which also analyses the gastric microbiota using this technique was found.

The stomach is a system of temporary storage and pre-processing of the food bolus for additional digestion and absorption; this system is directed by an integrated control (neural, hormonal, paracrine) which takes into account the different signals (chemicals, nutrients, xenobiota components) from the luminal content (Chu and Schubert, 2012). In the stomach three anatomic parts (fundus, corpus and antrum) and two functional regions -oxyntic (acid secretion) and pyloric (gastrin secretion) glandular- can be distinguished.

This anatomical and functional geography within the stomach has been investigated in several ways (Choi et al., 2014; Colombo et al., 2014).

In the pig, the oxyntic glands are found in the cardia gland and the fundic gland regions (OXY), while the antral-type mucous glands are found in the pyloric gland region (PYL).

Furthermore, in the pig stomach, regional differences were also observed in the protective layer of the mucus (Karlsson et al., 1997; Nordman et al., 1998) which represents the first line of interaction between bacteria and the gastrointestinal tract (Johansson et al., 2013).

The question arises as to whether the different gastric regions may represent distinct niches for diverse communities within that ecosystem; this possibility has been investigated for monogastric mammals in only a few studies, such as in humans (corpus and antrum) (Li et al., 2009) and in horses (squamous, glandular, antral) (Perkins et al., 2012) but the identification of a specific gastric microbiota, excluding *H. pylori*, requires additional investigation (Lopetuso et al., 2014).

The present study fits into the context of our other studies regarding the pig gastric mucosa (Colombo et al., 2014; Mazzoni et al., 2011; Priori et al., 2014), our aim was to contribute to the description of the gastric microbiota ,in particular, of the pig, and to identify possible differences of the bacterial community in different parts of the stomach.

For this purpose, Next generation semiconductor-based sequencing of the V6 hypervariable region of the 16S rRNA gene was used on gastric mucosal samples from the oxyntic area (acid production), the pylorus (gastrin secretion)and the gastric groove, a point in the small curvature close to cardia, (immunological function) (Mazzoni et al., 2011) and also from the gastric content.

Materials and Methods

Pigs and Sample collection

The procedures were conducted in compliance with Italian laws regarding experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna. Four crossbred (Large White *x* Landrace) healthy weaned pigs (6.5 weeks of age, 15.30 kg average body weight), normally fed a standard post-weaning diet (ingredient composition: corn 38.2 %, barley13%, wheat middlings 16%, soybean meal 50%, crude protein 13%, dried milk whey 9%, potato protein concentrate 4%, soybean oil 3%, vitamin-mineral premix 1%, dicalcium phosphate 1.2%, calcium carbonate 0.61%, salt 0.3 %, L-lysine HCl 0.38 %, Dl-methionine 0.11 %, L-threonine 0.15%, L-tryptophan 0.05%), were anaesthetised 1 h after the morning meal with sodium thiopental (10 mg/kg body weight) and were then slaughtered by intracardiac injection (Tanax, 0.5 mL/kg body weight; Intervet Italia, Peschiera Borromeo, Italy). For each subject, the stomach was removed, and

gastric mucosal samples from the oxyntic area, the pylorus, and the small curvature close to cardia (hereinafter called Groove*)* were collected. A sample of gastric contents was also collected from each pig; in total 16 samples were obtained (4 from each pig). The samplings in each pig and each stomach region were carried out using sterile instruments to avoid potential cross-contamination of the microbial DNA.

The samples were stored at -80 °C until use. The bacterial DNA was isolated and extracted using QiaAmp DNA Stool Mini Kit (Qiagen, Hilden, Germany). The protocol followed the manufacturer's instructions with a pretreatment step with TES buffer + Lysozyme at 37 °C for two hours. After isolation, the purified DNA was eluted in 50 µl of elution buffer. The quality and purity of the isolated DNA was checked using spectrophotometry on the NanoDrop (Fisher Scientific, Schwerte, Germany).

Library Generation and Sequencing

Polymerase chain reaction (PCR) amplification of the V6 hypervariable region from the 16S rRNA gene was carried out with a pool of 5 forward primers and 4 reverse primers pooled equimolar as described by Huber et al. (Huber et al., 2007). Phusion® Hot Start Flex 2X Master Mix (New England Biolabs Inc., Beverly, MA) was used following the manufacturer's protocol for a 25µl reaction; the PCR conditions were as follows: 98°C for 30 s, followed by 35 cycles at 98°C for 5 s, 61°C for 8 s, 72°C for 12 s, and a final elongation step at 72°C for 5 s.

Ion Torrent sequencing was obtained from 16 different DNA libraries. The libraries were constructed using the aforementioned amplified products, after ExoSAP-IT® (USB Corporation, Cleveland, Ohio, USA) treatment, and 16 equimolar pools of amplicons were obtained. The preparation of the libraries was carried out according to the instructions for the Ion Torrent Personal Genome Machine (PGM; Life Technologies,*Carlsbad, CA*) sequencing of short amplicons; for each library, 200 ng of amplified DNA was end-repaired and ligated with a specific barcode, in total 16 different barcodes were used, using the Ion Xpress™ Barcode Adapters 1-16 kits (Life Technologies). Subsequently, the protocol

included the following steps: quantification of each library with the Ion Library Quantitation Kit (Life Technologies) by quantitative polymerase chain reaction (qPCR), utilising a StepOnePlus™ Real-Time PCR System (Life Technologies); equimolar pooling of the 16 barcoded libraries, amplification by emulsion PCR with the Ion One Touch™ 200 Template kit (Life Technologies), and purification and sequencing with the Ion PGM™ Sequencing 200 kit using a Ion 314 chip (Life Technologies), following the manufacturer's protocols.

Raw reads of all the samples were deposited at the EBI Short Read Archive (SRA) under the study accession number ERP010584.

Data Analysis

A total of 353,656 Raw reads from sequencing were filtered for length ≥ 70 and average quality ≥ 20. The following steps were carried out in the Ribosomal Database Project (RDP) pipeline of the RDP release 11.3, using both 'unsupervised' and 'supervised' methods (http://rdp.cme.msu.edu/) (Cole et al., 2014); primer matching and trimming were performed by the 'Pipeline Initial Processor' of the RDP pipeline (Maximum number of Ns: 0; Max forward primer distance: 0; Max reverse primer distance: 0; Minimum sequence length: 50), chimera checking was carried out using the tool 'Find Chimeras' in DECIPHER (Database Enabled Code for Ideal Probe Hybridization Employing R) (Wright et al., 2012). Non-chimeric sequences were aligned by RDP Aligner and the sequence reads not covering the V6 region were eliminated. After quality control steps, 86,731 total sequences were obtained.

For bacterial taxonomy assignment, the RDP-classifier (version 2.2) (Wang et al., 2007) was used with 50% as confidence value threshold and gene copy number adjustment. Operational Taxonomic Unit (OTU) analysis was carried out on a clustering at the 97% identity threshold using the complete linkage clustering algorithm.

For the unsupervised approach 2961 reads (the lowest number of reads recovered in a single sample) were randomly subsampled from each sample (package GUniFrac in R) in order to minimise the

impact of the varied sequencing depth among samples. For the supervised approach, sequence tag data were normalised to relative abundance within the sample for analysis and visualisation. The statistical software PAST version 2.17 (Hammer et al., 2001) was used to analyse the abundance data of the reads assigned to taxa within the samples, with one-way ANOSIM (Analysis of Similarities) testing significance of the difference between the groups of samples based on differences in the gastric region. Cluster Analysis and Principal Components Analysis were used to generate graphical representations of the differences in community composition. The SIMPER (Similarity Percentage) analysis was used to identify the specific genera with the greatest contribution to the differences observed between the groups identified.

Results

Unsupervised Approach

After rarefaction, 2,020 OUTs were represented among the 47,376 reads from the16 samples in this study. The sequencing depth and the total OTU richness within individual samples are reported in Table 1.

The Shannon-Weaver diversity index and Eveness were calculated for each sample and the average indices at each of the points (Content, Groove, Oxyntic and Pyloric) showed a quite similar diversity, though with a lower equitability (eveness) of the OTUs within the Content samples (Fig. 1).

The Cluster analysis based on the abundance of the different OTUs in the samples (Fig. 2) showed two well-supported clusters; one consisted of all the samples from the content and the other consisted of all the samples from the mucosa. The clustering did not show an individual effect or an effect of the regions in the mucosa; however, 3 out of the 4 samples from pyloric region formed a sub-cluster poorly supported by bootstrap probability (BP = 41).

The null hypothesis, that there were no significant differences in community structure based on the sample type (Content, Oxyntic, Pyloric, Groove), was rejected by one-way ANOSIM with an R of 0.632 ($p = 0.0001$). The pairwise post hoc test showed significant differences ($p < 0.05$) between the

mucosal and the content samples, but no significant differences were seen for the comparisons between the different mucosal regions (Table 2).

Supervised Approach

The quality checked reads were analysed using the taxonomy-supervised method in the RDP pipeline, which consists of a 'taxonomy binning' of the reads on the basis of the existing bacterial taxonomy. This approach has some advantages, such as the least computational effort required, minor sensibility at the sequencing errors and easier handling of the data (Sul et al., 2011).

First of all the classification at the phylum level showed the dominance of the unclassified bacterial reads and chloroplast sequences, which represented chloroplast from the ingested vegetal matter, in the content samples and a uniform distribution of the phyla in the different regions of the mucosa (Fig. 3). In order to focus the study on the classifiable bacterial community, the unclassified and plastidial sequences were excluded from additional analysis.

The dominant phylum was Proteobacteria with 50% (on average) in the content samples and 60.9% (on average) in the mucosal samples, the second phylum was Firmicutes for the content samples (27.5% on average) and Actinobacteria for mucosal samples (30.8% on average) followed by Bacteroidetes (5.53% in the content samples; 2.78% in the mucosal samples). One-way ANOSIM showed weak ($R = 0.272$) but significant ($p = 0.0075$) differences in community structure; in the pairwise post hoc test, significant differences ($p < 0.05$) were reported between the mucosal and the content samples but not for the comparisons between the different mucosal regions.

The classification at the genus level identified 238 genera in total. The Cluster analysis, based on the relative abundance of the Genera (Fig.4), showed a situation similar to that seen in the unsupervised approach with a cluster for all samples from the content samples and another cluster for all the mucosal samples. No clusterings by subject or for different mucosal regions were found.

One-way ANOSIM again showed significant differences (R = 0.354; p = 0.0018) between the mucosal and the content samples but not for the comparisons between the different mucosal regions (Table 3).

The SIMPER analysis (Table 4) was carried out in order to identify the Genera wich most influenced the difference between the bacterial communities of mucosa and content. The overall average dissimilarity (Mucosa VS Content) was 51.43%. The SIMPER analysis showed that the differences between the bacterial communities of the gastric mucosa are primarily driven by the dominance of the genus *Herbiconiux* with an average abundance of 41.9% in the mucosal samples and 21.6% in the content samples. Other principal genera characterising the mucosa are *Brevundimonas* and *Moritella;* instead, *Pasteurella*, *Streptococcus*, *Lactobacillus* and *Lactococcus* characterise the contents.

Principal component analysis (PCA) regarding the relative abundances of the bacterial genera showed that the 78.5% of variance in the data could be explained by the first two principal components. This analysis confirmed the subdivision of the samples into two main clusters along the first component (Fig. 5), a cluster consisting of contents samples and a cluster containing samples obtained from the mucosa and the PCA biplot (Fig. 5) shows the influence on this clustering of genera also reported by SIMPER analysis.

Discussion

The current study revealed values of alpha diversity of the pig gastric microbiota similar in the different areas of the stomach anlysed (Oxyntic, Pyloric, Gastric Groove and Content), and comparable to those reported by Mann (2014). The bacterial community composition, analysed at different taxonomic levels (Phyla, Genera, OTUs), did not show differences in the distribution of the bacterial populations between the different areas of the gastric mucosa. This indicated that the anatomic and physiological differences in the different gastric areas (see Introduction) may not directly impact the bacterial community, which is probably more influenced by the outer mucus layer.

Unfortunately, however, the exact location of the microbiota in the stomach of mammals is still unknown (Yang et al., 2013).

Instead, significant differences in bacterial distribution at the phyla, genera and OTU levels are observed between the mucosa and gastric content. Even if our results should be treated with caution since they are based on a limited number of replicates, this differentiation between the mucosal and the luminal microbiota has already been described for the gastrointestinal tract of other mammals (Malmuthuge et al., 2014), indicating that the digesta bacterial community may not be sufficiently informative of the bacterial community associated with the gastric mucosa.

First of all regarding the taxonomic classification of the reads, the large number of unclassifiable bacterial sequences, approximately 55% in the content and 30% in the mucosa at the phylum level is first of all evident. This gives a general idea regarding the bacterial diversity still unexplored. When focusing on the reads taxonomically identified, it can be seen that the principal phyla (Proteobacteria, Actinobacteria, Firmicutes and Bacteroidetes) are those usually reported for the gastric ecosystem of mammals (Yang et al., 2013). In our study, however, the dominant phylum was Proteobacteria, in contrast to what was reported by Mann et al. (2014) for the non-glandular area of the pig stomach in which Firmicutes represented the dominant phylum with *Lactobacillus* genus. This difference could be due to the different gastric area analysed; in fact, the non-glandular stomach area is reported as that which hosts primarily lactobacilli (Sheh and Fox, 2013; Yuki et al., 2000).

In the present study, classification at the genus level identified *Pasteurella*, *Streptococcus* and *Lactobacillus* as those most characterising the gastric content. In the pig, *Pasteurella* is mainly reported for the upper respiratory tract (Kernaghan et al., 2012) and it is linked to diseases of the respiratory system (MacInnes et al., 2008). Streptococci and lactobacilli, for which our study found relative abundances slightly in favour of streptococci (6.79% vs. 5.58%), have already been described in the pig stomach as competitors in the post weaning period (Su et al., 2008).

Instead, the bacterial genera which characterise the gastric mucosa were *Herbiconiux* and *Brevundimonas*; *Herbiconiux* included strains associated with plant matter (Behrendt et al., 2011;

Hamada et al., 2012) which has never been described before in the gastrointestinal tract of mammals. The genus *Brevundimonas* includes opportunistic strains ubiquitous in the environment, and a strain with growth requirements typical of *Helicobacter* wich has been reported in the gastric mucosa of dogs (Buczolits et al., 2001). The presence of both *Herbiconiux* and *Brevundimonas* was also observed in a set of samples of the pyloric mucosa analysed, using the same technique, in another our study (unpublished data), but with lower abundances (about 4%), this could be explained by the association of these bacteria with the last meal ingested. Interestingly, *Herbiconiux* and *Brevundimonas* include strains which can degrade cellulose and xylan found in the gut of some insects (Hu et al., 2014; Kim et al., 2011) and it was noted that the bacteria associated with the plant matter of the meal could represent an inoculum of functionally similar strains in mammals (Van Gylswyk and Van Der Toorn, 1987). Furthermore, the action of cellulase and xylanase enzymes has already been the subject of interest in pig feeding studies (Bhat, 2000; Pedersen et al., 2012); the possibility of exogenous xylanase activity in the pig gastric environment has also been reported (Pedersen et al., 2012). Mann et al. (2014), for example, suggest for *Prevotella* the degrading activity of hemicelluloses in the stomach of pig; regrettably, microbiota profiling through the 16S rRNA gene cannot provide information regarding the functionality and vitality of the bacteria reported.

Finally, must not be forgotten that the bacterial community of the stomach is more directly influenced by the last meal ingested (Varloud et al., 2007) and by the sampling and the management of the animals (Perkins et al., 2012), and that the scarcity of studies in the literature explicitly addressing the gastric microbiota makes the formulation of stronger hypotheses regarding the impact of different gastric locations on microbiota composition difficult. The present results indicated that proper designs could be formulated for the additional identification and isolation of variables which modify gastric microbiota in the pig; nevertheless, the exploratory nature of this pilot study must be pointed out, and larger studies focused on the stomach would provide validation of the data presented herein. It is hoped, therefore, that the efforts now dedicated to the description of the gut microbiota will stimulate additional studies involving the gastric ecosystem.

Table 1 Distribution of reads and Operational Taxonomic Units (OTUs), before (pre rarefaction) and after (post rarefaction) normalization by subsampling to the lowest number of reads recovered in a single sample.

Individual	Sample	pre rarefaction		post rarefaction	
		Reads	OTUs	Reads	OTUs
1	Content	9273	813	2961	476
2	Content	7504	573	2961	366
3	Content	5967	562	2961	401
4	Content	9408	787	2961	450
1	Oxyntic	5660	401	2961	311
2	Oxyntic	4648	319	2961	264
3	Oxyntic	4891	396	2961	335
4	Oxyntic	6096	460	2961	351
1	Pyloric	4033	428	2961	381
2	Pyloric	2961	332	2961	332
3	Pyloric	4127	391	2961	335
4	Pyloric	4412	392	2961	338
1	Groove	3586	383	2961	356
2	Groove	4025	374	2961	337
3	Groove	4717	428	2961	365
4	Groove	5423	457	2961	370

Table 2 ANOSIM (analysis of similarities) post hoc test based on abundance of OTUs in samples.

	Content	Oxyntic	Pyloric	Groove
Content		0.029	0.033	0.028
Oxyntic	0.029		0.059	0.088
Pyloric	0.033	0.059		0.715
Groove	0.028	0.088	0.715	

Significant differences in pairwise comparisons are highlighted in gray. The one-way ANOSIM was performed on Bray-Curtis distance with 10,000 permutations, the samples were grouped according to the point of origin.

Table 3 ANOSIM post hoc test based on relative abundance of genera in samples.

	Content	Oxyntic	Pyloric	Groove
Content		0.031	0.027	0.031
Oxyntic	0.031		0.969	0.441
Pyloric	0.027	0.969		0.623
Groove	0.031	0.441	0.623	

Significant differences in pairwise comparisons are highlighted in gray. The one-way ANOSIM was performed on Bray-Curtis distance with 10,000 permutations the samples were grouped according to the point of origin.

Table 4 Similarity Percentage (SIMPER) genera contribution.

Genus	**Contribution**	**Mean ab. Cont.**	**Mean ab. Muc.**
Herbiconiux	10.15	21.60	41.90
Pasteurella	3.45	7.51	0.62
Streptococcus	3.09	6.79	0.64
Brevundimonas	2.77	8.29	12.90
Moritella	2.41	5.01	9.83
Lactobacillus	2.30	5.58	0.98
Lactococcus	1.96	4.23	0.32
Phenylobacterium	1.34	2.55	0.53
Ochrobactrum	1.19	3.31	4.62
Prevotella	1.18	2.74	0.42
Delftia	1.10	1.76	3.85
Stenotrophomonas	0.98	1.02	2.74
Curtobacterium	0.68	1.41	0.05
Pseudomonas	0.59	1.38	0.21
Cloacibacterium	0.55	1.01	0.17
Eikenella	0.55	1.17	0.15

“Contribution” represents the average contribution of a given Genus to the average dissimilarity between samples (overall mean = 51.43%). “Mean ab. Cont.” is the average relative abundance (in %) in content samples “Mean ab. Muc.” is the average relative abundance (in %) in mucosal samples. The list of genera is not exhaustive, an arbitrary threshold of a mean contribution of 0.5 was used as a cut-off.

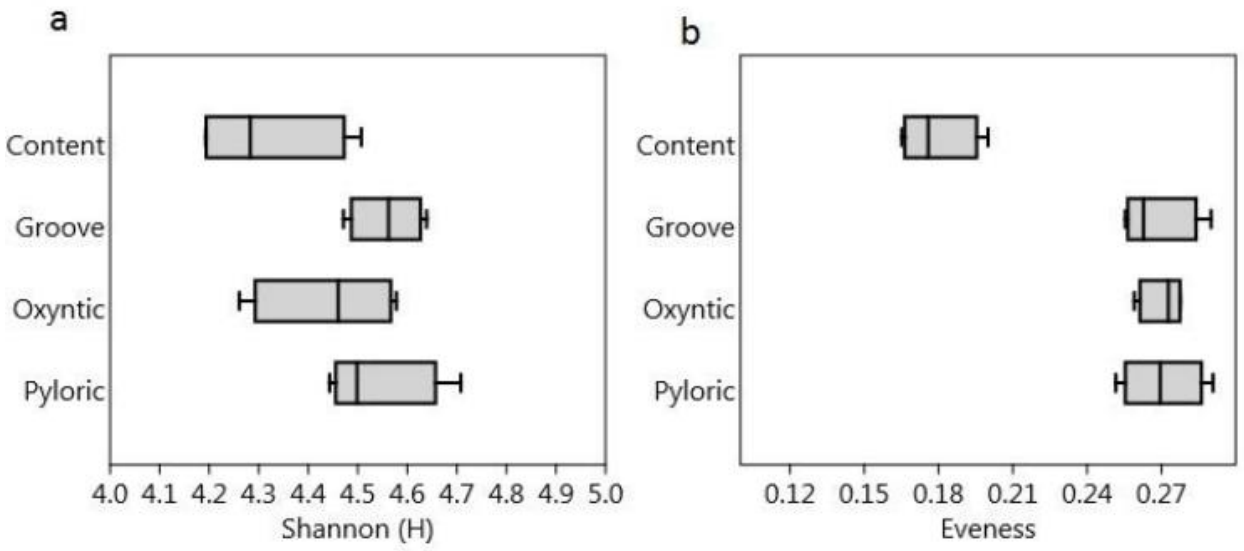

Figure 1 Box-plot of the Shannon-Weaver (a) and Eveness (b) index values in the samples from different regions of the stomach.

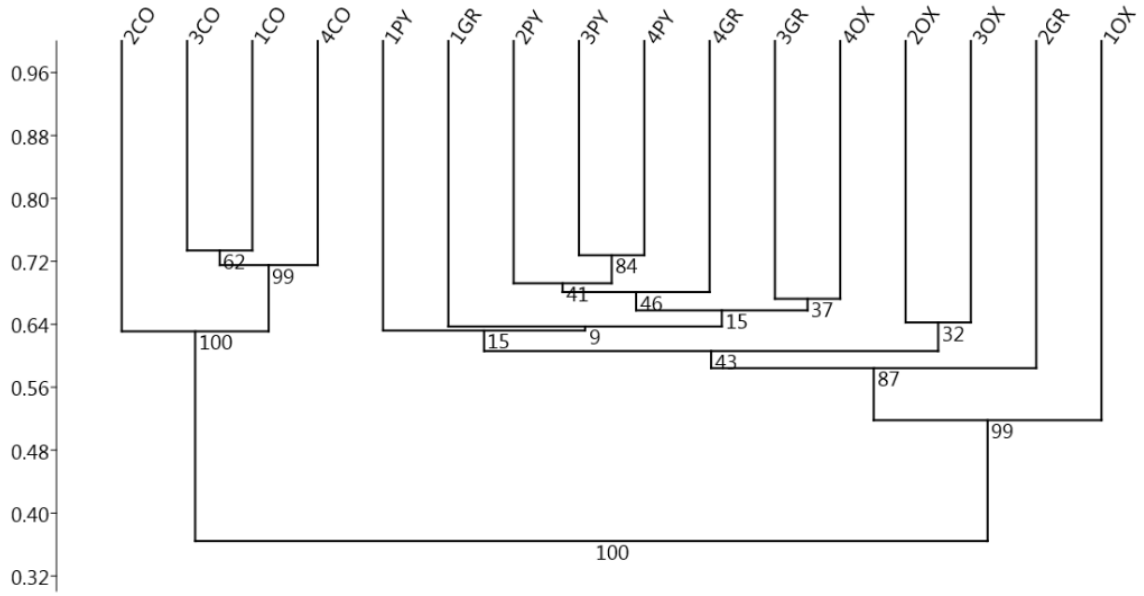

Figure 2 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Cluster analysis (Bray-Curtis distance) of the samples of the gastric mucosa based on the abundance of the OTUs. Labels indicate the pig identification number and the stomach region (CO: contents, OX: oxyntic, PY: pyloric, GR: groove) with 10,000 Bootstrap resamplings used: the bootstrap Probability values (BP) are shown at the nodes.

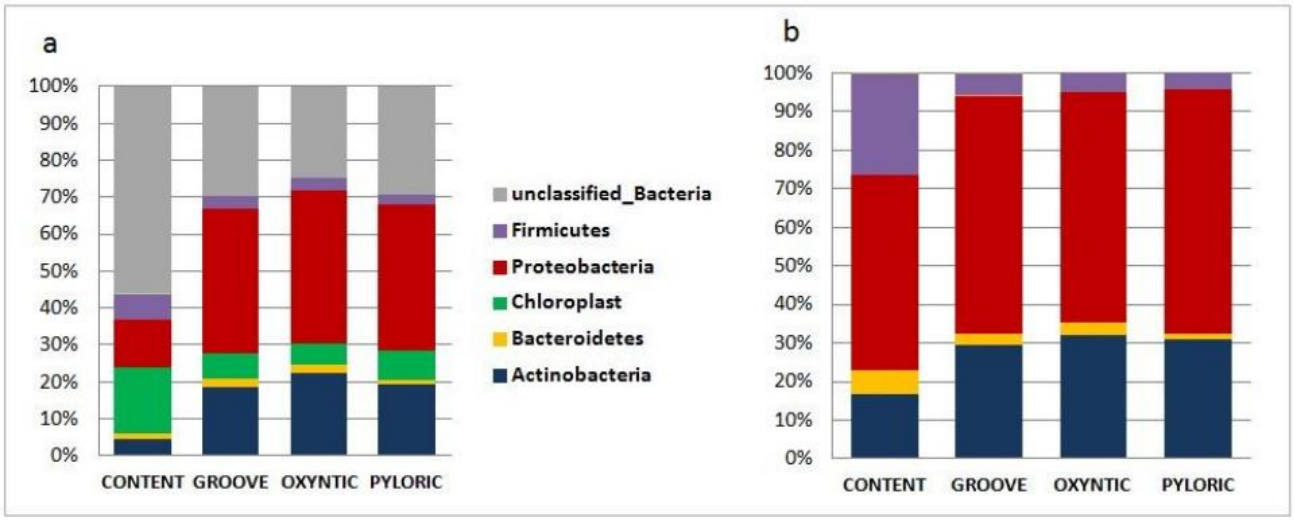

Figure 3 Relative abundance (average) of the principal phyla in the different stomach regions. Before and after removal of unclassified Bacteria and Chloroplast reads. Verrucomicrobia, Fusobacteria and Acidobacteria phyla (not visible in the Figure) were also found with abundances <1%.

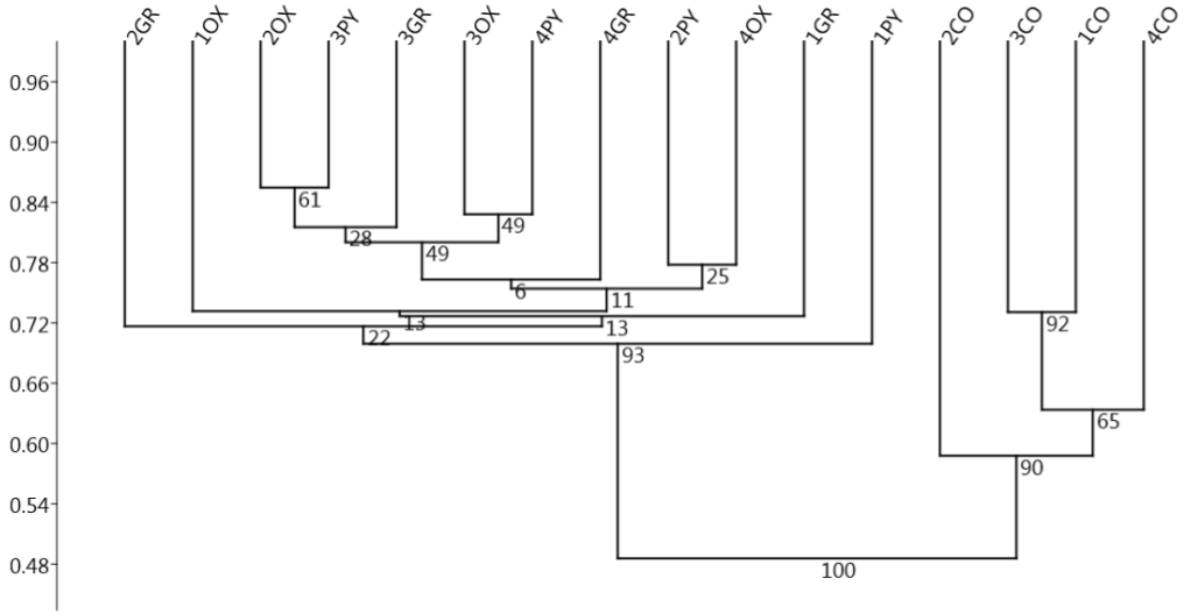

Figure 4 Unweighted Pair Group Method with Arithmetic Mean (UPGMA) Cluster analysis (Bray-Curtis distance) on the samples of the gastric mucosa based on the relative abundance of the sequence reads classified at the genus level. Labels indicate the pig identification number and the stomach region (CO: contents, OX: oxyntic, PY: pyloric, GR: groove) with 10,000 Bootstrap resamplings used; bootstrap probability values (BP) are shown at nodes.

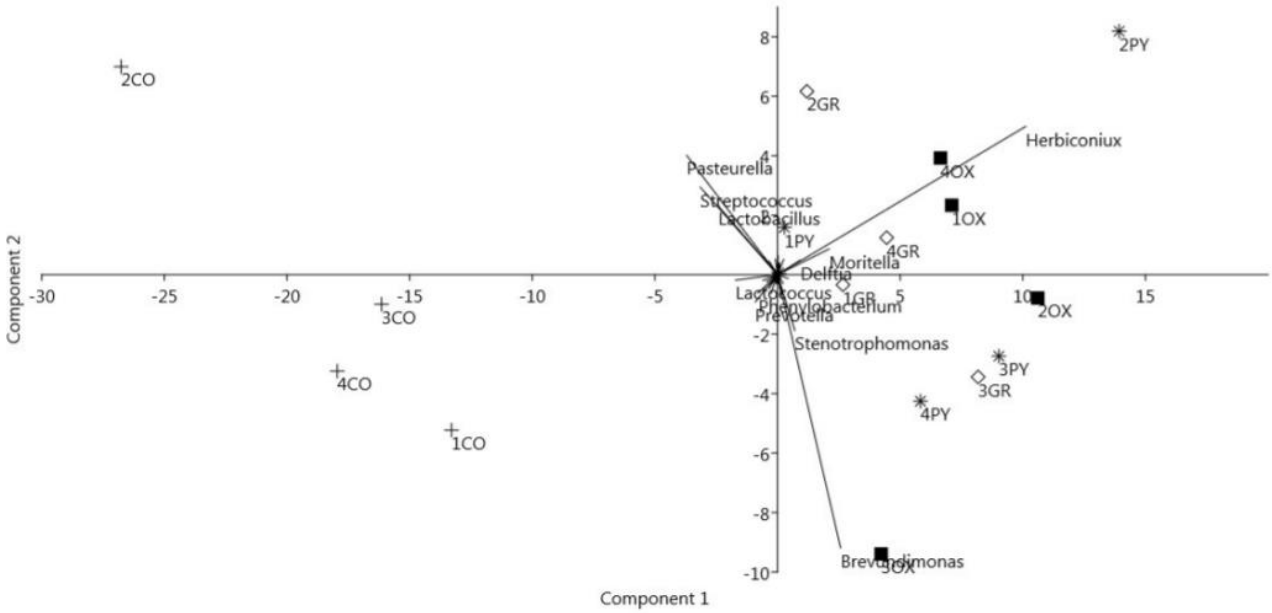

Figure 5 Biplot of the Principal Component Analysis of the stomach samples based on the relative abundance of bacterial genera per sample. Labels indicate the pig identification number and the stomach region (+ CO: contents, ■ OX: oxyntic, * PY: pyloric, ◊ GR: groove). Names of the bacterial genera with the highest loadings are plotted as vectors according to their correlation to the first two components.

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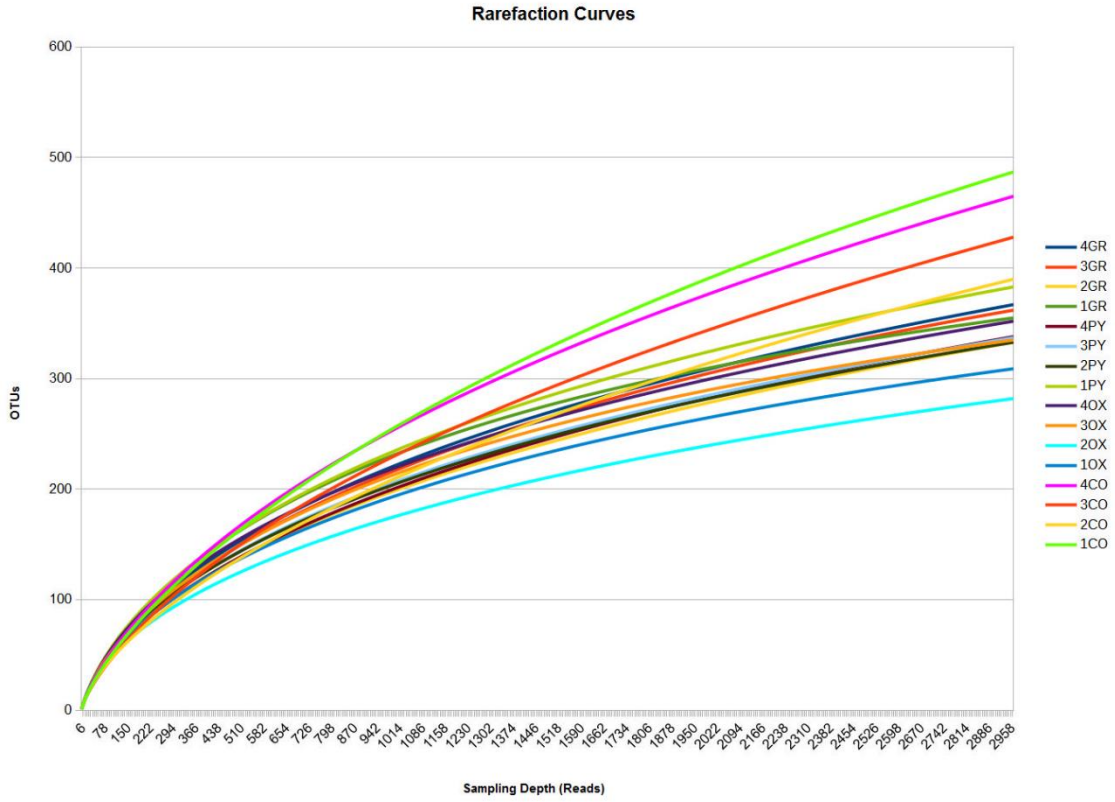

Figure S1 Per Sample Rarefaction Curves. Numbers indicate the pig identification number and the initials indicate stomach region (CO,contents; OX, oxyntic; PY, pyloric; GR, groove).

Long-term administration of formic acid to weaners: Influence on Intestinal microbiota, immunity parameters and growth performance

Abstract

The interest on the use of organic acids in diets for livestock animals is due to the potential benefit in maintaining gut homeostasis and controlling pathogenic bacterial growth. The objective of this study was to evaluate the influence of two doses of formic acid supplementation for six weeks to weaner pigs. The long-term effects of formic acid were tested for growth performance, microbiota composition in the jejunum using 16S rRNA, expression of marker genes of inflammation in the jejunum, and quantification of acid-secreting cells in the gastric oxyntic mucosa using immunohistochemistry. Three diets containing no (control; CO), 1.4 g/kg (low formic acid; LFA) or 6.4 g/kg (high formic acid; HFA) free formic acid were fed to 36 pigs for 42 d from 7 d post-weaning (7.1 ± 1.0 kg live weight) until the end of the trial (27.7 ± 5.5 kg live weight); they were housed in individual cages for six weeks. During the first three weeks, formic acid supplementation, at any of the dosage above, increased average daily gain (ADG) ($P = 0.004$), tended to increase average daily feed intake (ADFI) ($P = 0.08$) and tended to decrease g of feed to g of gain (F:G) ($P = 0.08$) while no effect was observed during the last three weeks. At the end of the trial, formic acid supplementation had only a minor effect on the microbiota composition in the jejunum. A higher microbiota diversity (Chao1 index $P < 0.05$) was found in the HFA group than in the CO group. The Control group showed a higher abundance ($P < 0.05$) of *Gemella*, *Lactobacillus* and *Parvimimonas* than the HFA and LFA groups, higher levels of *Acinetobacter*, *Fusobacterium*, *Leuconostoc* respect to the HFA group and of *Turicibacter* as compared to the LFA group. The abundance of *Streptococcus* was lower in the CO group than in the HFA group and higher than in the LFA group. The jejunal expression of C-C Motif Chemokine Ligand 2 (*CCL20)* was higher in the HFA group respect to the LFA group. Formic acid intake did not affect the thickness of the gastric mucosa, the number of parietal cells and stomach weight. This study showed that the addition of formic acid to piglet diets improved growth

performance during the first period after weaning and that the long-term supplementation of formic acid slightly affected the microbiota composition according to the dose.

Abbreviations: ADG, average daily gain; ADFI, average daily feed intake; F:G, g of feed to g of gain,

Introduction

Organic acids, weak acids having a carbon chain of one to seven carbon atoms and at least one carboxyl group, are supplemented in piglet diets owing to their ability to positively affect intestinal heath and function (Roth and Kirchgessner, 1998; Zentek et al., 2012). Organic acids are nowadays considered a suitable strategy for avoiding the use of antibiotics, thus providing a tool for the transition to the post-antibiotic era of livestock production systems (Casewell et al., 2003; Mroz, 2005). A reduction in gastric pH and in the buffering capacity of the diet (Mroz et al., 2000), and increases in pepsin activation and in the digestibility of proteins and amino acids are the main mechanisms whereby organic acids enhance animal performance (Mroz, 2005; Partanen and Mroz, 1999). Furthermore, lowering the intestinal pH prevents the instauration of pathogenic bacteria and promotes the development of acid-resistant microbiota such as lactic acid bacteria (Mroz et al., 2002). Of the different organic acids, formic acid is considered to be one of the most interesting additives due to its efficacy in reducing the development of coliform bacteria (Mroz, 2005) notwithstanding the fact that the divergent results regarding its effects on the different tracts of the gut have been reported (Gedek et al., 1992; Torrallardona et al., 2007).

Bosi et al. (2006) observed that dietary supplementation of calcium formate was associated with a reduction in parietal cells number in the mucosa of the stomach, leading to a negative effect on the gastric secretion of hydrochloric acid. However, the effect of a (lower) dose of free formic acid supplementation on gastric functionality has not yet been studied. Furthermore, no consistent results

are available regarding the prolonged use of formic acid in diets for post-weaning piglets. The objective of this study was to evaluate the impact of prolonged supplementation with two doses of formic acid on the gastro-intestinal mucosa, the microbial community in the jejunum and the growth performance of piglets.

Materials and Methods

The procedures complied with the Italian regulations pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna and the Italian Ministry of Health with the approval number 801/2015-PR.

Animals, housing and experimental treatments

One week after weaning, thirty-six pigs (35 d of age, 7.1 ± 1.0 kg average body weight) were housed individually in cages with a mesh floor and were kept in a temperature-controlled room for six weeks. The pigs were divided into three groups (12 pigs/groups) balanced for litter origin and body weight. Each group of pigs was consecutively given two diets during the experiment. The pigs received a pre-starter diet from the weaning (day -7, one week before their arrival at the experimental facility) to d21. This diet consisted of barley (25%), wheat (21%), cereal flakes (12%), lactoserum (11%), soy protein (11%) and cooked soy grains (8%) to provide 18.5% crude protein and 1.31% standardized ileal digestible lysine (SID Lys), 0.98% calcium and 0.39% digestible phosphorus. From d22 until the end of the trial (d42), pigs received a starter diet consisting of corn (56%), wheat bran (18%), and soybean meal (20%) to provide 17.3% crude protein and 1.18% SID Lys, 65% calcium and 0.22% digestible phosphorus.

The experimental diets were prepared in pellet form and were based on a standard control diet without antibiotics or other growth promoters which was either used as such (CO), or to which 1.4 g/kg (LFA) or 6.4 g/kg (HFA) pure formic acid (HCOOH) was added directly to the feed mixtures, substituting the same quantities of corn starch. During the first week after weaning, all the animals received the

CO pre-starter diet. The calculated net energy content of the diet was 10.36 kJ/g feed for the first formulation and 9.6 kJ/g feed for the second. The nutrient values were estimated using EvaPig® software (Noblet et al., 2008) together with information from the INRA-AFZ tables of feedstuff composition). The feed mixtures were produced at the INRA facilities in Saint Gilles (France).

Sampling and analytical procedures

The individual body weights of the pigs were recorded weekly. Individual feed intake was recorded daily to determine growth performance, and to record the incidence of diarrhea.

The pigs were sacrificed 4 h after their last meal by intracardiac injection of 0.5 mL Tanax/kg body weight (Intervet Italia, Peschiera Borromeo, Italy), after having been anaesthetised with sodium thiopental (10 mg/kg body weight). The stomach of each pig was removed and weighed, opened along the greater curvature, rinsed with sterile water, and weighed again. From the thickness of the oxyntic gland area near the greater curvature, a tissue specimen of ~1 cm^2 was collected, pinned tautly on balsa wood and then immersed in a 10% buffered formalin solution for 24 h. The samples were then removed from the fixative and washed in 5.14 mol/L ethanol. Samples were then dehydrated in a graded series of ethanol and embedded in paraffin for histochemical staining. At the end of the trial, two samples of the mid-jejunum were also collected. The first sample was opened and rinsed with sterile ice cold phosphate buffer saline solution (pH 7.3). The mucosa was scraped using a microscope slide, snap-frozen in liquid nitrogen and stored at -80°C for transcriptomic analyses. The second jejunum sample was opened, emptied by gravity, and the mucosa with the chime residue was gently scraped with a sterile microscope slide, snap-frozen in liquid nitrogen, and stored at -20°C for further analyses regarding the microbial composition.

Immunohistochemistry for parietal cells

The immunohistochemistry analyses of the gastric mucosa were carried out following the procedures described by Bosi et al. (2006). Briefly, the sections were treated with 90 mmol/L H_2O_2 in methanol for 30 minutes to block endogenous peroxidase activity, then treated with normal goat serum for 1 h,

followed by a primary antibody against the a-subunit H^+/K^+-ATPase incubated at 4°C overnight with biotinconjugated goat antimouse immunoglobulin G (IgG), and then with avidin-biotin complex (ABC) (Vector Laboratories). The immune reactions were visualised applying a 3–3'-diaminobenzidine chromogen solution (Vector Laboratories, Burlingame, CA). For each pig, all parietal cells were counted in 20 randomly selected glands located perpendicularly to the surface of the mucosa using an optical microscope. The depth of the glands, from the pits to the *muscularis mucosae*, was measured in the same area using a Zeiss Axioplan microscope (10 x objective) connected to KS 300 image analysis software (Kontron Elektronic, Eching, Germany).

Gene expression analyses

Total RNA was isolated from the mid-jejunum mucosa samples using Trizol reagent (Life technologies, Carlsbad, CA, USA) following the manufacturer's instruction. The RNA quantity and quality were evaluated using an ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA), and agarose gel electrophoresis, respectively. One thousand nanogram of RNA were reverse-transcribed using the ImProm-II reverse transcription system (Promega, Madison, WI, USA). The semi-quantitative polymerase chain reaction (qPCR) of interleukin-8 (*IL8*), glutathione peroxidase 2 (*GPX2*), regenerating islet-derived protein 3 gamma (*REG3G*), Trefoil Factor 3 (*TFF3)*, C-C Motif Chemokine Ligand 2 (*CCL20)*, ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 1 (*ST3GAL*) and Lipopolysaccharide Binding Protein (*LBPI*) and Solute carrier family 7 member 9 (*SLC7A9*) were performed with a Light Cycler instrument (Roche Diagnostics, Mannheim, Germany) using the primer and conditions reported in Table 1. Amplification was carried out in a 10-mL volume containing 2 mL of cDNA, 0.5 mmol/L of each primer and 5 mL of SYBR Green PCR Master Mix (Takara, Bio Inc., Japan). The specificity of each amplification was checked using a melting curve analysis. The data were normalised to the expression of the housekeeping gene Hydroxymethylbilane Synthase (*HMBS*), and the relative quantification of gene expressions was calculated using standard curve methods.

Microbiota analyses

Total bacterial DNA was extracted using a Qiamp Stool Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's instructions. The library formation and sequencing of the 16S rRNA gene were carried out using an MiSeq® Reagent Kit V3-V4 on an MiSeq-Illumina® platform.

Statistical analysis regarding biological and performance parameters

Performance data, gene expression data, histological measures and stomach weight, were analyzed by analysis of variance (ANOVA) using the GLM procedure of SAS (version 3.4 SAS Institute) with a 2-level full factorial design, including diet and litter. In addition, the following polynomial orthogonal contrasts: "With formic acid vs without formic acid" (CO vs LFA, HFA); "Low formic acid vs high formic acid" (LFA vs HFA) were tested using the Contrast statement of SAS. A probability level less than 0.05 was considered statistically significant while a value less than 0.10 was considered as a trend.

Microbiota bioinformatics and statistical analysis

For the microbiota data, the sequences generated were analysed using a subsampled open-reference OTU strategy with default settings in QIIME (v1.9.1). The reads of the 16S rRNA gene were paired-end and demultiplexed. Subsampled open-reference OTU-picking was carried out using UCLUST with 97% sequence similarity. Representative sequences were assigned taxonomy against the Greengenes database V13_8 using the UCLUST method with a 90% confidence threshold. Data were low abundance filtered by removal of the OTUs with a relative abundance ≤0.005% across all samples (Bokulich et al., 2013) and were chimera checked using the Blast fragments approach (Altschul et al., 1990) in QIIME. To remove sampling depth heterogeneity, rarefaction on the OTU table was performed with a cut-off of 9354, the lowest number of reads recovered in a single sample.

The alpha diversity index values (Chao1 and PD_whole_tree) were calculated in QIIME (v1.9.1). The effect of diet on microbial alpha diversity within each group was analysed using ANOVA with a Tukey honest significant difference (HSD) test. The beta diversity analysis was carried out utilizing the Vegan package of the R software (v3.3.0) using the Bray-Curtis distance on the OTU table; the

Bray-Curtis distance matrix was visualised with a non-metric multidimensional scaling (NMDS) plot, and the differences among the three treatment groups were tested with permutational analysis of variance (Adonis procedure with 999 permutations). In order to determine the Genera based on the OTU number and the single OTUs differentially abundant between the diet groups, the "multiple groups" procedure of the metagenomeSeq (v1.16.0) package in R 3.3.2 was applied (Paulson et al., 2013). The OTUs with more than the average number of effective samples per group and the adjusted *P* value FDR (false discovery rate) lower than 0.05 were considered statistically significant.

Cost-benefit analysis of the use of Formic acid

A simplified cost-benefit analysis of the use of Formic acid in the diets as compared to the control diet was calculated. For each one of the two feeding phases, the cost of 1 kg growth was obtained for each group considering the price of formic acid (1.2 € / kg), the prices of the basic diets (0.50 € / kg and 0.27 € / kg for the pre-started and started diets, respectively), and the feed to gain ratio. The price effect of the substitution of formic acid for price of corn starch was not considered because, in practice, the pure corn starch is not usually used.

Results

The animals remained healthy throughout the experiment. Growth performance traits were influenced by the diet (Table 2) in the initial phase of the trial (d0-d21). Animals fed diets supplemented with formic acid had higher ADG during the first three weeks (286 g/d and 268 g/d for the LFA and HFA diets) than those fed the CO diet (248 g/d) ($P = 0.004$). In addition, the ADG tended to be greater for treatment LFA compared with treatment HFA ($P = 0.090$). During the first three weeks, pigs fed the diets supplemented with formic acid tended to have a higher ADFI as compared to pigs fed the CO diet ($P = 0.08$). No significant differences between the groups were observed for the F:G.

During the second growing period (d22-d42) as well as for the entire experimental period, no differences in ADG, ADFI, and the F:G were observed between the treatment groups.

Formic acid supplementation did not change the relative abundance of the expression of genes tested in the jejunal mucosa of the piglet except for *CCL20* expression for which a higher level was observed in the HFA group (0.016) compared to the LFA (0.013) group ($P = 0.05$). The counts of the parietal cells in the gastric oxyntic mucosa and the depth of the glands, and the weights of the total and empty stomachs were not influenced by the diet (data not shown).

Two samples were eliminated from the analysis of the microbiota in the jejunum due to the low quality of the sequencing data. The 34 samples generated a total of 312,290 valid sequences reads, corresponding to 577 OTU (97% identity). The largest number of sequences was associated with the *Firmicutes* phylum (80.6% average), followed by *Proteobacteria* (8.8%) and *Actinobacteria* (7.3%) and *Chlamydia* (2.4%); other minor phyla (<1%) were *Bacteroidetes*, *Cyanobacteria* and *Fusobacteria*. Ninety-two different genera were identified in the genus aggregated data. At the genus level, *Lactobacillus* (38.1%), *Streptococcus* (18.7%), *Clostridium* (4.8%) and *Corynebacterium* (4.3%) were found to be the most abundant.

No significant effect on the alpha diversity was reported for PD whole three while significant differences were reported for the Chao index (ANOVA, $P = 0.039$). In particular, the Chao1 index was higher in the HFA diet than in the CO diet (Tukey's test, adjusted $P = 0.036$) (Table 3). The NMDS plot on the Bray-Curtis distance matrix (Figure 1) did not clearly distinguish clusters based on diet (Adonis $P = 0.225$). In the NMDS plot, each point represented the gut microbiota of a pig while greyscale and shapes visualised each diet. The multiple comparison analysis on the OTU abundance and Genera abundance indicated that the diets significantly influenced the relative abundance of 122 single OUTs in the 577 mainly belonging to Firmicutes Phyla (77.8%). Of the 122 OTUs, 59 OTUs significantly differed between CO and HFA, 44 OTUs significantly differed between CO and LFA and 19 OTUs significantly differed between LFA and HFA (Supplementary Table 1). Table 4 showed the significant differences regarding genera aggregated data. A significant lower abundance of *Acinetobacter* ($P = 0.028$), *Fusobacterium* ($P = 0.048$), *Gemella* ($P = 0.012$), *Lactobacillus* ($P < 0.0001$), *Leuconostoc* ($P = 0.023$) and *Parvimonas* ($P = 0.012$) genera was found

for the HFA diet than for the CO diet, while the relative abundance of *Streptococcus* was higher in the HFA group than CO group higher ($P < 0.0001$). A lower relative abundance of *Gemella* ($P = 0.029$), *Lactobacillus* ($P < 0.0001$), *Turicibacter* ($P = 0.045$), *Parvimonas* ($P = 0.034$) and *Streptococcus* ($P < 0.0001$) was observed in the LFA group than in the CO group. No significant differences were found on genera-aggregated data between the LFA and the HFA groups.

The calculated cost per kg growth was 0.655 €, 0.616 € and 0.632 for the first period and 0.408 €, 0.410 € and0.418 €, for the second period, for the CO, LFA and HFA groups, respectively.

Discussion

This study reports, for the first time, the effect of long-term supplementation of formic acid in post-weaning pigs. The two dosages of formic acid were used to compare the common supplementation dosage of formic acid in piglet diets with an "auxinic/low dosage". In intensive farming systems, pigs are exposed to notable discomfort. In particular, younger animal from birth to the start of the growing phase, are sensitive to dietary changes and mixing, two conditions which increase the risk of gut dysbiosis, which is associated with disease (Lallès et al., 2007; Pieper et al., 2008). After the ban of antimicrobial growth promoters, the prophylactic use of antibiotics has been the main strategy for preventing microbial unbalance. However, increasing awareness of the risks associated with their use has hastened the application of alternative strategies to prevent gastro-intestinal disorders. Supplementing the diet with formic acid or its salts is considered a valuable strategy. Formic acid has been shown to have antimicrobial properties with regard to specific pathogens in *in vitro* (Naughton and Jensen 2001; Knarreborg et al., 2002) and *in vivo* studies (Bosi et al., 2007) as well as being effective in sustaining pig performance after weaning (Bosi et al., 2007; Htoo and Molares, 2012) and during the growing-finishing period (Øverland et al., 2008; Øverland et al., 2013).

In our study, the animals were taken from a commercial farm, were delivered to the experimental facility one week after weaning, in order to not increase the stress already caused by weaning and to make sure that their ingestion would not be compromised. During the six weeks of our study, no major health problems occurred. The positive effect of formic acid supplementation on the growth performance of piglets was observed only during the first three weeks of the experiment. This confirmed the favourable effect of formic acid during the transition period from suckling to weaning as has been demonstrated to a large extent in the literature, and ascribed to its positive effect on reducing the pH along the gastrointestinal tract (in particular, in the stomach), and by increasing pepsin activation to improve protein digestibility (Mroz, 2005). However, during the last three weeks of the trial, the positive effects of formic acid supplementation disappeared without evident reason and, concurrently, the pigs fed with the control diet continued to grow steadily. After six weeks, when growth performance was similar between the three groups, the 16S rRNA sequencing data highlighted exiguos evidence that the inclusion of two doses of formic acid on piglets diets can affect the profile of the gut microbiota in apparently healthy animals. The supplementation with the highest dose of formic acid to the diet increased the alpha diversity of the gut microbiota, showing the importance of formic acid dosage on influencing the complexity of structure of the microbial community. A higher alpha diversity, defined also as biodiversity, has been proposed as indicator for the stability of gut microbial community (Zoetendal et al., 2004). This suggests that the HFA group had a more stable microbiota than the CO group, that could make piglets able to sustain eventual future risks of dysbiosis. Conversely the supplementation with the low formic acid dose was not sufficient to differentiate the alpha diversity from the control group, but also from the group with the higher dose. No data have previously been reported for the effect of formic acid on jejunal microbiota composition using the 16S rRNA technique, nevertheless, using other analytical technique or other dietary organic acid types, feed acidifiers have been found to affect the microbial community with divergent results. A supplementation of butyric acid (in proximal colon, Roca et al., 2014) and benzoic acid (in ileum, but not in cecum, Torrallardona et al., 2007; in cecum, but not with inulin supplementation, Halas et

al., 2010) increased microbial diversity, while a supplementation with formic acid (growing-finishing pigs, mid-colon, Canibe et al (2005) showed a reduction in microbial diversity. The different responses may be related to the different experimental conditions (age of pigs, type of diet, site of sampling etc.); nevertheless, it is interesting to observe that in several trials, including the present, the microbial diversity was increased by organic acids notwithstanding the fact that they are considered to control certain bacterial species (Mroz, 2005).

In previous studies, the influence of formic acid on gut microbiota has mainly been associated with coliform bacteria, yeast and lactic acid producing bacteria (Partanen and Mroz, 1999). A dose-dependent effect of formic acid on coliform bacterial population was reported by Gedek et al. (1992). In our study, only a low number of OTUs belonging to the *Enterobacteriaceae* family were observed and no significant effect of formic acid supplementations with respect to the CO group was found for this family. Conversely, common genera such as *Gemella*, *Parvimonas* and *Lactobacillus*, showed a lower OTUs abundance in HFA and LFA animals as compared to pigs belonging to CO group. *Streptococcus* genus was affected by the dose of formic acid supplementation, the HFA diet supporting the *Streptococcus* development in the jejunum while a lower relative abundance was found in the jejunum content of LFA pigs. The counts of *Lactobacillus* and of other common cultivable commensal bacteria in the small intestinal content were also reduced by various dosages of formic acid in previous research (Gedek et al., 1992). *Gemella*, *Lactobacillus* and *Streptococcus* genera are part of LAB sensu stricto (Leser et al., 2002) and are generally considered as safe commensals with no distinct pathogenic potential for animals. Furthermore, *Lactobacillus* is generally considered a beneficial bacteria due to its properties which include anti-inflammatory and anti-bacterial activities (Bauer et al., 2006; Lebeer et al., 2008). In our study *Lactobacillus* was the most abundant genus in all the groups and a sufficient level of these bacteria is supposed to be present in order to achieve its beneficial activities in all groups. Of the *Streptococcus* genus, the *S. suis* is considered to be an important pathogen which causes meningitis, septicemia and sudden death in piglets (Smith et al., 1997), and it can also infect humans (Arends and Zanen, 1988). Even if the gut is not the primary site

for the infection, piglets with a high number of *S. suis* in the gut can support the *S. suis* diffused in the environmental (Su et al., 2008). Other species belonging to *Streptococcus* such as S*treptococcus thermophilus* and *Streptococcus faecium* have been highlighted for their potential use as probiotics (Han et al., 2015; Perdigon et al., 1987). Nevertheless, the 16S rRNA technique applied in our study does not allow the assessment of the specific species belonging to the *Streptococcus* genus; thus, its relationship with the host intestinal balance cannot be described in detail.

The effect of formic acid on the pH in the gastrointestinal tract (GIT) and subsequentially on the lactic acid and coliform bacterial populations have been reported to be affected by the acid dosages (Gedek et al., 1992). In our study, in addition to the *Streptococcus* for which an opposite trend was found between the HFA and the LFA groups, a lower OTUs abundance of *Acinetobacter*, *Fusobacteria* and *Leuconostoc* in the HFA group and of *Turicibacter* in the LFA group with respect to the CO group confirms that the dosage of formic acid can affect the specific bacteria genera.

Taken together, the microbial results presented in this study suggested that even auxins dosages of formic acid led to a modification of the dynamics of the jejunal bacterial community decreasing both the number of lactic acid producing bacteria (*Lactobacillus*, *Leuconostoc* and *Gemella)* and butyric acid producing bacteria (*Fusobacteria*) (Pryde et al., 2002), in a dose-dependent way. These differences were observed after six weeks of supplementation and may represent a long-term adaptation of part of the microbiota to acidic conditions. Thus, the modification on the gut microbiota observed at the end of the trial cannot be directly associate with the better performance obtained in the first half of the trial with the supplementation of the formic acid.

Even though different genera in the jejunum were found to be influenced by formic acid supplementation, the growth performance during the last three weeks was not affected, and this could be ascribed to the absence of acute inflammatory conditions of the gastrointestinal tract since the gene expression of the array of genes involved in intestinal mucosa homeostasis was not different between the experimental groups.

The genes which were tested were selected on the basis of the transcriptome results of trials where pigs were acutely stimulated by the enterotoxigenic *Escherichia coli* (ETEC) (Trevisi et al., 2017; Loos et al., 2012; Bosi et al., 2004;). Interleukin-8 and CCL20 are typical inflammatory cytokine while REG3G is an intestinal defence C-type lectin (Soler et al., 2015). LPB is involved in the acute-phase immunologic response to gram-negative bacterial infections. The *GPX2* gene encodes for gastrointestinal glutathione peroxidase and is stimulated by hydrogen peroxide resulting from by inflammation; *TFF3* encodes for a stable secretory protein which protects the mucosa from insults. *ST3GAL1* have been shown to modulate the O-glycan biosynthesis regulating CD8+ T lymphocyte homeostasis (Priatel et al., 2000). Finally, the amino acid transporter $b^{0,+}$ AT (*SLC7A9*) was selected to represent the expression of a set of genes transcribing for amino acid transporters which were depressed after loop-perfusion with ETEC, compared with the control perfused loops in connection with a general rapid induction of inflammation. Therefore, it is possible that the modifications observed in the microbial profile were not sufficient to demonstrate differences in these gene markers of acute inflammation. Nevertheless, a higher level of *CCL20* expression in the HFA group than in the LFA group was observed suggested that the higher dose of formic acid was able to stimulate the immune response similar to the *CCL20* gene, which belongs to the subfamily of small cytokine CC genes, and encodes for CCL20 (C-C Motif Chemokine Ligand 20) protein which is responsible for the chemotaxis of dendritic cells (DC), effector/memory T-cells and B-cells (Ito et al., 2011). In pigs, higher levels of *CCL20* expression in the intestinal mucosa have been associated with *Escherichia Coli* infection (Trevisi et al., 2017; Zhou et al., 2012), suggesting that *CCL20* expression is affected by the intestinal microbiota. In our experiment, no evidence of *Escherichia coli* presence in the jejunal microbiota was observed using the 16S rRNA approach, and no significant differences between the HFA and the LFA groups were observed on the genera-aggregated data; nevertheless, considering the single OTUs, it was notated that the HFA group had a higher level of *Clostridiaceae* and *Enterobacteriaceae* and a lower level of *Lactobacillaceae* than the LFA group supporting the fact

that the stimulation of *CCL20* and, in general of the immune response in the HFA group could also be linked to the jejunal microbiota.

The potential of the gastric mucosa to secrete hydrochloric acid, indicated by the number of cells positive for the protonic pump H^+/K^+-ATPase in the oxyntic mucosa, was not altered by formic acid supplementation, nor was the weight of the stomach. This contrasts with our previous study where the inclusion of 1.2% calcium formate in the diet reduced the number of cells and the stomach weight after three weeks of supplementation to pigs at weaning (21 days of age) (Bosi et al., 2006). It can be hypothesized that the older pigs in the present experiment, with a proportionally higher feed intake, were able to produce a sufficient quantity of gastric juice so that the dietary acidification provided no additional benefit.

Concerning the cost-benefit of the use of formic acid in the post-weaning diets, this supplementation was cost-efficient for the first three weeks of the trial corresponding to the initial post-weaning period, but not for the second three weeks. This was mainly due to the absence of improvement in the feed to gain ratio in the second part of the trial. Furthermore, the prolonged administration of formic acid did not compromise the expression of inflammatory candidate genes in the jejunum and only mildly affected the microbiota composition. Our result supported the frequent use of formic acid (or formate) in commercial formulas for the immediate post-weaning period.

Conclusion

In healthy pigs, dietary supplementation with a low dose of formic acid exerts an auxinic effect and improves the ADG during the initial post-weaning period. On the other hand, the prolonged supplementation of formic acid e does not increase piglet performance, has no negative effect gene expression of the inflammatory candidate genes and slightly influence the microbial community in the jejunum affecting selected bacteria genera in a dose-dependent way. More specific studies are needed to clarify how the manner in which the modification observed in the gut microbiota could influence the health and performance of pigs in order to have a more complete evaluation of formic acid effectiveness as a long-term additive in post-weaning piglets.

Table 1. Expression primer sequence and analysis conditions.

Gene		Primer sequence (5'->3')	Amplicon length	Accession number	Temperature of annealing
IL8	Forward	CAAGCAAAAACCCATTCTCC	123	AB057440	61°C
	Reverse	TTTCTCTGGCAACCCTATGTC			
REG3G	Forward	ACCCAAAACCTGGATGGATG	102	NM_001144847.1	65°C
	Reverse	AGGGAGGACACGAAGGATG			
GPX2	Forward	GACATCAAGCGCCTCCTC	183	DQ898282.2	64°C
	Reverse	AGACCAGAAAGGCAAGGTTC			
TFF3	Forward	GTTGTTGCACTGCTCGGG	108	NM_001243483.1	62 °C
	Reverse	CTCGGCTTTGTCGCTTTGT			
CCL20	Forward	GGGTGAAACAAGCCGTGAAT	114	XM_005672261.2	60°C
	Reverse	CAGCACAGCGAGGTTCTTTT			
ST3GAL	Forward	AGGGTCTCCGCCTGGTTC	107	NM_001004047.1	61°C

	Reverse	AAGTTATTGGGCTGCTTCTCC			
LBP	Forward	AGGAACACAGCCGAATGG	161	NM_001128435.1	62°C
	Reverse	GAAGGTGCGGAAGGAGTTG			
SLC7A9	Forward	CTGGCCCAAGGAAATACAAA	106	NC_010448.3	60 °C
	Reverse	CATCATATGCCCAGAGTCCA			
HMBS	Forward	AGGATGGGCAACTCTACCTG	83	DQ845174	62 °C
	Reverse	GATGGTGGCCTGCATAGTCT			

IL8, Interleukine-8; *GPX2*, Glutathione peroxidase 2; *REG3G*, Regenerating islet-derived 3 gamma; *TFF3*, Trefoil factor 3; *CCL20* C-C Motif Chemokine Ligand 2; *ST3GAL*, ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 1; *LBPI*, Lipopolysaccharide Binding Protein; *SLC7A9*, Solute Carrier Family 7 Member 9; *HMBS*, Hydroxymethylbilane Synthase.

Table 2. Growth performance of pigs fed a control diet (CO) or with the CO diet supplemented with 1.3 g formic acid/kg feed (LFA) or with 6.4 g formic acid/kg feed.

Item	Diet					Significance of contrasts	
	CO [a]	LFA [b]	HFA [c]	SEM	P-value	CO [a] vs LFA[b] and HFA[c]	LFA [b] vs HFA[c]
Body weight (g)							
day 0	6994	7186	7226	228.7	0.41	0.46	0.90
Average daily gain (g/day)							
day 0 – day 21	247.8	286.0	267.8	7.27	0.004	0.004	0.09
Day 22 – day 42	699.1	716.00	727.6	25.7	0.73	0.46	0.74
Day 0 - day42	475.2	502.3	500.1	15.1	0.39	0.18	0.92
Average daily feed intake (g/day)							
Day 0 – day 21	320.7	350.5	333.9	9.5	0.10	0.08	0.23
Day 22 – day 42	1058.7	1079.0	1094.3	40.00	0.82	0.57	0.78
Day 0 - day42	692.6	716.9	717.9	22.9	0.68	0.39	0.985
Feed to gain (g/day)							
Day 0 – day 21	1.31	1.23	1.25	0.03	0.17	0.075	0.61
Day 22 – day 42	1.51	1.51	1.51	0.02	1.00	1.00	0.88
Day 0 - day42	1.45	1.43	1.44	0.02	0.70	0.44	0.77

[a] CO: Control diet (no addition of formic acid);

[b]LFA: Low formic acid (1.4 g formic acid/kg of feed);

[c]HFA: High formic acid (6.4 formic acid/kg of feed).

Table 3. Effect of long-term formic acid supplementation on the alpha-diversity index of the microbiota of the jejunum of post-weaned piglets.

Index	Diet					Significance of contrasts**		
	CO [a]	LFA [b]	HFA [c]	SEM	P-value*	CO [a] vs LFA[b]	CO [a] vs HFA[c]	LFA[b] vs HFA[c]
Chao1	221.88	271.06	278.08	10.76	0.04	0.14	0.04	0.78
PD_whole_tree	9.12	10.74	10.7	0.42	0.23	0.27	0.3	1

[a] CO: Control diet (no addition of formic acid);

[b] LFA: Low formic acid (1.4 g formic acid /kg of feed);

[c]H FA: High formic acid (6.4 formic acid /kg of feed).

*ANOVA P-value

**Tukey test, Benjamini-Hochberg adjusted P-value.

Table 4. Effect of long-term formic acid supplementation on jejunal genera-aggregated 16S rRNA data of post-weaned piglets.

Treatment comparison	Genus	Number of samples in COa	Number of samples in LFA[c]	Abundanced group CO[a]	Abundanced group LFA[b]	P value	adj.P valuee
CO[a] vs LFA[b]	Gemella	10	12	0.043	0.038	0.002	0.03
	Lactobacillus	10	12	0.416	0.413	<.000	<.000
	Parvimonas	8	12	0.016	0.009	0.003	0.034
	Streptococcus	10	12	0.185	0.165	<.000	<.000
	Turicibacter	6	7	0.007	<.000	0.005	0.045
Treatment comparison	Genus	Number of samples in CO[a]	Number of samples in HFA[c]	Abundanced group CO[a]	Abundanced group HFA[c]	P value	adj.P value[e]
CO[a] vs HFA[c]	Acinetobacter	7	10	0.016	0.004	0.003	0.028
	Fusobacterium	8	10	0.007	0.002	0.007	0.049
	Gemella	10	12	0.043	0.018	0.001	0.012
	Lactobacillus	10	12	0.416	0.315	<.000	<.000
	Leuconostoc	8	10	0.001	<.000	0.003	0.024
	Parvimonas	8	11	0.016	0.003	0.001	0.012
	Streptococcus	10	12	0.185	0.212	<.000	<.000

[a] CO: Control diet (no addition of formic acid);

[b]LFA: Low formic acid (1.4 g formic acid/kg of feed);

[c]HFA: High formic acid (6.4 formic acid/kg of feed);

[d]Data are expressed as OTU means of relative abundance;

[e]Adjusted P value by FDR correction.

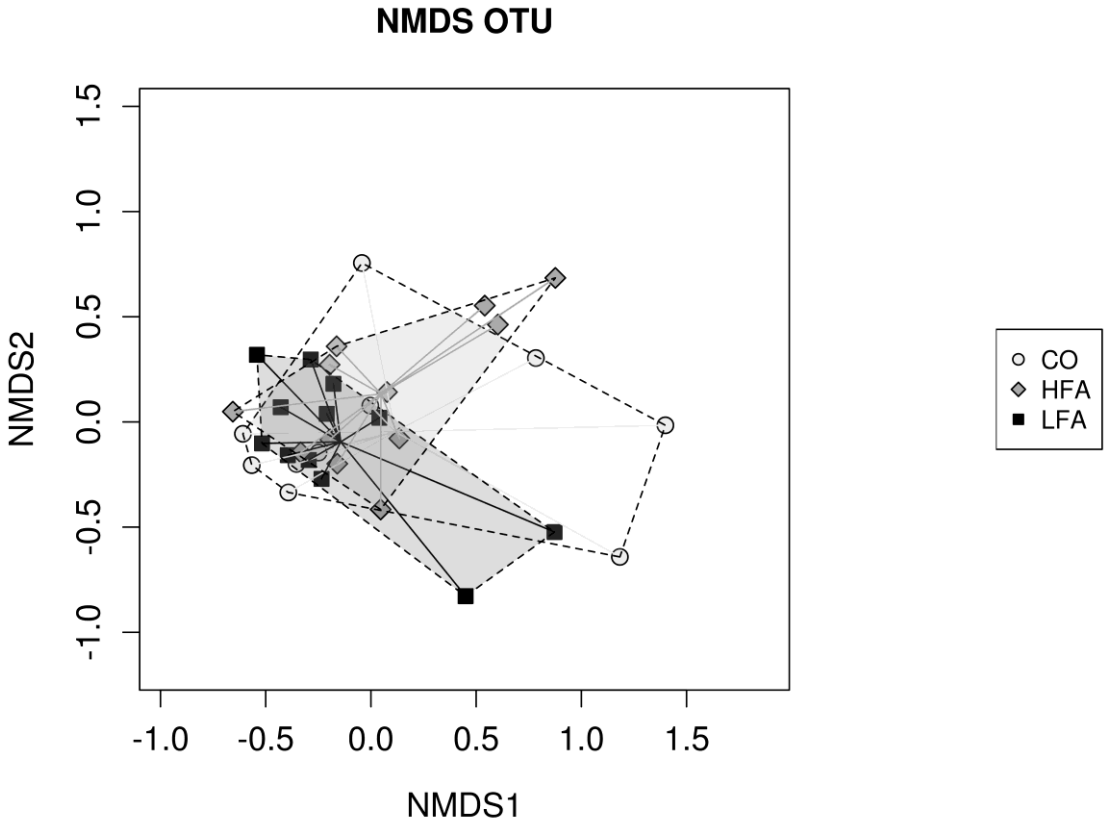

Figure 1. Non metric multidimensional scaling (NMDS) plot of Beta diversity (Bray-Curtis distance matrix) in jejunum content of pigs fed with diets supplemented with 0, 1.4 g and 6.4 g of formic acid per kg of feed (CO, LFA, HFA, respectively). In the NMDS plot, each point represents the gut microbiota of a pig and grayscale and shapes visualize each diet.

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Supplementary Table 1. Effect of long-term formic acid supplementation on OTUs relative abundance in the jejunum of post-weaned piglets.

A. Significant differenced OTUs in jejunal mucosal content between CO and LFA groups.

Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group CO[a]	Number of samples in group LFA[b]	OTUs counts in CO[a]	OTUs counts in LFA[a]	logFC[e]	*P* value	adj.*P* value[f]
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	NA	NA	8	12	246	138	4.16	2.03E-06	2.49E-05
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	NA	7	11	76	107	2.71	5.25E-03	2.29E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	8	72	36	3.17	5.02E-04	3.58E-03
	Bacteria	Firmicutes	Bacilli	Gemellales	Gemellaceae	Gemella	NA	10	12	3936	4096	3.95	3.08E-07	4.44E-06
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	NA	NA	7	11	306	234	2.42	6.56E-03	2.80E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	1437	1912	4.14	8.26E-08	1.54E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	minor	9	10	485	492	3.71	1.53E-05	1.70E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	918	1408	2.35	2.30E-03	1.22E-02
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	NA	8	10	142	121	3.36	1.13E-04	1.08E-03
	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	NA	NA	8	11	445	468	3.38	1.64E-04	1.42E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	10	1423	2279	7.9	1.26E-17	1.21E-15
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus	NA	6	9	610	398	2.83	3.14E-03	1.58E-02
CO[a] vs LFA[b]	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	1992	2449	3.73	1.42E-06	1.82E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	10	5919	2970	10.6	2.39E-35	6.90E-33
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	6	7	52	20	2.78	5.53E-03	2.38E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	7	12	123	1690	2.53	4.02E-03	1.87E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	12	548	854	2.98	1.50E-04	1.36E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	7	9	20	32	2.63	4.99E-03	2.24E-02
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella	dispar	7	11	142	196	3.35	2.53E-04	2.00E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	12	814	1275	4.16	3.88E-07	5.47E-06
	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA	7	9	382	187	2.9	9.98E-04	6.37E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	2826	3895	4.66	1.71E-09	5.80E-08
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	12	538	852	6.33	2.03E-14	1.67E-12
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	7	11	2233	640	4.54	1.92E-07	3.14E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	6	10	345	350	4.14	2.35E-05	2.56E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	9	10	144	140	3.04	4.10E-04	3.11E-03

Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group CO[a]	Number of samples in group LFA[b]	OTUs counts in CO[a]	OTUs counts in LFA[b]	logFC[e]	*P* value	adj.*P* value[f]
CO[a] vs LFA[b]	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	12	208	372	3.97	1.25E-06	1.64E-05
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	NA	NA	10	12	849	750	4.31	2.50E-08	6.28E-07
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	NA	8	10	96	96	2.9	1.74E-03	1.00E-02
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	NA	NA	6	8	1555	2434	2.89	3.42E-03	1.67E-02
	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA	7	11	243	194	4.85	2.08E-07	3.24E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	10	12	13456	1570	11.04	9.42E-46	5.44E-43
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	9	228	264	5.64	8.04E-12	3.86E-10
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	10	12	17793	32665	9.16	3.77E-32	7.25E-30
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	8	11	109	40	3.41	6.62E-05	6.58E-04
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	NA	7	10	933	226	2.83	1.18E-03	7.34E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA	8	12	1017	359	4.45	2.50E-08	6.28E-07
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	7	10	3900	275	8.13	2.18E-21	3.14E-19
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	12	204	1883	2.23	1.15E-02	4.59E-02
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Parvimonas	NA	8	12	1431	1012	4.77	4.49E-08	1.04E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	12	139	227	2.23	5.11E-03	2.25E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	mucosae	10	12	338	554	2.55	9.75E-04	6.32E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA	6	9	72	103	3.24	1.14E-03	7.12E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	NA	NA	6	8	198	96	4.05	1.17E-04	1.11E-03

B. Significant differenced OTUs in jejunal mucosal content between CO and HFA groups.

Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group CO[a]	Number of samples in group HFA[c]	OTUs counts in CO[a]	OTUs counts in HFA[c]	logFC[e]	*P* value	adj.*P* value[f]
CO[a] vs HFA[c]	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	NA	NA	8	12	246	241	3.53	3.78E-05	3.76E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	9	72	61	2.77	1.76E-03	1.05E-02
	Bacteria	Firmicutes	Bacilli	Gemellales	Gemellaceae	Gemella	NA	10	12	3936	1966	4.53	2.39E-09	8.10E-08
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	NA	NA	8	12	868	919	2.56	1.55E-03	9.54E-03
	Bacteria	Firmicutes	Bacilli	Bacillales	Planococcaceae	NA	NA	7	12	306	263	2.39	6.01E-03	3.07E-02

Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group CO[a]	Number of samples in group HFA[c]	OTUs counts in CO[a]	OTUs counts in HFA[c]	logFC[e]	*P* value	adj.*P* value[f]
CO[a] vs HFA[c]	Bacteria	Proteobacteria	Gammaproteobacteria	Pseudomonadales	Moraxellaceae	Acinetobacter	rhizosphaerae	6	8	133	139	2.76	3.80E-03	2.03E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	1437	2456	3.39	7.93E-06	9.94E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	minor	9	11	485	512	4.03	1.39E-06	2.16E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	918	1568	2.27	2.77E-03	1.57E-02
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Actinomycetaceae	Actinomyces	NA	8	11	142	194	3.48	3.95E-05	3.86E-04
	Bacteria	Proteobacteria	Alphaproteobacteria	Rickettsiales	mitochondria	NA	NA	8	10	445	355	4.04	5.32E-06	7.14E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	11	1423	3395	8.67	5.96E-22	8.60E-20
	Bacteria	Proteobacteria	Betaproteobacteria	Burkholderiales	Oxalobacteraceae	Cupriavidus	NA	6	7	610	506	3.27	6.44E-04	4.59E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	1992	3883	2.97	9.28E-05	8.50E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	5	7	268	523	5.95	3.36E-09	1.08E-07
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	5	11	51	68	3.44	3.96E-04	2.97E-03
	Bacteria	Chlamydiae	Chlamydiia	Chlamydiales	Chlamydiaceae	Chlamydia	NA	5	6	17	320	-2.49	9.16E-03	4.44E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	7	77	21	5.16	9.14E-08	1.95E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	8	10	5919	4632	10.67	6.61E-37	1.27E-34
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	6	8	52	20	2.87	3.23E-03	1.78E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	7	10	123	303	4.12	2.40E-06	3.55E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	12	548	724	3.94	3.37E-07	5.90E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	7	148	51	6.24	8.21E-11	3.95E-09
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	5	7	347	22	7.9	2.87E-16	2.76E-14
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	7	7	20	18	2.95	1.66E-03	9.98E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	Veillonellaceae	Veillonella	dispar	7	11	142	231	3.48	1.07E-04	9.48E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	12	814	1197	4.93	9.33E-10	3.59E-08
	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA	7	8	382	81	2.97	6.93E-04	4.88E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	10	12	2826	2857	4.77	3.49E-10	1.55E-08
	Bacteria	Firmicutes	Erysipelotrichi	Erysipelotrichales	Erysipelotrichaceae	NA	NA	6	7	76	22	3.31	3.13E-04	2.41E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	11	538	580	7.17	2.14E-18	2.47E-16
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	7	10	2233	403	6.17	7.78E-13	5.61E-11
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	6	6	345	155	5.82	4.41E-09	1.34E-07

	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Lactococcus	NA	7	10	360	283	2.97	8.62E-04	5.92E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	9	12	144	193	2.54	2.19E-03	1.27E-02
Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group CO[a]	Number of samples in group HFA[c]	OTUs counts in CO[a]	OTUs counts in HFA[c]	logFC[e]	*P* value	adj.*P* value[f]
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	9	12	208	243	4.52	1.90E-08	5.48E-07
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Micrococcaceae	NA	NA	10	12	849	1038	4.08	7.55E-08	1.74E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	Leuconostoc	NA	8	10	96	40	3.78	3.13E-05	3.35E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	6	27	11	3.23	1.63E-03	9.92E-03
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	NA	NA	6	6	1555	1347	4.32	1.35E-05	1.62E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	8	8	112	51	4.72	2.11E-07	3.93E-06
	Bacteria	Fusobacteria	Fusobacteriia	Fusobacteriales	Fusobacteriaceae	Fusobacterium	NA	7	8	243	115	5.16	3.86E-08	1.01E-06
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Streptococcaceae	Streptococcus	NA	5	9	198	448	4.75	6.19E-07	1.02E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	10	12	13456	4348	9.74	2.64E-37	7.61E-35
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	10	228	50	6.12	2.36E-14	1.95E-12
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	10	12	17793	24388	10.58	1.35E-43	7.81E-41
CO[a] vs HFA[c]	Bacteria	Proteobacteria	Gammaproteobacteria	Pasteurellales	Pasteurellaceae	Actinobacillus	porcinus	5	9	99	64	3.88	4.87E-05	4.68E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	7	8	89	29	3.97	6.25E-06	8.01E-05
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	7	12	3900	9066	5.9	1.09E-12	6.96E-11
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	12	204	298	3.58	3.49E-05	3.59E-04
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Tissierellaceae]	Parvimonas	NA	8	11	1431	384	5.69	3.71E-11	1.95E-09
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	5	7	12	21	2.65	7.00E-03	3.48E-02
	Bacteria	Proteobacteria	Gammaproteobacteria	Xanthomonadales	Xanthomonadaceae	NA	NA	5	9	95	70	3.29	5.92E-04	4.32E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	9	11	139	166	2.9	2.36E-04	1.89E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	mucosae	10	12	338	294	3.68	1.30E-06	2.08E-05
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	NA	NA	6	7	72	63	2.93	3.27E-03	1.78E-02
	Bacteria	Firmicutes	Clostridia	Clostridiales	[Mogibacteriaceae]	NA	NA	6	6	198	107	3.79	3.05E-04	2.37E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	Lachnospiraceae	Coprococcus	NA	7	12	177	190	2.34	6.48E-03	3.28E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	5	7	63	14	3.65	6.32E-04	4.56E-03

C. Significant differenced OTUs in jejunal mucosal content between HFA and LFA groups.

Treatment comparison	Kingdom	Phylum	Class	Order	Family	Genus	Species	Number of samples in group LFA[b]	Number of samples in group HFA[c]	OTUs counts in LFA[b]	OTUs counts in HFA[c]	logFC[e]	*P* value	adj.*P* value[f]
LFA[b] vs HFA[c]	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	6	523	12	3.54	1.65E-11	1.59E-09
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	10	12	303	1690	-1.59	2.23E-04	3.36E-03
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Propionibacteriaceae	Propionibacterium	NA	6	6	47	89	-1.43	5.10E-03	3.59E-02
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	10	11	403	640	-1.63	1.86E-04	2.90E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	6	10	155	350	-1.68	4.91E-04	6.03E-03
	Bacteria	Proteobacteria	Alphaproteobacteria	Rhizobiales	Phyllobacteriaceae	NA	NA	6	8	1347	2434	-1.43	3.50E-03	2.80E-02
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	NA	8	9	463	1135	-2.01	3.61E-05	8.33E-04
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	12	12	4348	1570	1.3	8.78E-04	9.56E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	12	12	24388	32665	-1.42	2.71E-04	3.63E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	10	11	216	40	1.75	3.96E-05	8.80E-04
	Bacteria	Actinobacteria	Actinobacteria	Actinomycetales	Corynebacteriaceae	Corynebacterium	NA	11	10	1129	226	1.27	4.05E-03	3.16E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Leuconostocaceae	NA	NA	6	6	587	5252	-4.36	5.01E-16	9.64E-14
	Bacteria	Firmicutes	Clostridia	Clostridiales	NA	NA	NA	12	12	7156	359	2.71	7.58E-11	6.25E-09
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	reuteri	8	10	29	326	-1.78	6.92E-05	1.37E-03
	Bacteria	Firmicutes	Clostridia	Clostridiales	Clostridiaceae	NA	NA	12	10	9066	275	2.23	2.73E-07	1.21E-05
	Bacteria	Proteobacteria	Gammaproteobacteria	Enterobacteriales	Enterobacteriaceae	NA	NA	8	11	2004	457	2.21	8.68E-07	3.13E-05
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	12	12	298	1883	-1.35	1.51E-03	1.47E-02
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	NA	7	6	21	169	-1.98	8.26E-05	1.49E-03
	Bacteria	Firmicutes	Bacilli	Lactobacillales	Lactobacillaceae	Lactobacillus	mucosae	12	12	294	554	-1.13	3.82E-03	3.02E-02

[a] CO: Control diet (no addition of formic acid);

[b]LFA: Low formic acid (1.4 g formic acid/kg of feed);

[c]HFA: High formic acid (6.4 formic acid/kg of feed);

[d]Data are expressed as OTU means of relative abundance;

[e]logFC: log2 fold change

[f]Adjusted P value by FDR correction.

Weaning transition and A0 blood types effect on faecal bacterial community of piglets

Abstract

The host-gut microbiota interplay is well recognized as a key factor for the homeostatic maintenance, for the pathological events control and for growth performances of the animals. The weaning transition represents a moment of drastic changes, which also have a strong impact on the gut microbial community leading to a high risk of dysbiotic events. The adhesion of bacteria on intestinal mucosa is mediated by molecules which compose the glycocalyx on epithelium surface and which act as specific receptors determining the structure of the mucosal bacterial community. Some of these receptors are the mucosal blood type antigens which are genetically determined in the host. The association between ABO blood groups and intestinal microbial profile has been tested in human with contrasting results. For the pig there are no studies on the relationship between blood groups and gut microbiota, however, in our previous study we reported some differences in the glycomic pattern of the jejunal mucosa and in the adhesion of *E. coli*, associated with the porcine blood groups A0. In the present study we followed the changes in faecal microbiota of piglets from the lactation to 2 weeks after weaning testing the hypothesis that the blood types may impact on its structure. No differences were reported for the A0 blood types. The metagenomic predictions revealed a shift from fatty acid degradation to fatty acid biosynthesis in bacterial community between pre- and post-weaning.

Introduction

Microbiota stability and colonization of the gastrointestinal tract (GIT) has a crucial role in preserving the host homeostasis and health (Wacklin et al., 2014). Nevertheless, GIT bacterial microbiota is variable and exposed to changes based on host genotype, age, exposure to microbes, diet, and many

other factors (Kim et al., 2011; Parks et al., 2013). In addition, the shaping of the early GIT microbiota by factors such as diet and genetic selection may impact the host's growth performance, immune response and the susceptibility to gastrointestinal disorders such as post-weaning diarrhea, which are key points for animal health and for productive outcomes in swine production (Dou et al., 2017; Mach et al., 2015).

The diet seems to be the most important factor affecting the gut microbiota in the short period, but the recent findings on the resilience of the microbiota or part of them, reinforce the idea that other factors can drive the microbiota settlement in the gut.

The modification of the gut microbiota is a dynamic event also driven by the continuous cross-talk between the host and the microbiota and it can be modulated by the presence of specific glycoprotein motifs in intestinal mucosa.

Since the detection of ABO blood groups, there has been a constant interest in its potential role in host susceptibility to infectious diseases in human. Indeed, differences on ABO blood groups can affect also in other tissues the antigen expression, which particularly could operate as receptor or co-receptor for bacteria in the intestinal mucus layer (Cooling, 2015). The association between ABO blood group variability and intestinal microbial profile has been analyzed in previous studies on human with contrasting results (Davenport et al., 2016; Wacklin et al., 2014). In pigs, contrary to human, the orthologous blood groups system consists of only one antigen (A) and two blood types (0, A); the 00 individuals express the H antigens which is the precursor, while the immunodominant structures of A (GalNAc α 1-3 (Fuc α 1-2) galactose) antigens characterize the A pigs (Coddens et al., 2009). In our previous experiences, the porcine A0 groups affected the jejunal mucosa glycomic pattern of young pigs infected or not with *Escherichia coli* F4 (ETEC), suggesting a role of A0 on microbiota colonization (Priori et al., 2016). However, very little is still known about A0 influences on bacteria community in pigs.

The aims of the present study are: i) test the hypothesis that the genotypes for A0 blood groups impact on piglets' fecal bacterial community long some crucial moments such as suckling and weaning. ii)

enrich the knowledge about the development of the early microbiota in piglets, contributing to identify potential key points in its shaping.

Material and Methods

The procedures were conducted in compliance with Italian laws on experimental animals and approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, ID number 704.

Animals and Sampling

Several sows were screened for the A0 genotype. Two multiparous sows with A0 and two with 00 genotypes were selected. For each sow, three female piglets with blood group genotype identical to the mother were chosen and followed until 2 weeks post-weaning. To limit the impact of other confounding factors, the sows and their litters were reared in the same batch during the lactation period, the same creep feed was provided after the second fecal sampling (day 14), and at the weaning (day 28) the piglets were moved to the same box. The room temperature was controlled and the access to water was guaranteed ad libitum.

From each piglet fecal-swabs were collected at day 7 (tI), 14 (tII) after birth and 2 weeks after weaning (tIII). Individual fecal samples were also collected by fecal-swab from the sows in the pre-weaning period (tI and tII) - in which the piglets were still breastfed and in contact with the mother's feces - in order to have a "maternal reference" microbiota.

The samples were immediately frozen in liquid nitrogen and stored at -80°C until use.

Blood groups genotyping

The porcine DNA was extracted from bristles, in brief: the bristle bulbs were incubated in Proteinase K solution (10 mg/mL of proteinase K in buffer [20 mM Tris HCl (pH 8.4), 50 mM KCl]) for two hours at 50°C, then the proteinase was inactivated at 95 °C, the samples were briefly spun in a microcentrifuge, the solution was transferred to a new tube and stored at -20°C until use. The multiplex PCR for A0 blood groups identification was performed as described in Nguyen et al.

(Nguyen et al., 2011), using the primers reported in supplementary file S1.

Bacterial DNA extraction and sequencing

Bacterial DNA was isolated and extracted with FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA) following the manufacturer's instructions. Quality and purity of the isolated DNA were checked by spectrophotometry on the NanoDrop (Fisher Scientific, 13 Schwerte, Germany).

The V3-V4 hypervariable region of the 16S rRNA gene amplicons was sequenced on MiSeq platform (Illumina Inc., San Diego, Ca, USA).

Bioinformatics & Biostatistics

Two piglet samples (one for genotype A0 and one for genotype 00 from timepoint I) were exclude from analysis for insufficient yield in sequencing process. The reads from remaining 42 samples were analyzed using subsampled open reference OTU strategy in QIIME v1.9.1 (Caporaso et al., 2010). The paired-end reads were merged, demultiplexed and quality filtered with a cutoff of Q20. Subsampled open-reference OTU-picking strategy was performed using uclust with 97% sequence similarity. The chimeric sequences were identified and removed using the "Blast_fragments approach". The representative sequences were assigned taxonomy against the Greengenes database V13_8 using the uclust with a 90% confidence threshold and the low count OTUs were removed with a threshold of 0.005% (Bokulich et al., 2013).

Finally, in order to infer the functional profile of the bacterial community, the OTU table was used to perform metagenome prediction in PICRUSt 1.1.0 (Langille et al., 2013). Briefly, starting from the open reference OTU table constructed in QIIME a new closed reference OTU table was generated using the gg_13_5_97 greengenes database as reference, the OTU table was normalized for 16S rRNA gene copy number and the metagenome functional prediction was obtained applying "predict_metagenomes" procedure, then, the "metagenome_contribution" procedure was applied to determine the OTUs contributing to particular functions.

The QIIME OTU table was imported in R 3.3.2 for the ecological parameters evaluation. The

variability within bacterial communities (alpha diversity) was assessed with the Shannon index in "phyloseq" package (McMurdie et al., 2013) and the effect of genotype and litter were tested with mixed model in "nlme" package fitting the models reported in supplementary file S1. The differences among bacterial communities (beta diversity) were assessed by Bray-Curtis distance matrices in "vegan" package (Oksanen et al., 2016), and plotted with Non Metric Multidimensional Scaling (NMDS), the effects of genotype, litter and time were tested with *adonis* procedure implemented in the same package fitting the models reported in supplementary file S1. In order to test taxonomic differential abundances the family aggregated data were normalized by cumulative sum scaling approach and analysed in MetagenomeSeq package (Paulson et al., 2013), the effect of Genotype was tested with the procedure for longitudinal data fitTimeseries (Paulson et al., 2017) and the fit-zig model implemented in the same package was used for the other pairwise contrast.

The level of significance was defined by p values (P) <0.05, for multiple comparison the Benjamini & Hochberg correction was applied (Padjust).

For the metagenomic predictions we focused our attention on the functional changes in microbial community of piglets, we analyzed the pathway aggregated data (level3) in order to have a general vision on the metabolic shift in bacterial community, then, the entire dataset of KEGG Orthology genes (Kos) was tested to have a deep resolution within the pathways.

The difference for Pathway aggregated data were tested in STAMP software with Welch's test, the level of significance was defined by Padjust <0.05. The table with predicted KOs was imported in R 3.3.2 and the differential abundances of KOs between pre and post-weaning period were assessed in "DeSeq2" package using the Wald test, the significance was defined by Padjust < 0.01, the resulting differences were plotted in a metabolic map with iPath2 (Yamada et al., 2011) application excluding the pathway conflicts.

The raw reads from the 42 samples analysed are deposited at the EBI Short Read Archive (SRA) under the study accession number ERP105637.

Results

Alfa and Beta diversity

A total of 3,445,968 reads were attributed to 1,439 total OTUs distributed among samples as shown in table 1**,** the relative rarefaction curves are reported in Supplementary Figure S1. The data show an increasing alpha diversity in piglet samples along the time (lm, r^2= 0.73, P<0.001), with after weaning values (timepoint III) comparable to the alpha diversity observed in sows microbial community (Figure 1). No significant differences were reported for genotype and litter factors on alpha diversity in piglets (Supplementary file S1). Concerning the beta diversity the evident variation of bacterial community over the time according with the age of the animals was observed (Figure 2) (adonis r^2=0.42, P= 0.001) but no significant differences were reported for the genotype and litter factors (Supplementary file 1).

Taxonomic composition

We found that the large part of the reads - in average 53% in sows and 41% in piglet samples - was not classified at the genus level, on the contrary the family level showed a better coverage -92% of reads in piglets and 71% of reads in sows samples- in taxonomic classification of reads (Supplementary figures S2-S4) and represents a good compromise to associate the taxonomic composition of a bacterial community with roles taxa-associated in bacterial ecosystem.

Furthermore, the beta diversity on family aggregated data revealed a pattern compatible with that observed at OTUs level (Supplementary figure S5). A total of 40 families were identified, for 30 of these differential abundances were reported between pre- and post-weaning period (Padjust <0.05), whereas, 17 families were found differentially abundant between bacterial community of weaned piglets and sows (Figure 3), and only 12 non-dominant families were differentially abundant between timepoint I and timepoint II in piglets (Supplementary figure S6), indicating that weaning causes the most significant change in the bacterial community. The fit timeseries test did not show significant differences for the genotype factor. The major shift (in term of relative abundances) due to the

weaning concerned the increase in Prevotellaceae (1.5% pre-weaning; 27% post-weaning) and the decrease of Bacteroidaceae (25% pre-weaning,; 0.08% post-weaning) and Enterobacteriaceae (15% pre-weaning; 0.05% post-weaning) families (supplementary figure S7). For the comparison between the faecal microbiota of weaned piglets (timepoint III) and that of the adult pigs (Sows), the principal changes in dominant families concerned a greater presence of Spirochetaceae (11.26% sows; 1.20% weaned piglets) and Ruminococcaceae (16.94% sows; 10.75% weaned piglets) in sows, conversely Prevotellaceae (27% weaned piglets; 5.10% sows) and Lachnospiraceae (8.35% weaned piglets; 1.63% sows) resulted with greater abundances in weaned pigs respect to the sows (supplementary figure S8).

Metagenomic prediction

In order to analyse the shift in metabolic potential of bacterial community, we focused our attention on differences related to the weaning transition in piglets. A total of 232 level3 KEGG pathways were reported, 165 of these revealed significant difference (Welch's T test, Padjust<0.05) between pre- and post-weaning, on the contrary, only eight pathways (most of which are not related to bacterial metabolisms) showed significant differences between timepoint I and II (Supplementary figure S9), confirming the major shift between pre- and post- weaning. No significant differences were reported between the two genotypes in the different timepoints.

Among the pathways data it is possible to note that the genes transcribing for proteins related to the fatty acids and galactose metabolisms are the most represented in the microbial communities during the lactation phase, whereas, in the post-weaning, an increase in those related to starch and sucrose metabolism is noted (Supplementary figures 10-12). To better dissect the effect of weaning within the pathways we analysed the entire set of KOs genes identified (4,697 KOs) testing for differences between pre- and post-weaning: 3,018 KOs reported significant differences (Padjust<0.01); 1,152 KOs mapped successfully in iPath2 maps and 406 of these belonged to the central metabolic pathway map (Supplementary Figure S13).

The representation through the central metabolic pathway map allowed us to visualize and isolate an

interesting pattern within the lipid metabolism (Figure 4) among this large dataset. The difference shown in figure 4 were determined by KOs associated to fatty acid degradation (*fad*), which were enriched in pre-weaning microbiome, and by KOs associated to fatty acid biosynthesis (*fab*) which were enriched in post-weaning microbiome (table 2). The identification of the OTUs contributing to these functions showed that Enterobacteriaceae is the family mainly involved in fatty acid degradation whereas Prevotellaceae, Lachnospiraceae and Ruminococcaceae are the main contributors to the fatty acid biosynthesis (Figure 4).

Discussion

In our previous work we showed the glycomic shift in the jejunal mucosa after the exposure to ETEC strains and we reported the influence of the porcine A0 blood group genotype in these changes, suggesting how the pig genetic background affecting the glycocalyx sugar motif may be relevant in the cross-talk between intestinal mucosa and bacterial community (Priori et al., 2016).

In the present study, the development of faecal bacterial community from suckling to weaned piglets was investigated taking in to account the potential effect of the porcine A0 blood type system.

The results clearly showed the dynamics of the modification in the faecal microbiota during weaning transition, however, no evidences were reported for the influence of the blood types on bacterial community structure as well as in its specific taxa abundances. Furthermore, the absence in literature of studies specifically focused on the blood groups/gut microbiota relations in pigs and the opposite conclusions reached by the two major studies concerning the association between ABO system and gut microbiota in humans (Davenport et al., 2016; Mäkivuokko et al., 2012) make the subject of study open to further insights. Nevertheless, we may speculate that changes in the mucosal glycomic pattern (proximal intestine) associated with the blood group genotypes may affect specific bacterial groups (Cooling, 2015) but does not lead to changes that can affect the whole gut microbial community with such magnitude to be detectable by analysing the faecal microbiota (Rangel et al., 2015). Alternatively, it is also possible that in a given population of piglets obtained from gestating-lactating

sows and presenting different genotypes related to the glycomic profile but cohabiting in the same farm environment, the best adapting way for the gut microbiota is to present the ability to grow with no dependence of one specific intestinal sugar motif. In other term the presence of cooperative strains and the functional redundancy in the gut microbiota of a pig population may counterbalance the advantage of increased competitiveness, associated to the better fitness of certain microbes to a single genotype (Foster et al., 2017).

We also tested the effect of the litter, on alpha and beta diversity of the bacterial communities, which can be a confounding factor (co-housing, maternal effect) in microbial community studies, but no significant differences associated with the litter effect were reported. The absence of this effect in previous studies was associated with the prevalence of stochastic factors in shaping the structure of early bacterial communities (Schloss et al. 2012; Mach et al. 2015).

Focusing the attention on the adaptation to weaning transition, evident changes in bacterial community were reported. In line with the literature (Frese et al., 2015a; Kim et al., 2011; Mach et al., 2015), the alpha diversity values showed an increasing trend reaching values (timepoint III) comparable to that of the adult pigs microbiota (sows). The increasing values in alpha diversity are considered as marker for a mature microbial community (Chen et al., 2017); are associated to functional redundancy, which contributes to a greater stability of the microbial ecosystem in contrasting stressful events that may lead to dysbiotic conditions (Konopka, 2009). More generally, a greater variability within communities is positively correlated with the health status of the host (Le Chatelier et al., 2013). It is interesting to note that in a recent study on miniature piglets (Hu et al., 2016) the alpha diversity decreased after weaning. As suggested by Chen (Chen et al., 2017) this may be due to the weaning age. Indeed, in the Hu's study, the piglets were weaned at 21 days of life (vs 28 days in our study). In general a greater weaning stress is associated with an earlier weaning age, thus this indicates that a more intense weaning stress may adversely affect the stability of the microbial community that is not yet 'mature' enough to face the new ecological conditions, promoting the proliferation of opportunistic pathobiont which can lead to typical disorders such as post-weaning

diarrhoea (Rhouma et al., 2017).

The dynamic pattern of the inter-individual variation in bacterial community is well represented by the beta diversity results: the samples are clearly clustered for timepoint and increased distances among individuals in timepoint II (day 14) are observed which then converge to timepoint III (post-weaning) showing a greater uniformity among the microbial communities of the different individuals. The same pattern in beta diversity is shown in a larger longitudinal study (Bian et al., 2016), this variation may reflect the interindividual difference in intestinal maturation during the lactation period, which settles in the late post-weaning phase (Montagne et al., 2007) allowing the establishment of a climax community (Isaacson and Kim, 2012).

Regarding the taxonomic shift, we found that the weaning transition is mainly characterized by a drastic reduction of Bacteroidaceae and Enterobacteriaceae paralleled by a dominance of Prevotellaceae in post-weaning, we also reported an increase in Lactobacilli in post-weaning but the differences were not significant. This shift has been highlighted by several studies and is generally correlated with the abrupt change from milk-based to cereal-based diet (Alain B Pajarillo et al., 2014; Frese et al., 2015; Mach et al., 2015). Studies on "milk-oriented microbiota" investigated the microbiome modifications in weaning transition focusing on the role of the sugar component of the diet. The rationale behind this hypothesis is that: the host, in proximal intestine, lacks metabolic capacity to completely digest the different glycans, these glycans reach the distal intestine shaping the microbial community composition, hence, the gut microbiome of suckling piglets shows metabolisms oriented to the milk oligosaccharides consumption, whereas bacteria able to degrade plant-derived carbohydrates like Prevotellaceae became dominant after weaning (Frese et al., 2015; Salcedo et al., 2016). This metabolic change was also reported by metagenomic prediction in our study, showing the decrease in “Galactose metabolism” and the increase in “Starch and sucrose metabolism” after weaning transition. In addition, the metagenomic predictions of our study also showed a shift in bacterial lipid metabolism during the weaning transition. In particular, the predicted fatty acids degradative (*fad*) enzymes were enriched in the microbial communities of suckling piglets

while the predicted fatty acid biosynthetic (*fab*) enzymes were enriched in the microbial communities of weaned piglets. It is known that the main source of energy in sow milk is fat, which mainly consists of long chain fatty acids (Le Dividich et al., 2005), it is also known that due to the lower pancreatic and intestinal lipase activities in the first part of the suckling period, the nursing piglet does not have a complete ability to digest fat (Gu and Li, 2003; Li et al., 2001); therefore, applying the same rationale used for glycans, we can hypothesize that un-digested fats can be used by intestinal bacteria capable of degrading fatty acids such as Enterobacteriaceae (Iram and Cronan, 2006). Conversely, the highest presence of enzymes involved in fatty acid biosynthesis (Cronan and Thomas, 2009), mainly due to Prevotellaceae, Ruminococcaceae and Lachnospiraceae in our study, can be linked to the highest fiber content in the post-weaning diet and to the ability to synthesize fatty acids by fermentation of complex carbohydrates of these bacteria. Although it is not easy to define the role of fats in microbial community modulation, there are some evidences that indicate the levels of fat in sow milk as one of the factors that may influence the composition of the faecal microbiota of the piglets (Bian et al., 2016). In addition, a recent study on germ-free mice inoculated with faecal microbiota from breast-fed infants showed that the administration of long chain fatty acid-rich emulsions resulted in an increase in Enterobacteriaceae, while the administration of medium chain fatty acid-rich emulsions resulted in an increase in Bacteroidaceae in faecal bacterial community of mice (Nejrup et al., 2017).

Furthermore, it has been reported that in *E. coli* the *fad* enzymes are induced by the long chain fatty acids but not by the short and medium chain fatty acids (Iram and Cronan, 2006) and that the genes fadJ and fadI, involved in anaerobic utilization of fatty acids, may play a key role in *E. coli* pathogenesis in environments with very low oxygen tensions (Campbell et al., 2003), therefore, the use of milk-derived long chain fatty acids as an energy source could represent an opportunistic strategy used by *E. coli* strains during the lactation period.

In conclusion, the present study has shown no evidence in favour of the hypothesis that the genotypes defining the porcine A0 blood groups can modulate the faecal bacterial community in piglets during

suckling and post-weaning phases. On the other hand, the data on detected bacterial metabolisms suggest a role of the fatty component of sow milk in the selection of Enterobacteriaceae in the microbiota of suckling piglets. Although supported by the literature, in the present study this hypothesis is based on metagenomic predictions, hence, specific studies aimed at testing it are needed.

Table1 Per sample metadata, sequencing yield, OTUs abundances and Shannon index values

Subject	Timepoint[a]	Category[b]	Genotype	Litter	Reads count	Observed OTUs	Shannon
216	I	p	AO	S2	47,878	394	3.04
211	I	p	AO	S2	63,472	466	3.80
212	I	p	AO	S2	59,272	456	3.90
227	I	p	AO	S3	136,943	486	4.32
224	I	p	AO	S3	69,831	311	3.42
237	I	p	OO	S1	100,801	369	3.80
236	I	p	OO	S1	115,145	384	3.19
254	I	p	OO	S4	126,739	485	3.46
257	I	p	OO	S4	42,252	325	3.64
256	I	p	OO	S4	51,381	368	3.74
211	II	p	AO	S2	80,263	534	3.77
212	II	p	AO	S2	95,286	464	3.78
216	II	p	AO	S2	88,498	539	3.68
231	II	p	AO	S3	94,577	533	3.73
224	II	p	AO	S3	44,729	594	3.80
227	II	p	AO	S3	68,069	665	4.64
238	II	p	OO	S1	94,572	467	4.04
237	II	p	OO	S1	52,949	597	4.58
236	II	p	OO	S1	73,944	487	4.17
256	II	p	OO	S4	107,435	426	4.08
254	II	p	OO	S4	110,306	343	3.00
257	II	p	OO	S4	40,715	325	3.46
216	III	p	AO	S2	60,494	734	4.77
211	III	p	AO	S2	87,581	732	4.61
212	III	p	AO	S2	52,175	602	4.57
231	III	p	AO	S3	58,497	786	5.01
224	III	p	AO	S3	61,965	832	4.96
227	III	p	AO	S3	65,919	766	4.81
237	III	p	OO	S1	69,650	837	5.13
238	III	p	OO	S1	95,203	923	5.07
236	III	p	OO	S1	66,477	750	4.88
256	III	p	OO	S4	66,259	879	5.18
254	III	p	OO	S4	94,765	863	5.03
257	III	p	OO	S4	120,230	846	5.30
135128	I	S	AO	S2	148,653	750	4.42
135130	I	S	AO	S3	135,259	777	4.88
135120	I	S	OO	S1	117,618	818	4.72
135136	I	S	OO	S4	77,230	753	4.86
135128	II	S	AO	S2	69,160	745	5.18
135130	II	S	AO	S3	62,834	683	5.02
135120	II	S	OO	S1	100,383	759	4.55
135136	II	S	OO	S4	70,559	725	4.42

[a] I= 7 days post-farrowing, II= 14 days post-farrowing, III= 14 days post-weaning;

[b] p= piglet; S= sow

Table 2 List of KOs involved in lipid metabolism significantly enriched in fecal bacterial community of weaned piglets (Post-Weaning) and suckling piglets (Pre-Weaning).

KO id	Gene	Module	Enzyme Definition	Group
K00645	fabD	M00082 (Fatty acid biosynthesis, initiation)	[acyl-carrier-protein] S-malonyltransferase	Post-Weaning
K09458	fabF	M00083 (Fatty acid biosynthesis, elongation)	3-oxoacyl-[acyl-carrier-protein] synthase II	Post-Weaning
K00059	fabG	M00083 (Fatty acid biosynthesis, elongation)	3-oxoacyl-[acyl-carrier protein] reductase	Post-Weaning
K01782	fadJ	M00087 (Fatty acid degradation, beta-Oxidation)	3-hydroxyacyl-CoA dehydrogenase / enoyl-CoA hydratase / 3-hydroxybutyryl-CoA epimerase	Pre-Weaning
K01825	fadB	M00087 (Fatty acid degradation, beta-Oxidation)	3-hydroxyacyl-CoA dehydrogenase / enoyl-CoA hydratase / 3-hydroxybutyryl-CoA epimerase / enoyl-CoA isomerase	Pre-Weaning
K00632	fadA, fadI	M00087 (Fatty acid degradation, beta-Oxidation)	acetyl-CoA acyltransferase	Pre-Weaning

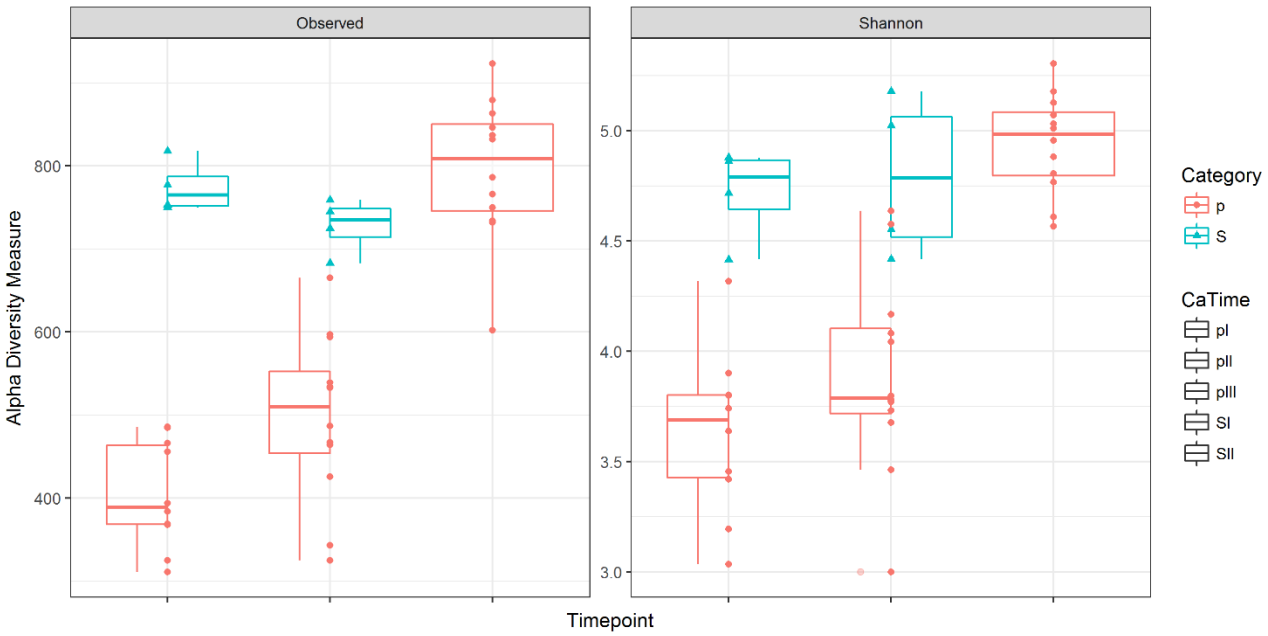

Figure 1: box plot charts of observed OTUs abundances and Shannon index values for piglets and sows samples at the different timepoints.

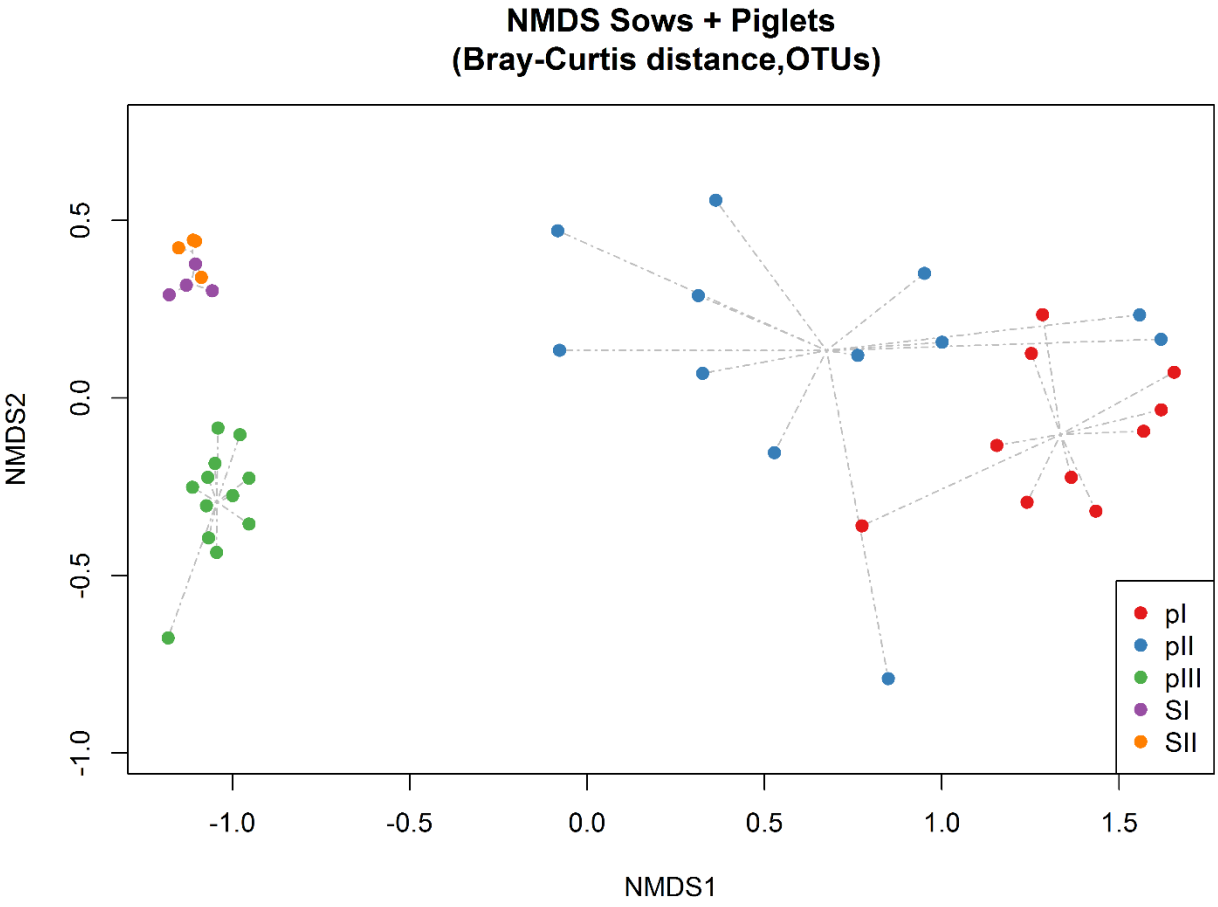

Figure 2: Non-metric multi dimensional scaling (NMDS) on Bray-Curtis distances at OTUs level. pI= piglets timpoint I (day 7 post farrowing), pII= piglets timepoint II (day 14 post farrowing), pIII= piglets timepoint III (day 14 post weaning), SI= sows timepoint I (day 7 post farrowing) SII= sows timepoint II (day 14 post farrowing).

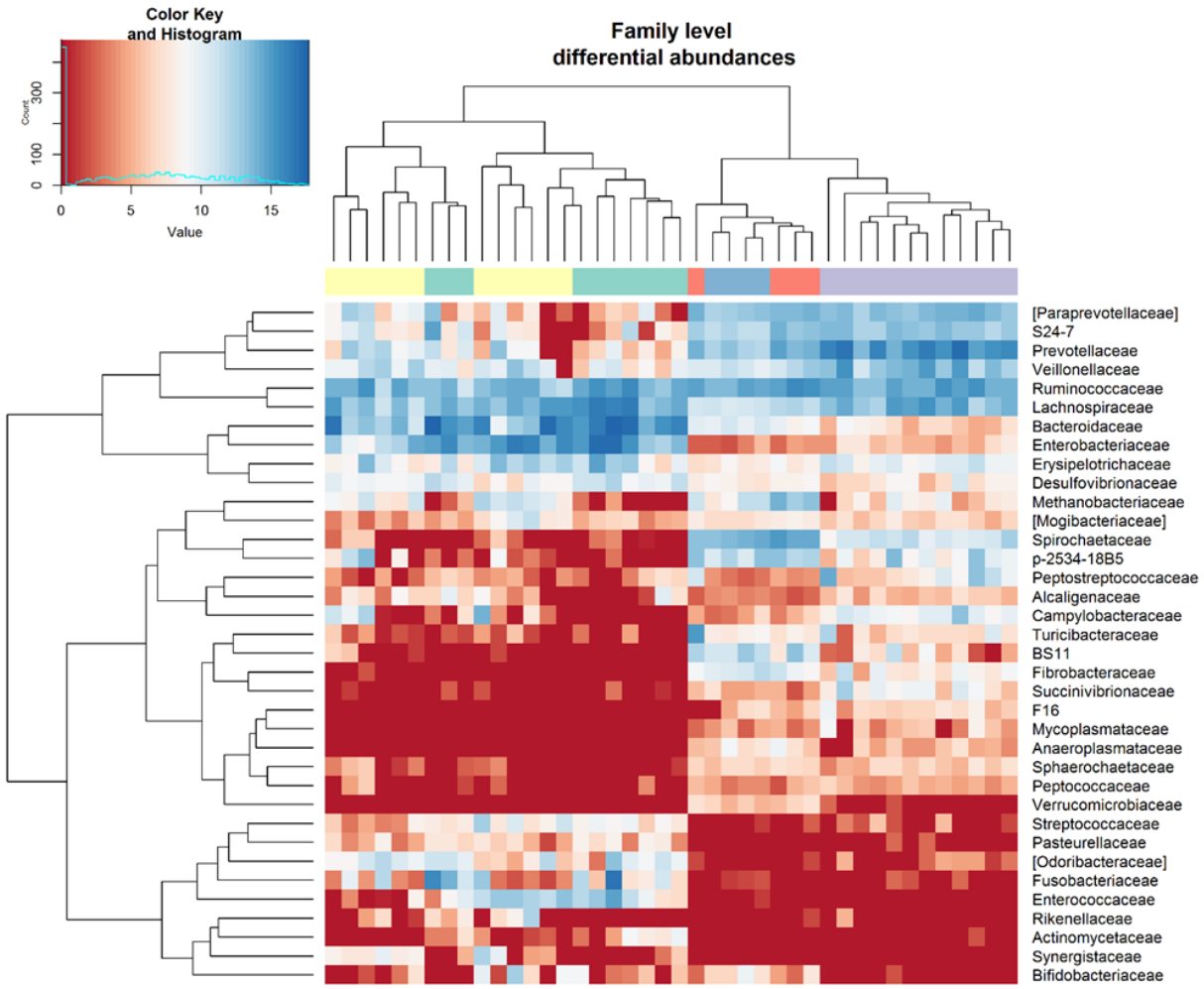

Figure 3: Heatmap and hierarchical clustering of families which showed differential abundances between suckling piglets and weaned piglets and between weaned piglets and sows. Upper bar: green= piglets timepoint I; yellow= piglets timepoint II; purple= piglets timepoint II, orange= sows timepoint I; blue= sows timepoint II. The family abundances were normalized by cumulative sum scaling and log2 transformed in metagenomeSeq package.

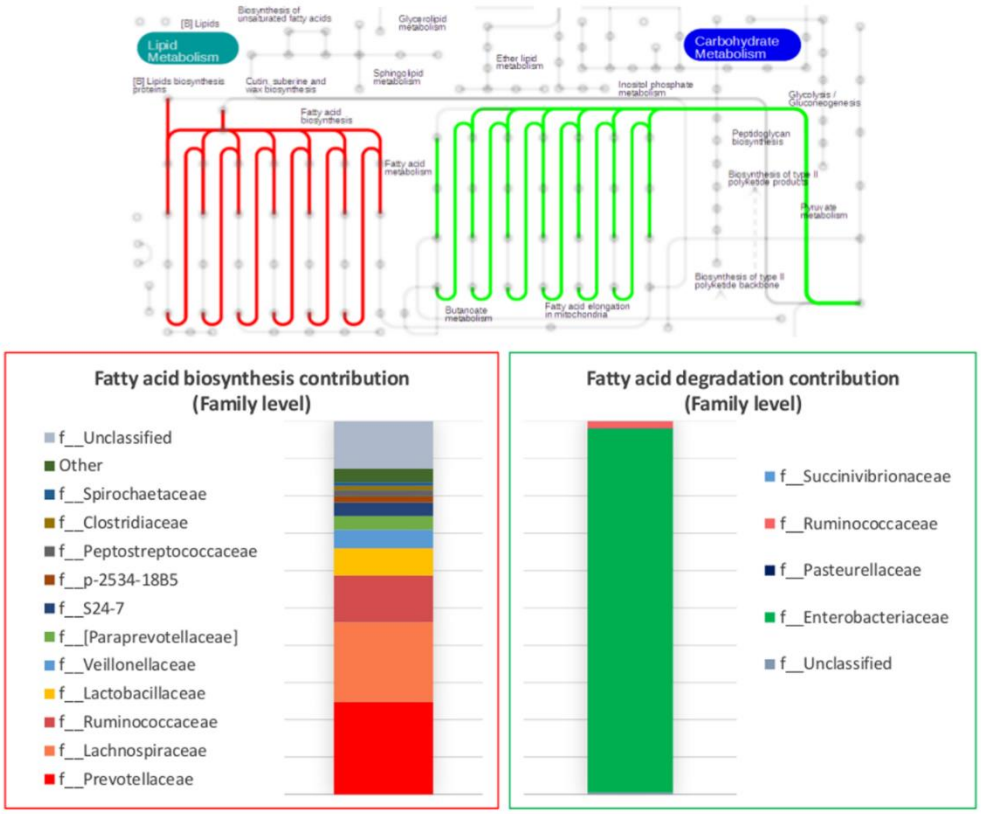

Figure 4: Lipid metabolism in bacterial community of the piglets, differences between pre-weaning (green lines and square) and post-weaning (red lines and square) and taxonomic contribution at family level.

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Supplementary file

Primer used for blood groups A0 genotypes screening

Primer sequence (5'->3')		Amplicon length	Accession number	Reference
Forward	CGCCAGTCCTTCACCTACGAAC	1076 -> allele 0	GU256574	Nguyen et al. (2011)
Reverse	CGGTTCCGAATCTCTGCGTG			
Forward	AATGTCCTTATGCTGGCCTGG	1076 and 340 -> allele A	GU256573	
Reverse	AACAACACACTCCTGAACAACAGA			

Models fitted in nlme package to test the effect of genotype and litter factors on alpha diversity (Shannon index) in piglets

Shannon Piglets ~ Genotype, random = ~ 1|Timepoint/Subject

	numDF	denDF	F-value	p-value
(Intercept)	1	30	107.56	<.0001
Genotype	1	30	0.30	0.5861

Shannon Piglets ~ Litter, random = ~ 1|Timepoint/Subject

	numDF	denDF	F-value	p-value
(Intercept)	1	28	110.38	<.0001
Litter	3	28	1.43	0.2537

Shannon Piglets ~ Litter, random = ~ 1|Timepoint/Genotype/Subject

	numDF	denDF	F-value	p-value
(Intercept)	1	25	110.38	<.0001
Litter	3	25	1.44	0.2557

Models fitted with Adonis procedure in vegan package to test the effect of genotype and litter factors on beta diversity (Bray-Curtis distance) in piglets

Bray-Curtis Piglets ~ Timepoint

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Maturity	2	4.2092	2.10460	8.7301	0.3603	0.001
Residuals	31	7.4733	0.24107		0.6397	
Total	32	11.6825			1.0000	

`Bray-Curtis Piglets ~ Litter, strata = Timepoint`

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Litter	3	0.864	0.28801	0.79866	0.07369	0.090
Residuals	30	10.819	0.36062		0.92604	
Total	33	11.682			1.00000	

`Bray-Curtis Piglets ~ Genotype, strata = Timepoint`

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Genotype	1	0.2597	0.25967	0.72743	0.02223	0.333
Residuals	32	11.4228	0.35696		0.97777	
Total	33	11.6825			1.0000	

`Bray-Curtis Piglets ~ Litter %in% Genotype, strata = Timepoint`

	Df	SumsOfSqs	MeanSqs	F.Model	R2	Pr(>F)
Litter: Genotype	3	0.864	0.28801	0.79866	0.07369	0.117
Residuals	30	10.819	0.36062		0.92604	
Total	33	11.682			1.00000	

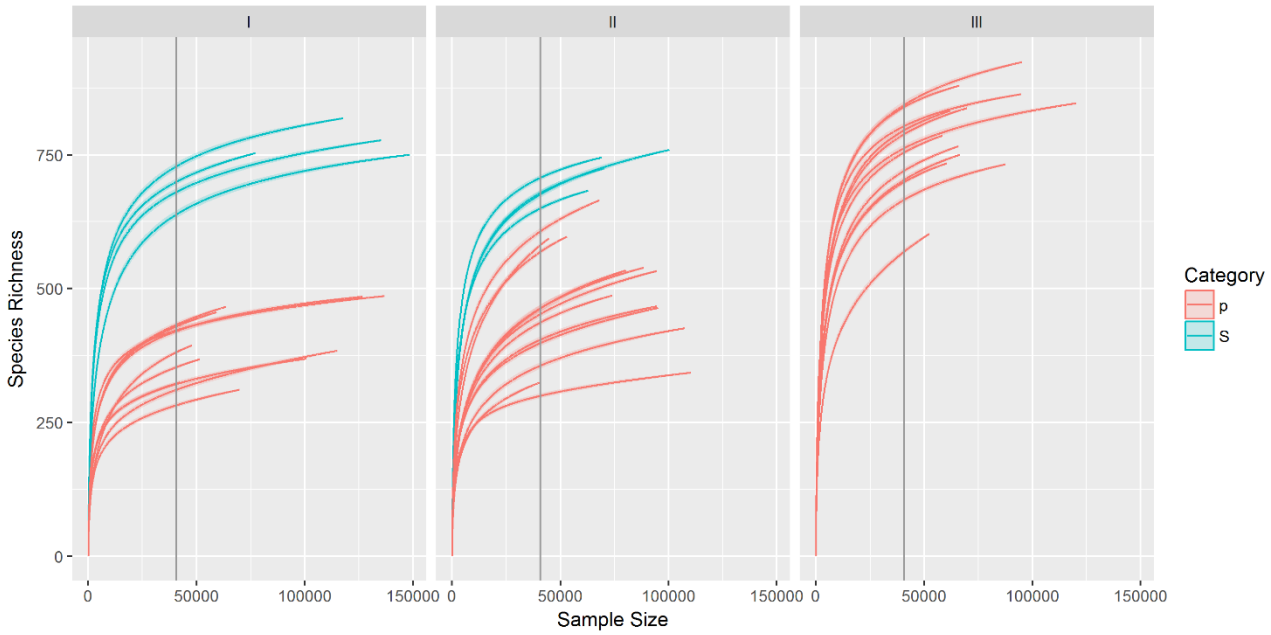

Figure S1: Per sample rarefaction curves; p= piglets, S= Sows, I= day7 post farrowing, II= day14 post farrowing, III= day14 post weaning.

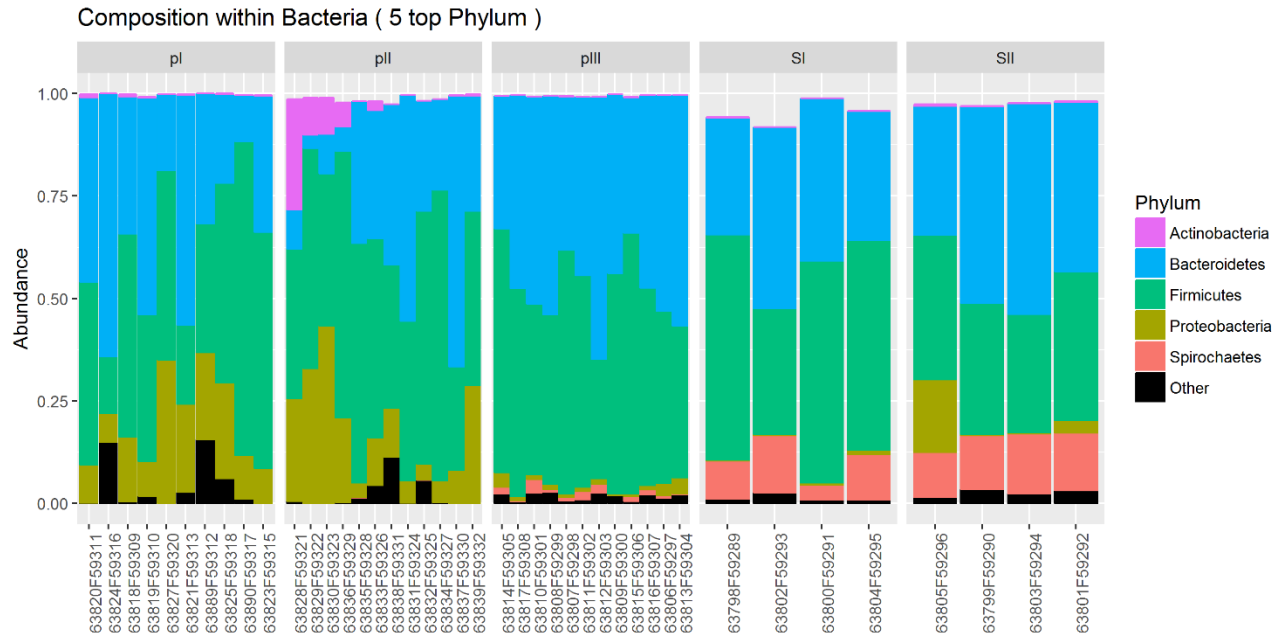

Figure S2 Per sample taxonomic classification top 5 Phyla (relative abundances); p= piglets, S= Sows, I= day7 post farrowing, II= day14 post farrowing, III= day14 post weaning.

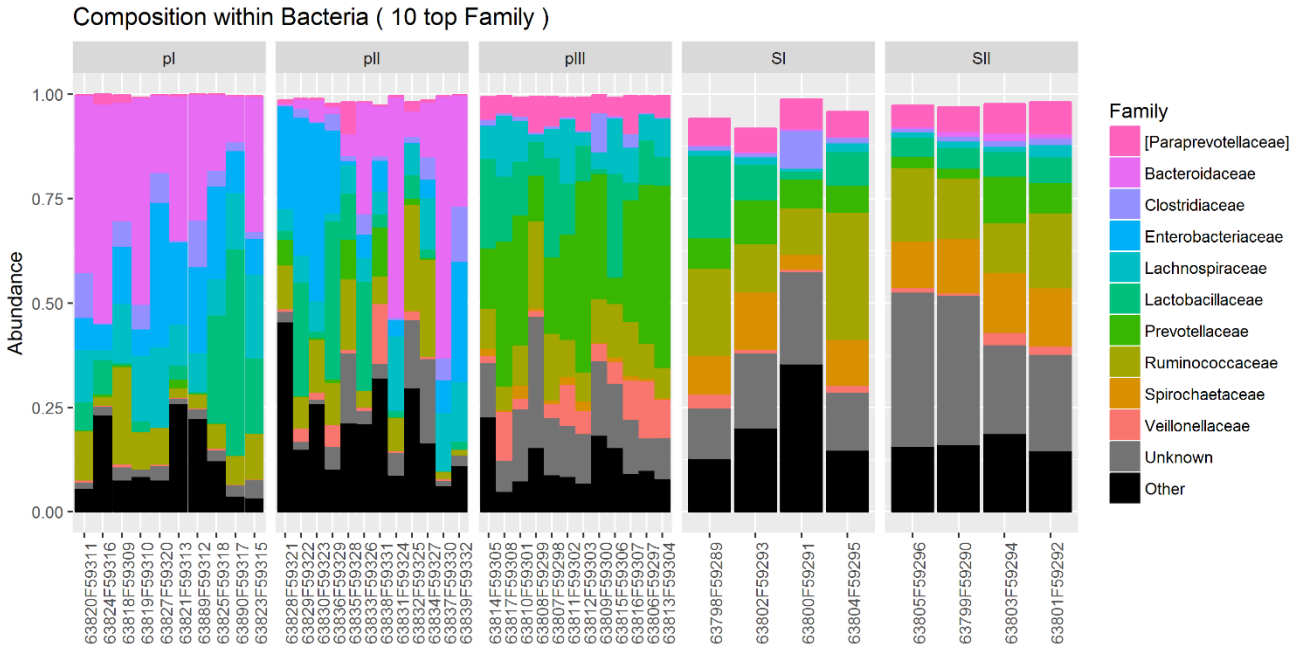

Figure S3 Per sample taxonomic classification top 10 Families (relative abundances); p= piglets, S= Sows, I= day7 post farrowing, II= day14 post farrowing, III= day14 post weaning.

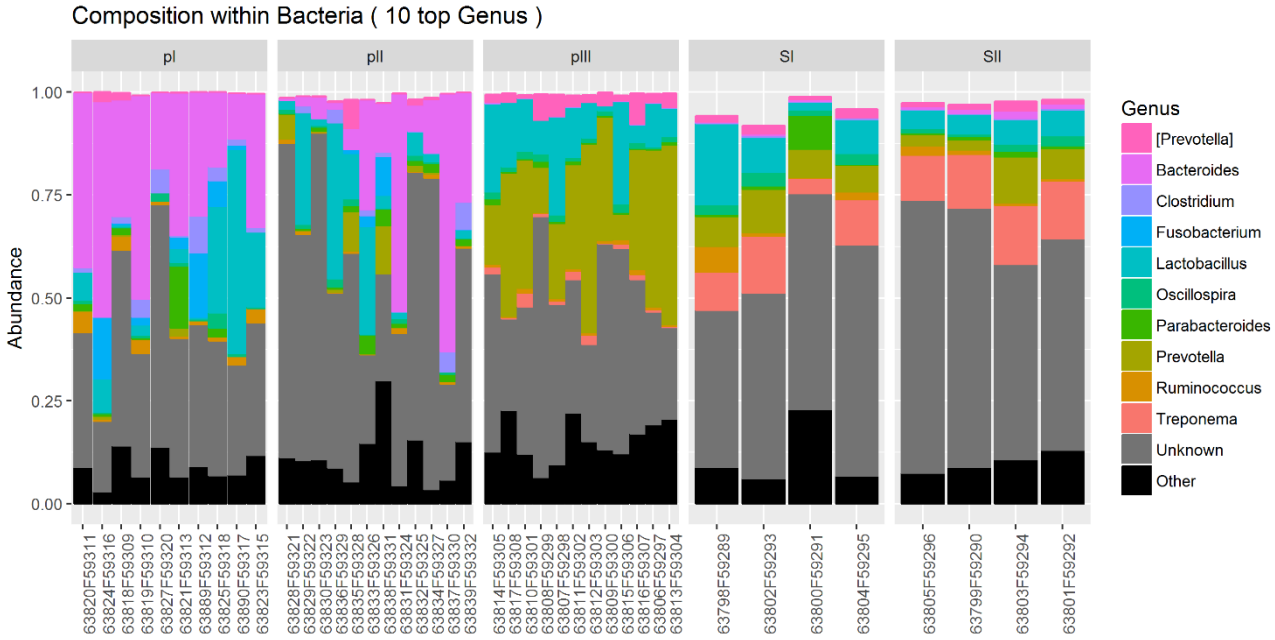

Figure S4: Per sample taxonomic classification top 10 Families (relative abundances); p= piglets, S= Sows, I= day7 post farrowing, II= day14 post farrowing, III= day14 post weaning.

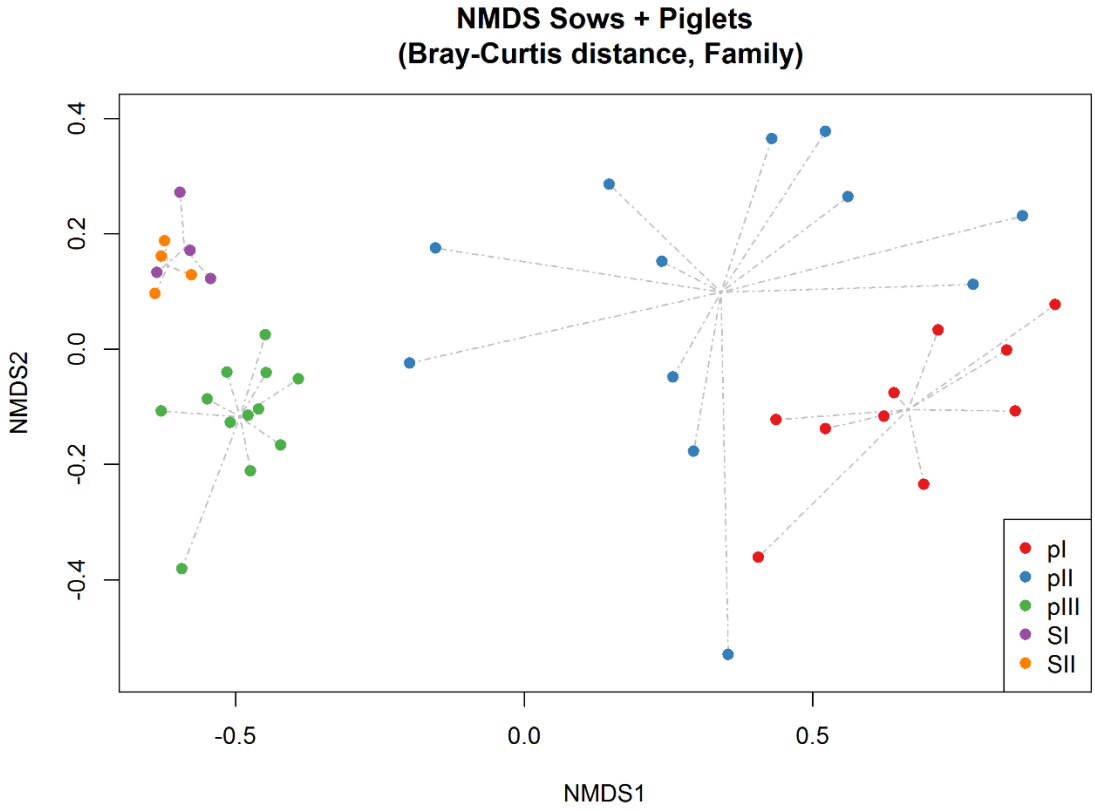

Figure S5: Non-metric multidimensional scaling (NMDS) on Bray-Curtis distances at Family level. pI= piglets timepoint I (day 7 post farrowing), pII= piglets timepoint II (day 14 post farrowing), pIII= piglets timepoint III (day 14 post weaning), SI= sows timepoint I (day 7 post farrowing) SII= sows timepoint II (day 14 post farrowing).

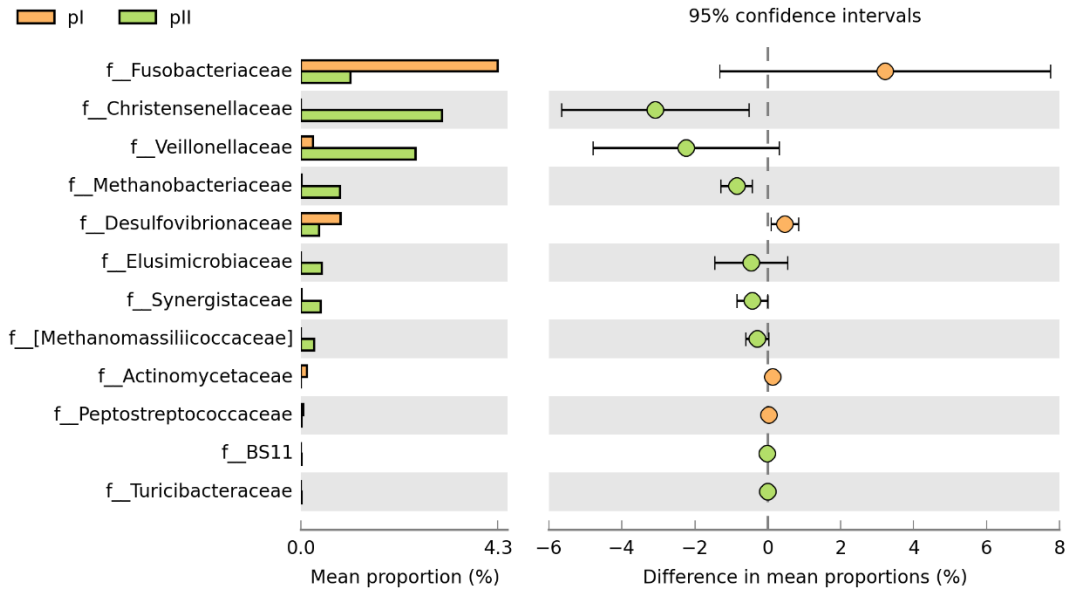

Figure S6: Extended error bar plot showing the families that have significantly different abundances between piglets at the timepoint I and piglets at the timepoint II. pI= piglets timepoint I (day 7 post farrowing), pII= piglets timepoint II (day 14 post farrowing). The differences were tested in metaGenomeseq package as reported in M&M section.

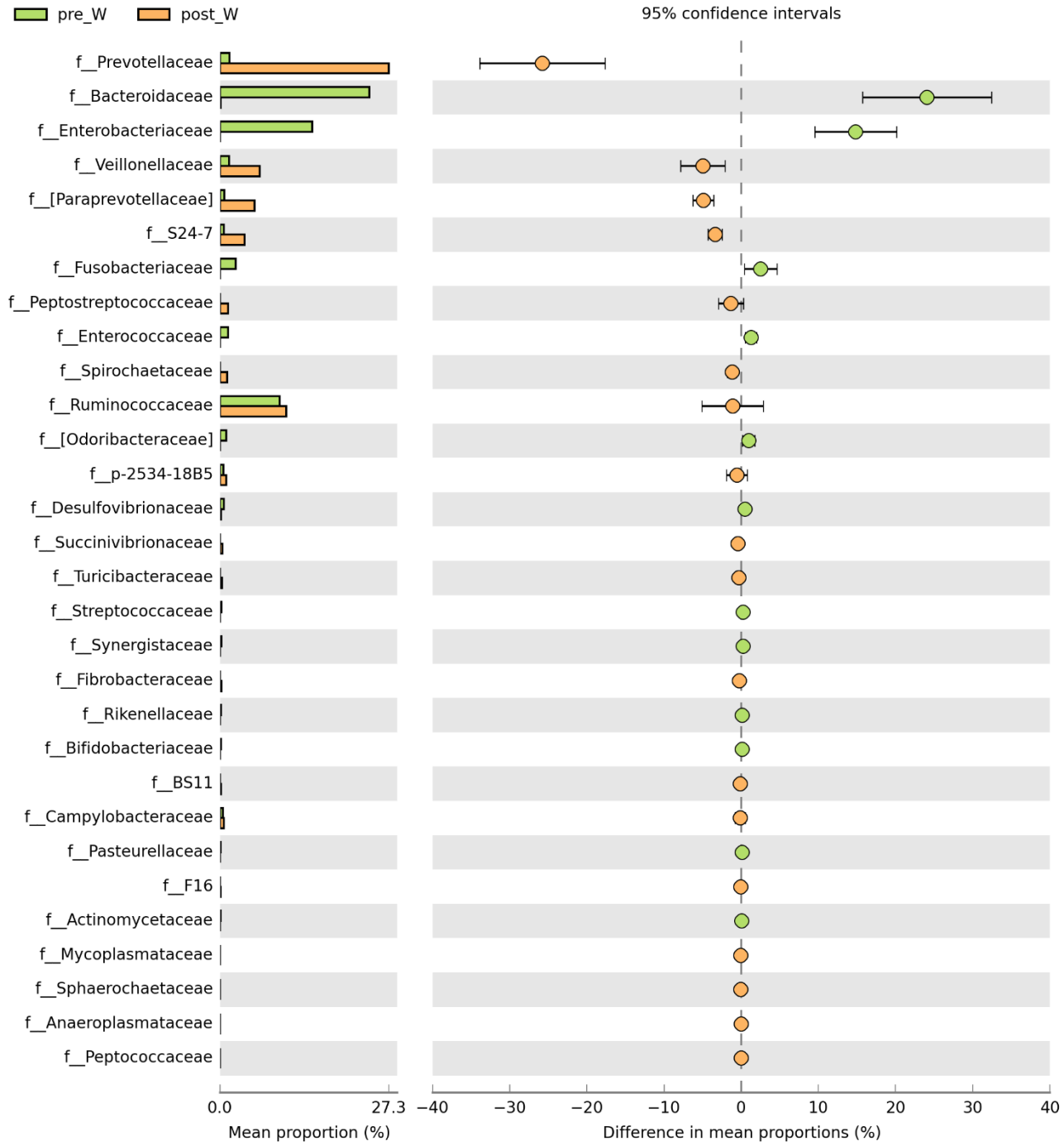

Figure S7: Extended error bar plot showing the families that have significantly different abundances between suckling piglets (pre_W) and weaned piglets (post_W). pre_W= piglets timepoint I (day 7 post farrowing) + piglets timepoint II (day 14 post farrowing), post_W= piglets timepoint III (day 14 post weaning). The differences were tested in metaGenomeseq package as reported in M&M section.

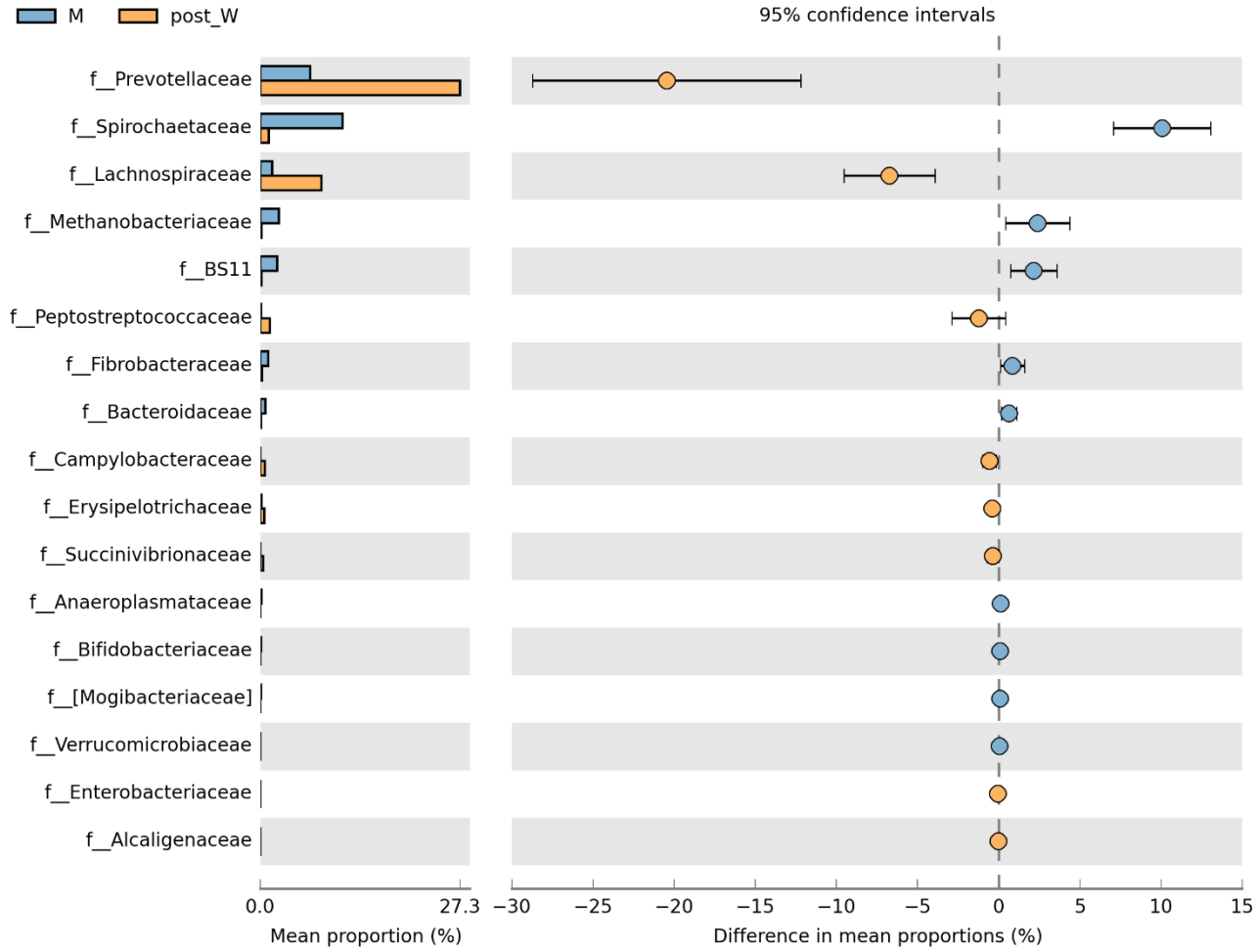

Figure S8: Extended error bar plot showing the families that have significantly different abundances between Sows (M) and weaned piglets (post_W). M= mature microbiota (Sows day 7 post farrowing + sows day 14 post farrowing), post_W= piglets timepoint III (day 14 post weaning). The differences were tested in metaGenomeseq package as reported in M&M section.

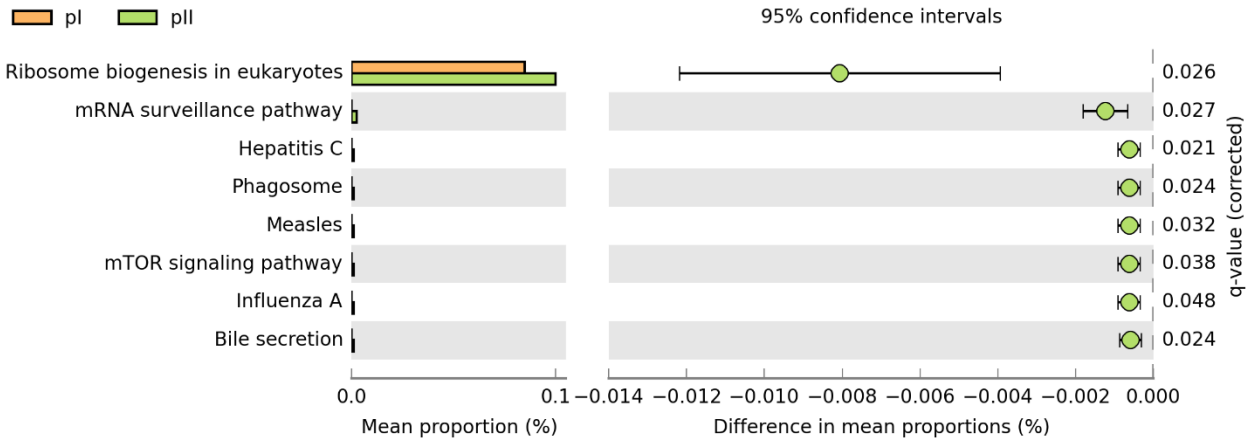

Figure S9: Extended error bar plot showing the Level3 KEEG pathways that have significantly different abundances between piglets at the timepoint I and piglets at the timepoint II. pI= piglets timepoint I (day 7 post farrowing), pII= piglets timepoint II (day 14 post farrowing). The differences were tested in STAMP as reported in M&M section. Most of these pathways have not biological meaning for prokaryotes.

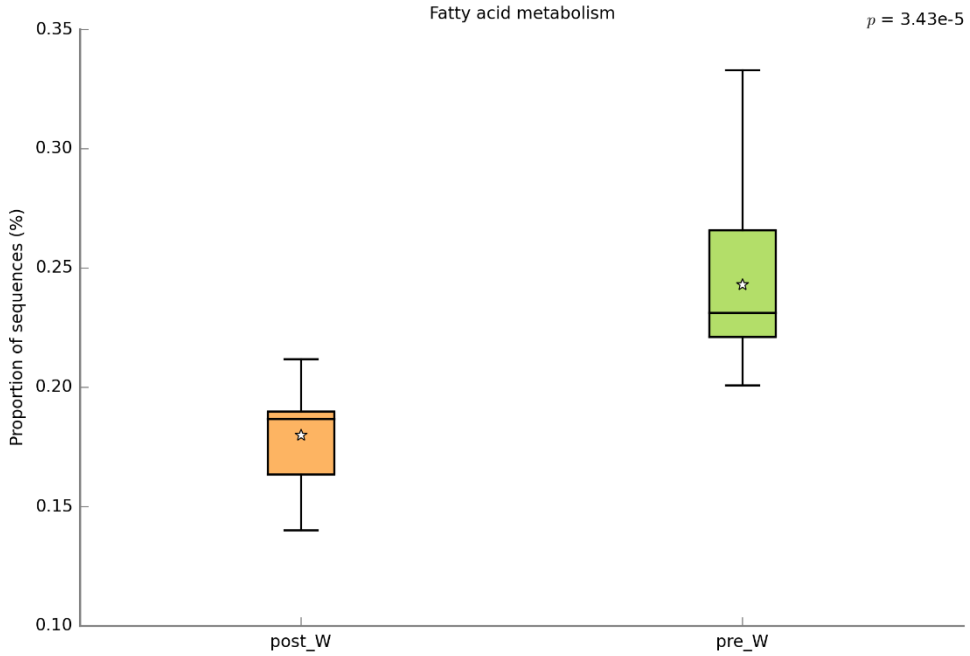

Figure S10: Boxplot showing the differences in "Fatty acid metabolism" (Level3 KEEG pathway) between suckling piglets (pre_W) and weaned piglets (post_W). pre_W= piglets timepoint I (day 7 post farrowing) + piglets timepoint II (day 14 post farrowing), post_W= piglets timepoint III (day 14 post weaning).The differences were tested in STAMP as reported in M&M section.

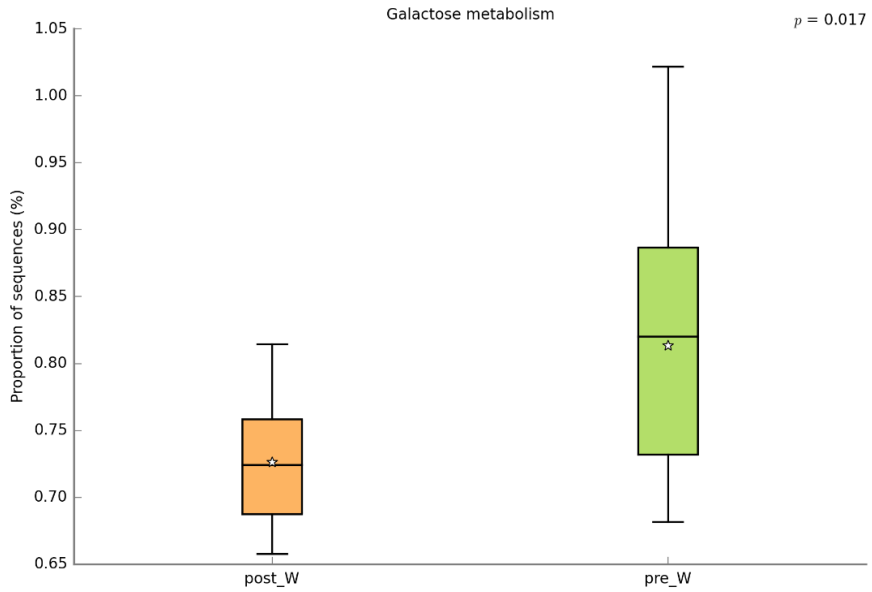

Figure S11: Boxplot showing the differences in "Galactose metabolism" (Level3 KEEG pathway) between suckling piglets (pre_W) and weaned piglets (post_W). pre_W= piglets timepoint I (day 7 post farrowing) + piglets timepoint II (day 14 post farrowing), post_W= piglets timepoint III (day 14 post weaning). The differences were tested in STAMP as reported in M&M section.

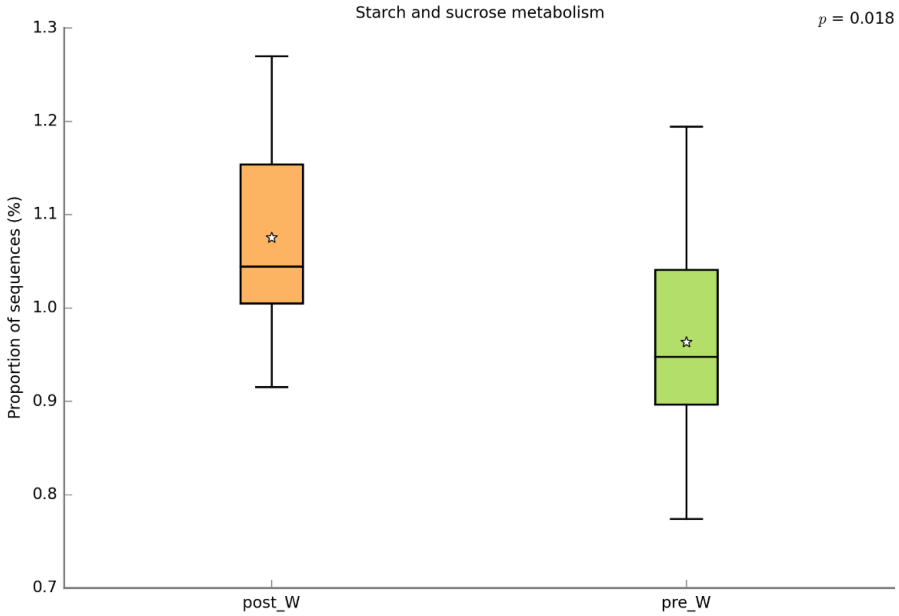

Figure S12: Boxplot showing the differences in "Starch and sucrose metabolism" (Level3 KEEG pathway) between suckling piglets (pre_W) and weaned piglets (post_W). pre_W= piglets timepoint I (day 7 post farrowing) + piglets timepoint II (day 14 post farrowing), post_W= piglets timepoint III (day 14 post weaning). The differences were tested in STAMP as reported in M&M section.

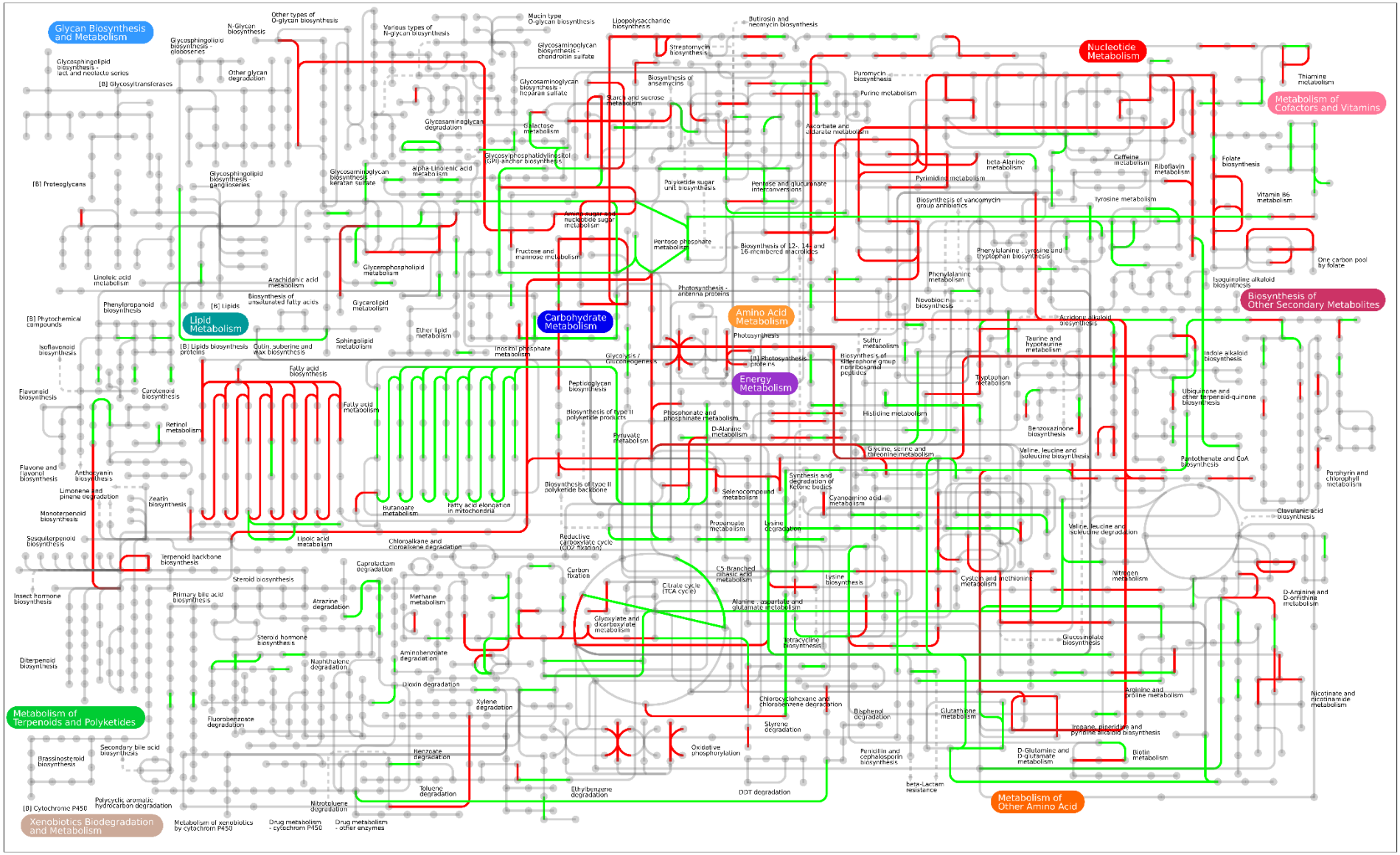

Figure S13: IPath2 metabolic map; in green are highlighted the pathways significantly enriched in bacterial community of suckling piglets, in green are highlighted the pathways significantly enriched in bacterial community of weaned piglets. The differences were tested in DeSeq2 package as reported in M&M section

General discussion

In the present thesis, it was analysed the structure of the bacterial communities in different sections of the gastrointestinal tracts of pigs taking into account the potential effect of different factors that may play a role in their shaping.

In the first work, we studied the less characterized GIT bacterial community, that of the stomach, placing the emphasis on the potential microhabitats that can be found within the gastric environment. The results showed that the gastric mucosa may "select" a microbial community that differs from that found in the luminal content, which showed lower variability levels than the mucosal community. On the one hand, this result shows how the gastric mucosa, considered an environment hostile to the bacterial settlement, may actually represent an ecological niche for a resident microbiota, and on the other hand it shows the filtering property of the stomach. In fact, the less variable luminal community was found to be characterized also by genera like *Lactobacillus* and *Prevotella*, which are known to be among the dominant taxa in the small and large intestine respectively.

Furthermore, the quite high presence of plastidial DNA and bacterial taxa associated with the plant-derived matter, the high number of reads missing taxonomic classification and the aforementioned differences between mucosa and digesta, pose also methodological questions on the role of the diet as inoculum and/or as confounding factor, on the choice of the hypervariable region (V6 in this case) of the 16S rRNA gene to be used for a reliable profiling of the bacterial community and on the representativeness of the luminal portion of the microbiota for what is instead the portion attached to the mucosa.

In the second work we tested the effect of a long-term administration of formic acid to healthy pigs monitoring the growth performances of pigs during the six week following the weaning and evaluating the impact on GIT at the end of the experiment through the counts of cells positive for the protonic pump H^+/K^+-ATPase in the oxyntic mucosa (hydrochloric acid secretion), the expression

level of a set of genes (*IL8; GPX2; REG3G; TFF3; CCL20; ST3GAL; LBPI; SLC7A9*) related to the inflammatory response in the jejunal mucosa, and through the profiling of the microbiota attached to the jejunal mucosa.

The results showed that the dietary supplementation with formic acid exerts an auxinic effect and improves the ADG during the early post-weaning period (2-4 weeks post-weaning). On the other hand, the prolonged supplementation of formic acid had no effect on the growth performances in the late post-weaning, did not affect the count of the protonic pump in oxyntic mucosa of stomach, and no effects were reported also for the inflammatory candidate genes expression. Overall, these data may indicate the adaptation of the host to the administration of the formic acid, which shows a greater effectiveness of this additive in the immediate post-weaning, when the risk of dysbiosis is greater.

Also with regard to the bacterial community, the prolonged administration of formic acid did not show a strong impact on its structure, however, the less presence of lactic acid producing bacteria (*Lactobacillus*, *Leuconostoc* and *Gemella*) and butyric acid producing bacteria (*Fusobacteria*) in the treated groups may represent the result of an adaptation of the bacterial community to the presence of formic acid through a negative feedback mechanism.

In the case of the present study the main objective was to test the long-term administration of formic acid, for this reason it was decided to use a week of diet adaptation, in order to mitigate the variability due to the weaning, before administering the experimental diets. Based on the results obtained, and on the data reported in the literature, it seems that it is worth focusing on the mechanisms of action of organic acids in the immediate post-weaning with further studies that can clarify the dynamics involved in this phase.

In the last work the rearrangement of the faecal bacterial community of piglets from the lactation to the post-weaning phase was analysed taking into account the potential effect of blood group (genotypes).

The results clearly showed the change in the structure of the microbiota that occurs during the weaning transition, the observed differences are mainly correlated to the change of diet that has a strong effect in the shaping of the microbiota. The milk oriented microbiota dominated by Bacteroidaceae and Enterobacteriaceae showed a gradual increase in variability to reach levels comparable to those of mature microbiota two weeks after weaning, when we find a faecal bacterial community dominated by Prevotellaceae and Ruminococceae which are families normally associated with fiber fermentation. Besides this evident taxonomic shift, the metagenomic predictions also showed an interesting change in the metabolic potential of the bacterial community, in particular, the Enterobacteriaceae seem to have a role in the degradation of long chain fatty acids in the lactation phase, this could be related to the high fat content in sow's milk and could represent the path of energy supply for pathogens such as *E. coli* in the intestine of the suckling piglets.

Conversely, effects of the blood group on the structure of the microbiota were not been found, this can lead to the exclusion of the impact of blood groups but also to a procedural consideration. The rationale behind the blood groups hypothesis concerns the different intestinal glycomic patterns associated with the different blood groups; different sugary motifs in glycocalyx of the intestinal epithelium may select different bacterial communities that use these sugars as an energy source and/or as specific anchors for mucosal adhesion. In our previous study we found clear differences in the glycomic motif of the mucosa of the jejunum in relation to the two pig blood groups, however, these differences concerned, in fact, the mucosa of the jejunum.

As already considered for the study on the stomach, and from what is known in literature, the luminal bacterial communities are not necessarily representative of what happens on the mucosa, moreover, the faecal microbiota is certainly more representative of the large intestine community than that of the small intestine, so the absence of detectable differences could also be due to this gap in representativeness. The studies on the microbiota are often focused on the faecal bacterial community, and while this may represent a limit, on the other hand, the increase of knowledge in this direction is

necessary for the identification of detectable biomarkers with minimally invasive techniques such as faecal sampling.

Furthermore, even though the present thesis is not a methodological study, it should be noted that in the three works presented here, different techniques and methods have been applied; that involved the bacterial DNA extraction method (QiaAmp DNA Stool Mini Kit; FastDNA SPIN Kit for Soil), the choice of the hypervariable region of the 16S rRNA gene (V6; V3-V4), the sequencing methods (Ion PGM; MiSeq Illumina), the reference database for taxonomic assignment (Ribosomal Database Project; Greengenes) and different approaches in data analysis (e.g. Rarefaction; Cumulative Sum Scaling normalization), which are also reflecting the research work carried out during the PhD program. The use of different techniques and approaches and the resulting differences are central subjects of discussion in literature (McMurdie and Holmes, 2014; Paulson et al., 2014; Rintala et al., 2017; Salipante et al., 2014; Weiss et al., 2017) and, as already reported in the literature review part of this work, the "study effect" is currently one of the strongest variability factors in microbiota studies. The biological interpretation of complex data such as those deriving from the "omics" studies therefore requires careful considerations on the existing methodological gaps and represents one of the scientific challenges open for the coming years.

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