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The influence of the genetic background and feeding strategies on the health, performance and gastrointestinal homeostasis of weaned pigs

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“We cannot fathom the marvelous complexity of an organic being;

but on the hypothesis here advanced this complexity is much increased.

Each living creature must be looked at as a microcosm--a little universe, formed of a host of self-propagating organisms, inconceivably minute and as numerous as the stars in heaven.”

— Charles Darwin

I want to thank my family, friends, supervisors, colleagues, collaborators,

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ABSTRACT

In pig intensive production system, the early weaning (26 ± 2 days of age) is generally considered the most stressful phase during pig growth and represents the main challenge for the pig farmers. During weaning, piglets have to face several stress factors including the social stress, the change of diet and of environmental, as well as the drop of acquired immunity level. In addition, host factors including the low capacity to absorb fluid in the gut, the age-related expression of specific receptors for bacteria adhesion and lack of a complete immune competence contribute to the occurrence of the post-weaning diarrhea (PWD) syndrome mainly supported by enterotoxigenic *Escherichia coli* (ETEC) F4 and F18. In this phase, the host-microbial cross-talk play a key role in driving the immune system development as well as the host metabolism. In this context, the new omics approaches (e.g. genomics, metabolomics and microbiota NGS sequencing) allow to deeply discern the host-microbial interaction. The intestinal microbiota interact with the host and this interplay can be influenced both by external factors, first of all, the diet and by internal (host-dependent) factors including the age, sex and genetics.

Concerning this aspect, the present thesis reports three studies involving genomics and feeding strategies influences in piglets immune and metabolic response. The first two studies focus in disclosing the role of the genetic background of piglets in shaping the gut microbiota as well as the host response in term of metabolic profile and gene expression of the gut mucosa in healthy conditions. The results of the first study demonstrate that *MUC4* genetic variants previously associated to ETEC F4ac susceptibility influence the characteristics of jejunum mucosa by affecting the degree of jejunum fucosylation, the expression of genes involved in the intestinal homeostasis such as *CCL20*, *REG3G* and *TFF3* and the blood metabolic profile of healthy piglets. Furthermore, taking together the first and the second studies, the results evidence the role of *FUT1* genetic variants in affecting the jejunal microbial balance profile by increasing the level of non-beneficial bacteria such as *Veillonella*, *Fusobacterium,* Enterobacteriaceae and haemolytic bacteria in the susceptible genetic variants. Furthermore, *FUT1* genotype affects the blood metabolic profile of healthy piglets during suckling and post-weaning periods. The effect of *FUT1* gene on gut microbial population and host metabolism could be related to its ability to influence the intestinal fucosylation degree. Finally, the third study evaluates the effectiveness of a probiotic strategy to counteract the ETEC F4ac infection in the post-weaning pigs selected for the susceptible *MUC4* genetic variant for ETEC F4ac. The results confirm the effectiveness of using *MUC4* gene as a marker to select pigs for ETEC F4ac infection model, indeed all the selected pigs included in the study showed the specific receptors for this pathogen on the intestinal villi. In relation with the effect of *Saccharomyces cerevisiae* supplementation at the concentration of $5x10^{10}$ CFU/kg of feed in weaning piglets, our findings show that

both preventive and competitive ways of administration contain the detrimental effect of ETEC F4ac infection as confirmed with the results of the intestinal mucosal transcriptomics profile. Collectively, the work presented in this thesis provides new insight about the interplay between the host-genetics, gut microbial composition and host physiological status. Furthermore, it provides confirmation that the use of known genetic markers for ETEC F4 and F18 could represent a potential tool to stratify the animals in the trials both in healthy or challenge-based protocols.

LIST OF ORIGINAL MANUSCRIPTS INCLUDED IN THE THESIS

The presented thesis is based on the following three original manuscripts:

1. **Luise D.**, V. Motta, M. Bertocchi, C. Salvarani, M. Mazzoni, P. Bosi, P. Trevisi. "Effect of DNA polymorphisms for gut homeostasis on microbiota composition, genes expression, glycomic binding pattern profile in jejunum, blood serum metabolic profile and growth performance of healthy post-weaning pigs" prepared for the submission to Journal of Animal Science and Biotechnology

2. Riis Poulsen A.-S. *, **D. Luise***, M. V. Curtasu, N. Canibe, P. Trevisi, C. Lauridsen. "Effects of alpha-(1,2)-fucosyltransferase genotype variants on the plasma metabolome, the gastrointestinal microbial population, and epithelial immunological parameters of healthy piglets pre- and post-weaning". Submitted to PLOS ONE

3. Trevisi P., R. Latorre, D. Priori, **D. Luise**, I. Archetti, M. Mazzoni, R. D'inca and P. Bosi. 2017. "Effect of feed supplementation with live yeast on the intestinal transcriptome profile of weaning pigs orally challenged with *Escherichia coli* F4". Animal 11(1): 33-44.

BACKGROUND

1. PIGLET GUT EUBIOSIS AND DYSBIOSIS

The gut microbiota is composed mainly of bacteria, but also by archaea, viruses, fungi and other microbial eukaryotes. The number of bacteria cells inhabiting the gastrointestinal tract (GI) have been recently estimated to be composed of ~ 10^{13}-10^{14} cells, which is a very close to the number of host cells and encompasses ~100 times the amount of genomic content (microbiome) as the human genome (Bäckhed et al., 2005; Sender et al., 2016). The microbial community has a critical role in host wellbeing, comprising host nutrition, immune function and metabolism and is organized in a complex dynamic and breakable ecosystem (Fouhse et al., 2016).

A definition of a gut microbiota in an eubiotic status is not completely clear. It is associated with the idea that the microbial community is mainly constituted by potentially beneficial species while potentially pathogenic species are present with a lower percentage (Iebba et al., 2016); furthermore, it is widely accepted that more the microbial population is variable and more the intestinal microbiota could be considered stable and beneficial (Zoetendal et al., 2004).

Any perturbation of the microbial-host ecosystem results in a dysbiosis, that from a microbiological point of view has been characterized by a gut microbial imbalance and by an increase in the number of Enterobacteriaceae that replace the obligate anaerobic bacteria (Winter et al., 2013). From a more comprehensive point of view, taking into account the interplay between the gut microbiota and the host response, dysbiosis is also defined as an alteration of the composition or function of the microbial community, giving metabolic or immunological consequences to the host (Iebba et al., 2016)

In pigs, the "core-healthy" gut microbiota among all gastrointestinal sites is mainly constituted by Firmicutes and Bacteroidetes phyla, accounted for nearly 85%, and Proteobacteria represent the third more abundant phylum. Considering the genera classification level *Clostridium*, *Blautia*, *Lactobacillus*, *Prevotella*, *Ruminococcus*, and *Roseburia* are recognised as the most common in pigs (Holman et al., 2017; Xiao et al., 2016), however, gut microbiota vary along the gastrointestinal tract (Isaacson and Kim, 2012) and according to the animal maturation. In addition, the microbial population vary according to host depended factors such as the age, intestinal maturation, sex and breed, as well as by host-independent factors including litter, diet, use of antibiotics and the sanitary condition of the farm (Xiao et al., 2016).

1.1. Piglet microbial colonization and the post-weaning dysbiosis

The microbial colonization of the gastrointestinal tract (GIT) starts at birth and it shows to play a critical role also in the post-weaning and adult life (Dou et al., 2017; Mach et al., 2015). During the perinatal period, the GI microbiota is mainly constituted by the *Escherichia coli* (*E. coli*), *Streptococcus* sp., *Bacteroides*, *Bifidobacterium*, *Clostridium*, and *Lactobacillus* (Petri et al., 2010). During suckling, the faecal microbiota is mainly composed of *Fusobacterium*, *Lactobacillus*, *Bacteroides*, *Escherichia/Shigella*, *Megasphaera*, *Oscillibacter and Ruminococcaceae* genera. At weaning is observed a reduction of microbial variability, associated to a decrease in the number of *Latobacillus* and *Clostridium*, and an increase of *Enterobacteriacae* genera (Chen et al., 2017; Mach et. al., 2015). This lower variability can also predispose to the occurrence of dysbiosis that is one of the predisposing factors to the post-weaning diarrhoea l (PWD) syndrome. The dysbiosis that occurs at weaning, is generally associated with the increasing of the intestinal permeability due to a transient inflammatory status, especially in the small intestine, that allowing the passage rate of antigens (e.g. pathogen) through the intestinal barrier. This inflammation process induces the production of nitric oxid (NO) that in the gut can be quickly converted to nitrate. The presence of nitrate favours the *E. coli* development as those bacteria are able to use nitrates because they have the nitrate reductase genes. In addition, the inflammation increases also the blood flow and thus the presences of oxygen in the gut mucosa. The higher oxygen concentration can favour the facultative anaerobic bacteria such as the Enterobacteriaceae instead of the obligate anaerobic bacteria such as bacteria belonging to Bacteroidia and Clostridia classes (Gresse et al., 2017; Winter et al., 2013).

Then, after weaning, the microbiota composition achieves stable level after 10 days post-weaning and it is characterized by higher level of *Prevotella*, *Acetivibrio*, *Dialister*, *Oribacterium*, *Succinivibrio* genera respect to the composition during the pre-weaning phase (Chen et al., 2017; Mach et. al., 2015). This is mainly ascribing to the capacity of these bacteria to degrade polysaccharides of the cereals in the diet through their xylanases, mannanases, β-glucanases enzymes. An additional reason is also that after weaning the host acquired more efficiency in the digestion of the protein in the diet (Pluske et al., 1997), resulting in a reduced level of nutritional substrate available for the opportunistic pathogens.

1.2. The role of *Escherichia coli* in pig dysbiosis around weaning

Among the several pathogens that can develop and colonize the GIT of weaners, *E. coli* is considered one of the most common. *E. coli* is an enterobacteria Gram- which is generally present as commensal bacterium in the pig gut microbiota.

The taxonomy differentiation of *E. coli* in the 200 serotype is based on the difference of its antigens of the cell wall (O), of the capsule (K), of the fimbriae (F) and of the flagella (H). The terminologies "fimbriae," have been introduced by Dugui and Anderson (1967). This structure allows the bacteria to adhere the mucosa surface with host cell-specific receptors (Karlsson, 1989) and is responsible for the hemagglutinating activity. Fimbriae are antigenic thermolabile proteins sticking out from the bacterial cell wall and in the *E. coli* numerous variants have been observed (Table 1)

Table 1. ETEC characteristic: fimbriae, enterotoxins serogroups and respective phase of pig infection Adapted by Francis (2002).

ETEC characteristics			Pig phase of infection
Fimbriae	Toxin(s)	Serogroup(s)	
K99	Sta	O8, O101	Neonatal
K99, 987P	Sta	O9	Neonatal
987P	Sta	O20, O141	Neonatal
F4	LT, STb +/- Sta	O8,O149,O157	Neonatal and weaned
F18ab	Sta, STb +/- Stx2e	O139	Weaning
F18ac	Sta, STb +/- Stx2e	O141, O157	Weaning
F18ab, F18ac	Sta, STb +/- Stx2e	O138	Weaning

Among the reported variants, in pigs a particular interest has been attended to the F4 and F18 fimbriae as those are the most prevalent in the Enterotoxigenic *Escherichia coli* (ETEC) infection associated with the PWD worldwide (Chen et al., 2004; Frydendahl, 2002; Vidotto et al., 2009), while F5 and F6 and F41 have been more often associated with ETEC diarrhoea occurring during the neonatal period (between 4 and 14 days of old) (Toledo et al., 2012; Wilson and Francis, 1986). This is partially due to the fact that the host presents the specific receptor for the different ETEC adhesion in an age-dependent way (Nagy and Fekete, 2005) (Table 1).

The ETEC infection is characterized by the presences of two major classes of toxins: the heat labile toxins (LT) and heat-stable (STa and STb) toxins. The ETEC F4 produces the LT, STa and STb toxins, while the ETEC F18 produce STa, STb and the more rarely the Stx2E (named also VT2e toxin) that is associated with the oedema disease pigs (Table 1). The porcine LT variant belong to the LTI group and is composed by 5 B-subunits and one A subunit that, after the bacteria-binding, is responsible for the activation of adenylate cyclase which increased the production of cyclic AMP (cAMP) and thus a stimulation of cAMP-dependent protein kinase A. This mechanism increased the secretion of Cl^- and reduces the absorption of Na^+ causing an imbalanced trans epithelial osmotic gradient that leads to the diarrhoea (Barrett and Keely, 2000). The STa and STb are small peptides that activate the cGMP-dependent protein kinase and the calmodulin-dependent protein kinase II respectively. The first causes an impair of transepithelial osmotic gradient by the inhibiting the Na/Cl transport system, while the second affects the calcium regulation and actives the production of prostaglandin E2 (PGE2) and 5-hydroxytryptamine (Peterson and Whipp, 1995). The Stx2E presents a subunit A and five B subunits. The subunit A inhibits the protein synthesis in target cells by cleaving a specific adenine residue on the 28S rRNA of the 60 S ribosomal subunits. The subunits B are able to bind to the glycolipid receptor on the host epithelium and release the holotoxin, which is absorbed through endocytosis by the host (Lo et al., 2014).

1.2.1. F4 fimbria and putative receptors

Three antigenic variants of F4 fimbriae have been identified, namely F4ab, F4ac, and F4ad showing differences in hemagglutination characteristics (Bakker et al., 1992; Faeg et al., 2009). The a antigenic region seems to be common to all the variants, and in addition, some subunits are associated to all the three F4 fimbriae variants (FaeG, FaeC, FaeF, FaeH and probably FaeI and FaeJ). At genetics level, *faeG* gene has been identified as the key gene responsible for the differences among the three variants indeed this differences have been ascribed to the diverse distribution of the a, b, c and d epitopes in the FeaG subunit (Van den Broeck et al., 2000). Moreover, among the variants, seems that F4ac is the most common variance worldwide (Gyles and Fairbrother, 2005).

Many putative receptors have been identified for the F4 adhesion belonging to carbohydrates of glycoproteins that are shown in the intestinal epithelial cells and intestinal mucus. Focusing on the F4ac it seems that putative receptors are two intestinal mucin-type sialoglycoproteins (IMTGP-1 and IMTGP-2) (Francis et al., 1999) linked by a galactose (Grange et al., 1998). However, a number of additional putative receptors with a glycosphingolipids nature such as lactosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, globotriaosylceramide, lactotetraosylceramide, and lactotetraosylceramide have been described and characterized (Coddens et al., 2011; Grange et al., 2002, 1999).

1.2.2. F18 fimbria and putative receptors

The F18 fimbria is composed of multiple copies of the major subunit FedA and by minor subunits FedE and FedF (Imberechts et al., 1992). F18 fimbriae present two antigenic variants F18ab (previously known as F107) and F18ac (previously known as 2134P, or 8813), where the F18ac present an extra CCG triple linked to the FedA subunit (Imberechts et al., 1994). The "a" is considered the common antigenic factor, while "b", "c" are the specific factors (Francis, 2002; Sarrazin and Bertschinger, 1997). All the F18+ ETEC strains are able to produce heat-stable enterotoxins including STa and STb, (Francis, 2002), while the ability to produce the Shiga toxin-producing have been associated more commonly only to F18ac (Wittig et al., 1995). The F18 fimbriae bind to glycoproteins on microvilli of the small intestine via the FedF protein (Nagy and Fekete, 2005; Smeds et al., 2001). The F18 host receptor has not been completely characterized and confirmed yet, however, it showed a glycosphingolipids nature and have been observed a positive correlation between F18 bacteria adhesion and the presences of H-2 histo-blood (r= 0.57)group antigens (HBGAs) or its derivative A-2 HBGAs (r=0.77) in the porcine gut epithelium (Coddens et al., 2007). It also seems that the amount of glycosphingolipids and of non-acid glycosphingolipids vary with piglet age; glycosphingolipids it is 10 times higher in new-born piglet respect to the adult and the non-acid glycosphingolipids are seven times higher. The higher non-acid glycosphingolipids amount in new-born piglet could mask the F18-binding glycosphingolipids or inhibit adhesion by sterical hindrance explaining why the ETEC F18 infection is more associated to the post-weaning piglets' respect to new-borns (Coddens et al., 2008).

1.3 Feeding Strategy to prevent the post-weaning dysbiosis

Diet is recognized as one of the main factors able to modulate the gut microbial profile and function. Manipulation of the animal diet by changing its composition, the protein and fibre ratio, the quality of dietary fibre, the type and quantity of resistant starch, and quantity of non-digestible and endogenous proteins have been widely studied. However, not only the composition of the diet but also its forms (e.g. liquid, fermented liquid, solid), as well as the use of several feed additives showed the ability to modify the gut microbial composition and the taxa abundance.

With reference to the post-weaning period, different feeding strategies are unravelled by research studies and in the following paragraph, is presented a comprehensive description. A high protein diet has been associated with an increase of *E. coli* and *Clostrium perfringens* abundancy and a reduction of beneficial bacteria such as *Bifidobacteria* (de Lange et al., 2010; Rist et al., 2013). Furthermore, these bacteria are

also positively associated with the occurrences of PWD. This could be due to the fact that high level of crude protein increases the buffering capacity in the stomach, that coupled with the already high pH in the stomach of weaning pigs, concur to favourite the proliferation of Enterobacteriaceae and/or Clostridia, able to produce detrimental substances such as ammonia, amines and phenols (Rist et al., 2013). In addition, not only the amount of crude protein but also the source of dietary protein has been observed to modulate the intestinal bacteria community at weaning. Indeed, the use of high digestible proteins has been proposed as a feeding strategy to reduce the PWD occurrence. For instance, casein, recognised as high digestible protein, can be easily absorbed in the small intestine and can reduce the protein fermentation and then the production of the detrimental substances (Pieper et al., 2012). Nevertheless, the protein fermentation takes place mainly in the large intestine while PWD are localized in the small intestine, thus a clear explanation is not completely available (Pieper et al., 2016). A possible reason is that high crude protein diet could increase also the presence of bacteria able to directly damage the mucosal barrier in small intestinal that can cause enterobacteria translocation across the lumen or favour the detrimental effect toxins produced by the *E. coli* and resulting in PWD.

An additional strategy that has been tested for increasing the piglet gut health by favourable modulation of intestinal microbiota is the use of liquid feed (LF) or fermented liquid feed (FLF). The LF is prepared by mixing the dry raw material with water or with the liquid by-product of food industries. While FLF can be prepared by mixing and fermenting a complete feed with water at a ratio included in the range of 1:1.5 and 1:4 or by the fermentation of the grain fraction and a subsequential mixing with additional ingredients (Missotten et al., 2010). The FLF is characterized by a high presence of lactic acid bacteria that thanks to their lactic acid production reduce the intestinal pH, resulting in a control for *E. coli* and *Salmonella* overgrowth. In addition, the FLF is also rich in yeasts such as several *Pichia* species, *Candida milleri* and *Kazachstania bulderi* that act directly on some Enterobacteriaceae by blocking their binding to the gut epithelium (Missotten et al., 2015; Van Winsen et al., 2001).

Modulation of microbiota composition at weaning is also possible by the use of prebiotic, probiotic or btheir synergistic use (symbiotic). Prebiotics are defined by Gibson and Roberfroid, (1995) as “non-digestible food ingredients that beneficially affect the host by selectively stimulating the growth and/or activity of one or a limited number of bacteria in the colon that have the potential to improve host health”. The use of prebiotic diet is widely studied in post-weaning-piglets, for instance, one of the possible feeding strategies at weaning is to supply a low protein diet with a reduced content of crude protein coupled with a higher level of fermentable carbohydrates that increase the number of probiotic bacteria such as *Lactobacillus* and *Bifidobacterium (*Rist et al., 2013). In addition, a large number of prebiotics have been tested in piglets including no digestible oligosaccharides such as galacto-oligosaccharides (GOS)

(Bouwhuis et al., 2017), fructo-oligosaccharides (FOS) (Letellier et al., 2000), inulin (Metzler-zebeli et al., 2017). No digestible oligosaccharides showed beneficial effects on the host gut health by increasing the growth and/or activity of *Bifidobacteria* and *Lactobacillus*, recognised as beneficial bacteria, and reducing the potential pathogens overgrowth. More recently additional categories of prebiotics such as isomalto-oligosaccharides (Wu et al., 2017), milk-oligosaccharides (Salcedo et al., 2016), seaweed-derived polysaccharides (Bouwhuis et al., 2017) have been suggested.

Probiotics are defined as "live microorganisms that, when administered in adequate amounts, confer a health benefit on the host" (FAO/WHO, 2001). Probiotics belong to different bacteria genera or yeast and their effect is considered specie-specifics, for this reason some effective probiotic for human are not effective in pigs. They can be beneficial for the host thanks to several modes of actions as revived by Roselli et al., (2017): i) for their ability to produce antimicrobial substances including hydrogen peroxide, lactic and acetic acids, bacteriocins; ii) for their role in competing with pathogens by occupying their sites of adhesion or for nutrients competition, iii) for their capacity to enhancement of the mucosal barrier integrity and immune modulation (Roselli et al., 2017; Lo Verso et al., 2017). A wide number of studies focusing on the effect of different beneficial microbes recognised as probiotics for pig are available, however, the research in this field is never been consider completely exhaustive as the effect the specific probiotic can change accordingly to a huge number of factors including the probiotic dose, the period of administration, the animal age and maturation, and the environmental conditions in general (Kenny et al., 2011). Thus in Table 2a,b a collection of the most recent researches on the effect of probiotics administration to piglet is presented, with special emphasis on the gut microbial changes. As shown in Table 2a,b, the most promising bacteria strains belong to *Lactobacillus* sp. (*L. casei* and *L. reuteri*), *Bacillus* sp. (e.g. *B. subtilis* and *B. cereus*) and to yeast as *Saccharomyces cerevisiae*. *Lactobacillus* sp. are able to produce antimicrobials to reduce the detrimental effect of some Gram-positive and Gram-negative species, furthermore, they can produce the lactic acid and regulate the intestinal pH and reduce the enterobacteria overgrowth. In the studies included in this collection, the supplementation with 1.7×10^{10} CFU *L. reuteri* to neonatal piglets reduced the pH, increased the lactic acid and reduced the non-beneficial bacteria such as *E. coli* in the small intestine (Hou et al., 2015b). In the same way, the supply of *L. casei* coupled with *Enterococcus faecalis* to neonatal piglets decreased the pH in stomach and duodenum, improved the cecum microbial variability and decreased the piglet diarrhoeal and mortality (Liu et al., 2017). *Bacillus* spp. is a spore anaerobic/facultative anaerobic bacteria which is recognised to act as beneficial microbes in the gut. In the finding studies, *B. subtilis* ($4x10^9$ CFU) supply to weaners increased the microbial diversity and the number of faecal *Lactobacillus* and reduced the number of *E coli* bacteria (Hu et al., 2014). *B. cereus* ($1x10^9$ spore/g) showed the ability to reduce the diarrhoeal scores

and diarrhoea incidence and level faecal *E. coli* when administrated continually after weaning for 35 days (Papatsiros et al., 2011). *Saccharomyces cerevisiae* supplementation in weaning pigs showed a favourable stimulation of the immune response (Cui et al., 2017; Jiang et al., 2015) and an increase in small intestine of bacteria generally consider beneficial such as the *Lactobacillus* (Brousseau et al., 2015; Cui et al., 2017) and Ruminococcaceae in large intestine (Brousseau et al., 2015). Additionally, the administration of *Saccharomyces cerevisiae* showed a positive effect on piglet health when tested in challenge condition. The supplementation with 182 g/t of *Saccharomyces cerevisiae boulardii* reduces piglet mortality and improves the immune response of weaners challenged with *E. coli* lipopolysaccharide (LPS) (Collier et al., 2014) and when *Saccharomyces cerevisiae* (strain CNCM I-4407) was supplied to sows and piglets to contrast the ETEC F4 infection, it revealed the capacity to reduce the diarrhoea scores and diarrhoea duration and to decrease the shedding of pathogenic ETEC bacteria of weaners (Trckova et al., 2014). Hence, probiotics administration directly to piglet in the post-weaning diet or in an early stage by supplying them during the suckling period or in the late gestation of sow's diet could be a possible strategy to improve the piglet health and robustness, however, the mode of action is specific to each probiotic and further studies will be desirable to improve the knowledge in this field.

Table 2a. Studies on probiotic(s) administration in suckling and weaned piglets and its effects on microbiota composition and intestinal health.

Probiotic (s)	Dose	Period of administration	Challenge	Microbiological sample	Probiotic effect	Reference
Bacillus subtilis KN-42	2×10^9; 4×10^9; 20×10^9 CFU/g	Post-weaning period. From weaning for 28-days	No	Faeces	> Lactobacillus; < E. coli and > microbial diversity in 4x109 diet	(Hu et al., 2014)
Bacillus licheniformis and Saccharomyces cerevisiae	1.5×10^{10} cfu/*g Bacillus licheniformis* and 0.3×10^{10} cfu/g *Saccharomyces cerevisiae*	Post-weaning period. From weaning for 12-days plus	*E. coli* F4 challenge at d9 post-weaning	Cecum	<fecal score; < cecal E. coli count; < endotoxin and diamine oxidase concentration; >ADG and ADFI	(Pan et al., 2017)
Bacillus cereus	$1x10^9$ sprore/g feed	Post-weaning period. From weaning for 35 days	No	Faeces	< diarrhoea scores; < ETEC	(Papatsiros et al., 2011)
Lactobacillus casei and Enterococcus faecalis	$1 \cdot 10^9$ CFU	Suckling period 1, 7, 14 and 21 d of age	No	Cecum	> average daily gain; <diarrhoea ; <mortality; > blood igA; > villus length and > microbiota variability and stability	(Liu et al., 2017)
Lactobacillus reuteri I5007	1.7×10^{10} CFU	Suckling period from day1-4 or for 4 days from 1 to 17	No	Ileum	> lactic acid; <pH; < number of *E.coli;* > expression of TGF-β and < expression of IFN-γ in the mesenteric lymph nodes	(Hou et al., 2015a)
Bland of Bifidobacterium animalis (DSM15954) and 4 Lactobacillus species: acidophilus (DSM13241), casei(ATCC55544), p entosus (DSM14025), and plantarum (DSM13367)	5×10^9 CFU	Suckling period: Immediately after birth every 6 h during the parenteral nutrition and every 3 hours in the enteral phase. Animals were sacrified after 40/48 hours	No	Distal Small Intestine and Colon	< *Clostridium*; > *Lactobacillus;* < *necrotizing enterocolitis scores;*	(Siggers et al., 2008)
Bifidobacterium longum subsp. infantis (CECT 7210) and B. animalis subsp. lactis BPL6 (B. lactis BPL6)	10^9 CFU	Post-weaning for 16 days	*Salmonella Typhimurium* challenge at day 8 post-weaning	Faeces	< fecal excretion of *Salmonella*; > improvements in the villous:crypt ratio	(Barba-Vidal et al., 2017b)

Table 2b. Studies on probiotic(s) administration in suckling and weaned piglets and its effects on microbiota composition and intestinal health.

Probiotic (s)	Dose	Period of administration	Challenge	Microbiological sample	Probiotic effect	Reference
Bifidobacterium longum subsp. Infantis CECT 7210	2.5×10^8 CFU	Post-weaning for 16 days	*Salmonella Typhimurium* or E. *coli* F4 challenge at day 8 post-weaning	Faeces	< faecal excretion of *Salmonella* and *E.coli*	(Barba-Vidal et al., 2017a)
Saccharomyces cerevisiae	4.3×10^9 CFU	Post-weaning piglet. Weaning at 14d of age	No	Ileum and cecum	> ratio of Lactobacilli to E. coli; > mucosal sIgA	(Cui et al., 2017)
Pediococcus acidilactici strain MA18/5M and *Saccharomyces cerevisiae subsp. boulardii* strain SB-CNCM I-1079	In sows: 2.5×10^9 cfu per day from d –28 to –14, 3.5×10^9 CFU per day from d –14 to 0 (parturition), and 6×10^9 CFU per day from d 0 to 21 (nursing). In piglets: 1×10^9 at birth and 2×10^9 CFU/kg after weaning for one week	I sow from 28 before parturition + at birth + post-weaning	No	Ileum and colon	***Pediocuccius*** < microbiota diversity and >Firmicutes in ileum ; ***Saccharomyces cerevisiae*** > *Porphyromonadaceae* and *Ruminococcaceae* in the colon.	(Brousseau et al., 2015)
Saccharomyces cerevisiae, strain Y200007	3.00 g/kg	Post-weaning piglet. From weaning at 14-d old for 21 days	No	Duodenum and Jejunum	> G:F; > villus height and villus-to-crypt ratio in duodenum and jejunum; > IgA, IL-2, and IL-6 in blood serum	(Jiang et al., 2015)
Saccharomyces cerevisiae (strain CNCM I-4407)	In sows: 1 g of live yeast/kg feed; in suckling piglets 1 g of live yeast for three times; in post-weaning piglets 5 g of live yeast/kg feed for 12 days	Sows and the late gestation; Piglets at suckling, and postweaning.	ETEC F4 challenge at day 1 post-weaning	Feces	< diarrhoea scores, < diarrhoea duration; < shedding of pathogenic ETEC bacteria; > IgA in blood serum	(Trckova et al., 2014)
Saccharomyces cerevisiae boulardii	182 g/t	Post-weaning for 16 days.	Challenge with *E. coli* lipopolysaccharide (LPS) at d 16.	NA	< Mortality; < IL-1β and IL-6 and > TNF-α and IFN-γ in blood after LPS challenge	(Collier et al., 2014)

2. THE HOST-MICROBIOTA CROSS-TALK

According to the high abundance of cells and the role that the gut microbiota plays in relation with the host, the gut microbiota is generally considered the host's largest, metabolically active entity, called also "microbial organ" (Turroni et al., 2008). However, from an ecological point of view, since the microbiota and host mucosa are in contact, it can influence the host and vice-versa. Therefore, pig and its gut microbiota are recognised to live in a mutually beneficial or mutualistic status (Camp et al., 2009).

Gut microbiota communities are able to produce a wide range of metabolites according to nutrients availability and luminal environment (eg. pH), it concurs to develop the immune system and to modulate the host metabolism and physiology; while the host concurs to modify the gut environmental (e.g. pH, oxygen level) and thus to influence the development and the metabolism of the bacterial community. This continuous communication between the host and the microbiota named "cross-talk" could affect the following aspects: i) Intestinal immunity and integrity ii) Food processing and nutrients and fluids uptake and iii) Gut-Bain axis.

2.1. Intestinal immunity and integrity

The immune system has a crucial role in maintaining the host in a healthy status by preserving an equilibrium (or tolerance) and eliminating the potential pathogens. It is recognized that the intestinal immune system of the neonate is less developed and organized compared to the ones of adults. The initial development of the immune system is induced by different factors including the maternally-derived antigen and antibody, the host genetic background, the diet and the interaction with the gut microbiota (Stokes et al., 2017). The pivotal role of commensal bacteria for the development of the intestinal immune system has indeed been observed particularly during the neonatal period, when the gut starts to be colonized by bacteria and in the meantime the gut immune system starts to develop, thanks to the studies using the axenic animal models (Smith et al., 2007). In pigs, the intestinal immunity development that occurs during the neonatal period and the post-weaning period is particularly important as piglets at birth are immunodeficient. A defined programming for intestinal immune system development has been identified in piglets during this period to reach the well-organized structure observed in animals (Stokes et al., 2004). Furthermore, the lack of a correct intestinal immunological development and the disturbance of intestinal integrity, coupled with the complex physiological situation that occurs at weaning, can lead to the occurrence of PWD (McLamb et al., 2013). In the following paragraphs, an overview comprised of

piglet mucosal immune system, its intestinal barrier function and the signals involved in the host-bacterial cross talk is presented, with special attention to piglets weaning phase.

2.1.1 The organization of piglet gut mucosal immune system

The mucosal immunity is a subject of great interest because of the vast surface area of the GIT and its role to create a co-operation with the intestinal lumen. Its then responsible for the cross-talk with the commensal and pathogenic bacteria and for the GIT absorptive function and regulate the maintenance of tolerance towards the commensal microbiota (Burkey et al., 2009).

The mucosal immune system in the gastrointestinal tract is composed of the organised and diffuse tissues.

The organized tissue includes the mesenteric lymph nodes (MLNs) and the Peyer‘s patches (PPs) which in piglet are localised in the terminal part of the ileum (large ileocecal patch) and along the jejunum and in the proximal part of the ileum (small PPs) (Dvorak et al., 2006). The PPs present the follicle associated epithelium which contributes to the antigens uptakes through the microfold (M) cells or by the paracellular route. The M cells are specialized in the phagocytosis and transcytosis of particulate antigens and microorganism and once that the antigens or microorganism are transported inside the intraepithelial pocket of M cells, it takes contact with lymphocytes and mononuclear phagocytes (macrophages and dendritic cells) and the activation of T cells starts. (Mabbott et al., 2013). Once that the specific antigen is recognised by the naïve T cell, the T cells will differentiate into CD4+ or CD8+. The CD4+ cells are immune response mediators and they allow to maximise the adaptive immune response capability (Janeway et al., 1988; 2001). CD4+ cells initially become CD4-Th0 cells, then according to the different needed adaptive response, they can differentiate to Th1 or Th2. Th1 are stimulated by macrophages and dendritic cell signals and one activated their express IL-2 to induce the clonal expansion and IFN-γ to activate the cytotoxic T cells and macrophages. Th2, once activated by extracellular antigens, start to stimulate the production of several interleukins such as IL-4, IL-5, IL-10 and IL-13 which induce the B cells differentiation and thus the antibodies production (Janeway et al., 1988; 2001). The CD8+ cells cause direct cytotoxicity of infected target cells. When the infected cell is recognised, CD8+ cells can cause cellular apoptosis by producing perforin and granzymes, the fas-ligand triggers and cytotoxin cytokine to damage the plasma membrane of the infected target cells allowing ions and water to get inside (Koretzky, 2010). The second way for the antigen sampling is directly through the dendritic (DC) cells which extend dendrites into the intestinal lumen through opening of tight junctions (TJ) in the epithelial cells (Rescigno et al., 2001). Once that DCs uptake the antigen, travel to the MLNs, where they can present antigen to T cells (Burkey et al., 2009).

The diffuse lymphoid tissue involves the lamina propria and the epithelium. The pig lamina propria has a good level of organization and is rich in leucocytes indeed CD4+ T cells and CD8+ T cells showed a specific distribution. A prevalence of CD4+ T cells was observed within the villi, while the CD8+ T cells are more prevalent in the mucus production by the Goblet cells under normal physiological conditions, is the epithelial basement membrane (Vega-López et al., 1993). In addition, the lamina propria presents also the Antigen-presenting cells (APCs), categorised as functional, immature dendritic cells, that express the MHC II complex (Haverson et al., 2000).

The intestinal epithelial layer is constituted by a panel of different cells, the intestinal epithelial cells (IEC), that make up a thickness of 20 µm. As shown in Figure 1, it includes Microfold (M) cells, Goblet cell, Paneth cells (PC), and absorptive cells that are classified as enterocytes in the small intestine and colonocytes in the colon.

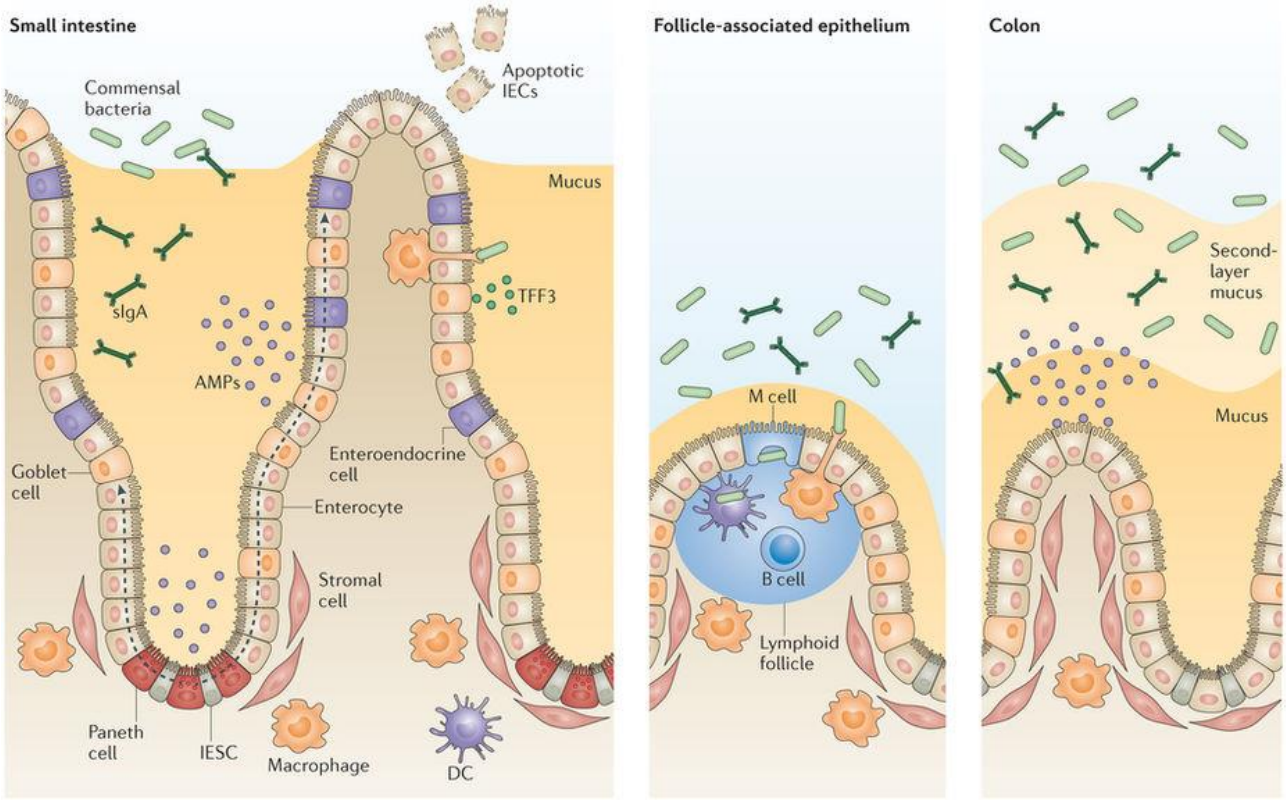

Figure 1. The intestinal mucosa and the organization of its cells in the small intestine and colon.

*Intestinal epithelial cells (IECs) includes Microfold (M) cells, Goblet cell, Paneth cells (PC), and absorptive cells (enterocyte in the small intestine and colonocytes in the large intestine. With the exception of Paneth cells, the IECs once differentiated migrate up the crypt–villus axis. Secretory goblet cells and Paneth cells secrete mucus and antimicrobial factors). In addition, enterocytes contribute to the realise of secretory IgA. Microfold cells (M cells) and goblet cells contribute to the transport of antigens and live bacteria across the epithelial barrier to dendritic cells (DCs) and macrophages. Publishers Ltd: Nature Reviews Immunology:(*Peterson and Artis, (2014). *The IEC barrier, copyright (2014); LICENCE NUMBER 4242510766419).*

Goblet cells are localized along the crypt and villus of both the small and large intestine. These cells are known to be the mucus and trefoil factor producer cells, thus they contribute to the formation and maintenance of the mucus layer and to influence the mucosal barrier function. The mucus production by the Goblet cells under normal physiological conditions is continuous and any variation or disturbance by specific factors such as microbes, microbial toxins and cytokines can alter the mucin production and secretion both in quantity and in its chemical composition (Cornick et al., 2015; Peterson and Artis, 2014).

Paneth cells (PCs) are mainly localized in the crypt of the small intestine. Those cells are involved in the barrier faction as they take part to the innate immune response by the secretion of granules which release antimicrobial peptides to protect by non-beneficial microbes (Bevins and Salzman, 2011). In addition, PCs have been also linked to the production of cytokine (Porter et al., 2002) and to the regulation of immature dendritic cells development (Ito et al., 2012).

Enterocytes and colonocyte are the main representative cells in the small and large intestine respectively as they comprise the 80% of the IECs (Goto and Kiyono, 2012). These cells have the absorptive and digestive functions but are also able to produce some antimicrobial peptides such as β-defensins and (Cunli et al., 2001), C-type lectin regenerating islet-derived protein IIIγ (REGIIIγ) (Peterson and Artis, 2014) and alkaline phosphatases (Bates et al., 2007). In addition, enterocytes also present the polymeric immunoglobulin receptor (pIgR) necessary for the activation of secretory immunoglobulin A (SIgA) (Goto and Kiyono, 2012).

The mucosal immune system develops with age, particularly in pigs that at birth present an immature immune system, its development is intensive during the perinatal period. Furthermore, since the placenta of pig is epithelial-chronicle, molecules of high mass weight such as the maternal antibodies cannot cross the barrier so the foetus cannot obtain them. The immune system starts to develop during the postnatal period when the piglets began to take contact with new antigens and continue at weaning with a second wave of nutritional antigens enter in the intestinal tract (Stokes et al., 2004). At birth, only not organised clusters of lymphocytes, in the areas that will subsequently develop into Peyer's patches, are present; in 2 weeks' lymphoid cells expressing the CD2 markers and the Peyer's patches start to colonise the mucosa; after 4 weeks the T cells start to express the CD4+T markers and very low B cells began to produce the IgG: After 5 weeks of age, the intestinal epithelium and the basement membrane start to show the CD8+ cells while in the crypt, B cells producing the IgA appeared. Finally, the pig can have a mature immune system after the 7th week of age (Stokes et al., 2004). In early weaned pigs (21-28 days of age) the intestinal immune system is not completely developed, and the loss of passive immune protection arising by the Igs, the non-specific antimicrobial protection and the active compounds coming from mother milk,

concur to cause the occurrence to the PWD syndrome (Everaert et al., 2017). For this reason, the mucosal immunity and the cross-talk with commensal bacteria play an important role for the piglet to prevent the infection, in particular at weaning.

2.1.2 The piglet intestinal integrity

The intestinal integrity is defined to deal with a maintaining of intestinal barrier whole and its assembling. The mucus layer, the IECs, and the connection between the IECs by tight junctions (TJ), desmosomes, adhesion junctions, and gap junction are the main responsible for intestinal integrity. Therefore, alterations in mucus layer, IECs proliferation and death ratio and changes in the TJ paracellular transfer can affect the in intestinal integrity, allowing the passage of non-beneficial bacteria across the intestinal lumen (Camilleri et al., 2012).

The mucus layer has a gel-like structure and consists of mucins, IgA, trefoil factor, antimicrobial peptides, water and ions. The main component of mucus layer are mucins, complex agglomerates of structural glycoproteins secreted by Goblet cells (Forstner, 1995; Lang et al., 2007). The mucins can be released in the mucus (MUC2 - the most abundant in the small intestine, MUC5AC, MUC5B and MUC6) or can be membrane-bound glycoprotein adherent to the epithelial wall (MUC1, MUC3, MUC4, MUC12, MUC13, MUC16, MUC17, and MUC20) (Lang et al., 2007). The mucins present specific O-linked glycans mainly constituted by repetitions of proline, threonine and serine sequences (PTS sequences). The PTS sequences allow also the attachment of polysaccharides, that are widely O-glycosylated. The core part of this polysaccharides are generally constituted by galactose, N-acetyl-galactosamine and N-acetyl-glucosamine and, attach to them, a glycan that usually terminates with a monosaccharide such as a fucose or a sialic acid of a galactose is present (Sicard et al., 2017). The structural diversity of these carbohydrates and its organization in the mucin constitution are responsible for bacteria adhesion, both pathogens (as described in the previous chapter 1.2 for the ETEC F4 and F18) and commensals and plays an important role in the microbial and host cross-talk.

In addition to the mucins, the goblet cells secret the trefoil factor peptides. In mammalian, three main the trefoil factor peptides have been recognised: TFF1, TFF2 and TFF3. They present a common trefoil domain that is composed by a sequence of 38 amino acid (AA) rich in cysteine residue that forms a characteristic disulphide bond, giving to those peptides the resistance against gastric acid and proteases (Scholven et al., 2009; Thim, 1994). The TFFs proteins show anti-apoptotic properties to the epithelial cells (Taupin et al., 2000), through the activation of several mechanisms including the stimulation of HT-29 that induced the phosphorylation of Epidermal Growth Factor Receptor (EGFR) and the activation of mitogen-activated protein kinase or protein kinase B, which favour the survival of the cells. TFFs have

also a central role both in the modulation of immune response and the tissue repair by the regulation of pro and anti-inflammatory cytokines (Scholven et al., 2009) and in the reduction of the intestinal permeability by the down-regulation of tight junction proteins in pathogenic condition such as the inflammatory bowel disease (Xu et al., 2012). Furthremore, the interaction between mucin and TFF increase the mucus viscosity and elasticity increasing the intestinal protection to the adhesion of potential pathogen bacteria (Thim et al., 2002). The antimicrobial factors that protect the crypt cells from pathogens are mainly produced by Paneth cells and include alpha-defensins, lysozyme and Reg3 proteins (Salzman, 2010).

In addition to these antimicrobial factors, the secretory IgA (SIgA) provides support the host defence against potential pathogens and to the regulation of intestinal homeostasis (Goto and Kiyono, 2012). The SIgA are produced by the plasma cells in the dimeric form and are secreted in the intestinal lumen through the epithelial cells that express the polymeric immunoglobulin receptor (pIgR). SIgA are composed of a dimer of IgA subunits that are joined by a small polypeptide (J chain) and bound to a glycoprotein, the secretory component (SC). SIgA can neutralize bacteria toxins and prevent the pathogens translocation, or they can have a direct effect on the pathogen virulence capacity such as in the case of the interaction between the murine monoclonal IgA (IgAC5) to the O-Ag of *Shigella flexneri.* After the bond, the *Shigella flexneri* type 3-secretion (T3S) system activity, that serves the pathogen for entry into intestinal epithelial cells is suppressed (Forbes et al., 2011). SIgA do not interact only with the pathogenic bacteria but also with the commensal bacteria; indeed, experiment in germ free mice (GF) that were appendectomized and colonized with bacteria, showed that after 8 weeks the level of fecal IgA and SIgA cells in cecum reach the same level found in conventional mice (Masahata et al., 2014). The mechanisms by which the SIgA cells interact with commensal and pathogenic bacteria are summarized in the Figure 2. Briefly the SIgA cells present three main mechanisms: the first regards the stimulation of the intestinal epithelium. The SIgA-bacteria complex stimulates the production of pIgR, and of the TJ protein and reduces the NF-kB signalling resulting in pro-inflammatory responses. The second mechanism involves the M cells. The M cell can recognise the SIgA-bacteria complex, phagocy the complex and direct it to the DC cells. The third mechanism regards the capacity of SIgA cells to select specific and potentially beneficial bacteria, bond and anchor them within mucus to create a beneficial biofilm closed to the intestinal epithelium that helps to exclude potential pathogenic bacteria (Mantis et al., 2011).

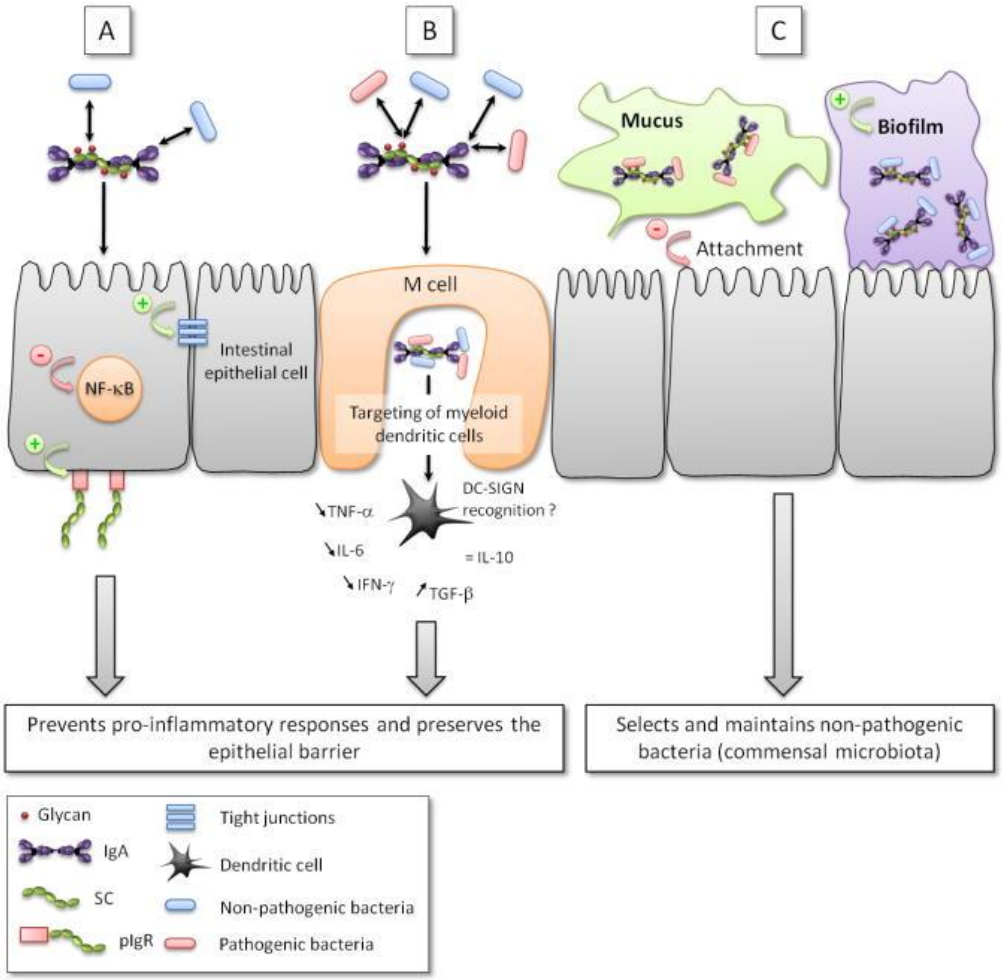

Figure 2. Mechanisms by with the SIgA interact with the commensal and pathogenic bacteria.

A) After the SIgA contact with bacteria the intestinal epithelium barrier is reinforced by the stimulation of TJ, a stimulation of pigR and a reduction of NF-kB. B) The complex of SIgA and bacteria are recognised and uptake by M cells and direct to the DC cell. C) The SIgA can exclude the bacteria through anchoring within mucus the only the beneficial bacteria. Publishers Ltd: Mucosal Immunology: ((Mantis et al., (2011), *Multi-functional interactions of between SIgA and pathogenic and nonpathogenic bacteria in the intestinal mucosa copyright (2011) LICENCE NUMBER 4242021059217).*

Not only the composition but also the thickness of mucus layer is associated with the development of intestinal inflammation as the mucus has the main role to protect the mucosa against digestive secretion, physical or chemical damage and pathogens presences. The mucous secretion needs to be continuous as its constant replacement allow to eliminate bacteria and favour the mucus layer renewal (Cornick et al., 2015).

The IECs proliferation, their death and the level of their turn-over is crucial for the maintenance of the intestinal barrier as IEC act as a physical barrier to selectively regulate the absorption of nutrients and against antigens. The surface of the intestinal epithelium is continually renovated by the differentiation of pluripotent intestinal epithelial stem cells (pluripotent IESCs). The pluripotent IECs originate from stem

cells placed in the crypts and once differentiated IESCs start to migrate from the crypt to the top of the villi. A perturbation of IECs turn-over can ultimately result in an alteration of villus/crypt ratio.

Tight Junctions serve to control the paracellular flux between the IEC cells, while desmosomes and adhesion junctions are central for communication and binding between the IECs (Sharma et al., 2010; Suzuki, 2013). As shown in Figure 3 TJ are organized and composed of several proteins including Occludin (OCL), Claudins (CLDN) and the Zonula occludens (ZO-1). Occludin is a transmembrane protein that allows forming a barrier between IECs and was the first TJ proteins that had been identified. Thus its role was related to the TJ formation, however, a further study of Saitou et al. (2000) reported that wild-type and occludin -/- mice did not show a different epithelial resistance or different morphology in the small and large intestine demonstrating that not only OCL is implicated in TJ formation. However, OLC is implicated in the intestinal integrity and permeability, as it plays has a role in influencing the expression of the other TJ proteins as well as the assemblage of the TJ complex (Rao, 2009; Al-Sadi et al. (2011). CLDN have a central role in the regulation of the intestinal integrity because CLDN interact with the ZO-1in the intracellular environment, while in the extracellular space CLDN serve to create the barrier or pores. CLDN 1, 3, 4, 5, 8, 9, 11, and 14 are involved in the barrier-forming and CLDN 2, 7, 12, and 15 in the pore-forming claudins. The pores in the TJ serve to allow molecules or ions of specific charge and size to pass through IECs and they are generally more present in the small intestine, where the absorption of nutrients takes place, respect to the large intestine (Suzuki et al.2013). ZO-1 proteins are intracellular proteins mainly involved in the intestinal integrity because they are able to interact both with different proteins in the TJ (CLDN) and with the actin skeleton (Suzuki et al.2013)

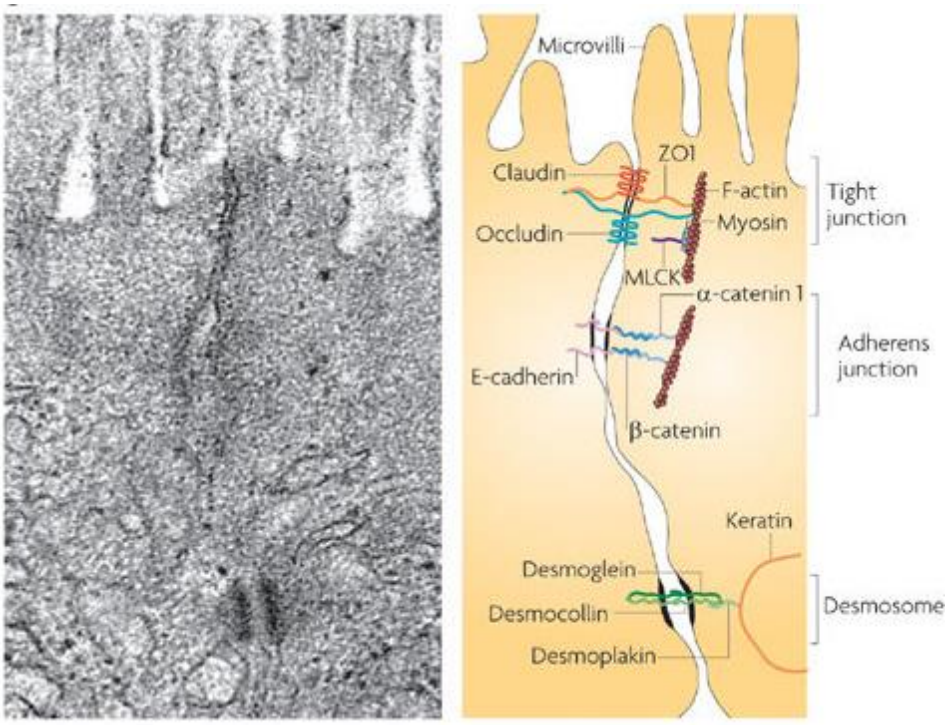

Figure 3: IEC, TJ, Adherens Junctiona and desmosome.

Below the base of the microvilli, the adjacent IECs are linked by the tight junction that are composed by a complex interaction between claudins, zonula occludens 1, occludin and F-actin. The adherens junction is composed by E-cadherin, α-catenin 1, β-catenin. While in the beneath the apical junctional complex, the desmosome allows the IEC junction by its structure composed by desmoglein, desmocollin, desmoplakin and keratin filaments. Published in Nature Reviews Immunology: ((Turner, 2009), *Intestinal mucosal barrier function in health and disease copyright 2009; LICENCE NUMBER 4242521482402*).

Particularly after weaning the intestinal barrier function is damaged. The low feed intake/transient anorexia that characterizes the immediate period after weaning, concur in te onset of inflammation in the gut and in favour the occurrence of diarrhoea; indeed, more than 10% of piglets do not ingest any feed immediately after weaning. This cause a not sufficient enteral nutrient, especially in the upper part of the intestine, that concurred to reduce the proliferation of epithelial cells, as villus atrophy in the small intestine is usually observed. The intestinal mucosa reduces its relative weight by 20-30% during the first two days after weaning and its full recovery is estimated to take from5 to 10 d after weaning (Lalles et al., 2004). The villus height during the first 24 hours post-weaning can be reduced by the 75% (Hampson 1986).

In the same way, the TJ proteins expression (ZO-1 and OCL) both in ileum and jejunum after weaning are downregulated than their expression pre-weaning, showing a damage of barrier function. Their mRNA expression generally increases slowly after the weaning (Wang et al., 2016). Furthermore, different approaches based on the new omics techniques evidenced that at weaning and immediately after weaning, the epithelial cell proliferation is damaged. Transcriptomics studies on IECs after weaning evidenced that

the genes involved in the cell proliferation pathway are downregulated compared to the pre-weaning phase, while genes involved in the apoptosis and pro-inflammatory are up-regulated (Wang et al., 2008; Zhu et al., 2014). According to that, the study of IECs using a proteomic approach evidenced that after weaning the expression of proteins involved in cell cycles are down-regulated as well as the protein involved in the mTOR pathway while protein involved in the cycle arrest, protein catabolism and amino acid metabolism are more enriched (Yang et al., 2016).

Literature shows that different factors influence the barrier function in the post-weaning period, among them, the age of weaning, the level of stress and the level of feed intake as well as the diet form and composition (Wijtten et al., 2011). The early weaning can compromise the barrier function as the younger piglets have a lower intestinal maturity (Smith et al., 2009). In the same way, the stress due to weaning induces an activation of the immune response. The corticortropin releasing factor (CRF), produced in stress condition can activate the mast cells through a mechanism that release proteases and TNFα are able to cause a loss of barrier function and a damage of TJ and permeability.

2.1.3. Microbial recognition and immune signals

The continuous cross-talk between host and microbiota required immune recognition and immune regulation. In addition to physical barriers such as mucus layer and immune structures such as PPs, organised lymphoid tissues described above, the mechanism that involved the Pattern Recognition host-receptors (PRRs), present both in the epithelial and DC cells, in the recognition of bacteria (Microbe-Associated Molecular Patterns – MAMPs) is shown as one of the key mechanism by which the host and the microbiota act a continuous cross-talk (Schroeder and Bäckhed, 2016; Stokes, 2017). The PRRs can be classified in trans-membrane protein as the Toll-Like Receptors (TLRs) and the Nucleotide-binding Oligomerisation Doman (NOD)-like Receptors (NODs) (Table 3). The immune response activated by the TLRs involved the mitogen-activated protein kinase (MAPK) and the Nuclear Factor B (NF-kB) (Stokes, 2017). The MAPK and NF-kB cascade cause the activation of the T cells and immune response genes, with an increased cytokine production. Once that the inflammatory response is activated, it can be stopped both by the host through the regulatory T cells and by the commensal bacteria. The commensal bacteria can stop the inflammation process by the inhibiting the of the NF-kB expose activation of PPAγ signals, transforming growth factor- (TGF-) and MMP3K pathways (Sharma et al., 2010). The microbial activation of the immune response is crucial to stimulate/initiate the development of the intestinal immune system, that at weaning is not mature yet. Indeed, the absences of enteric microbiota reduce the TLRs family member. Comparison between germ free mice and conventional mice shows that the intestinal mucosa of conventional mice have higher villi, a more organised Gut-Associated Lymphoid Tissue

(GALT) higher antibody production, a thicker lamina propria (Macpherson and Harris, 2004). More in details, some specific bacteria have been linked to immune system development. For instance, cytophaga-flavobacter-bacteroidetes shows the ability to stimulate the T cells production of IL17 in the lamina propria of mice (Ivanov et al., 2008), *Bacteroides fragilis* shown the ability to stimulate the TLRs pathway in mice colon (Round et al., 2011) and Lactobacilli have been associated to the stimulation of IL-22 through the Aryl Hydrocarbon Receptor (AHR) by its bacterial production of e indole- 3-aldehyde (Zelante et al., 2013). Very few data are reported for pig, however, a study in gnotobiotic pigs, delivered by caesarean section into fully germ-free bubbles, and supplemented with different mix of bacteria showed that they had higher plasma level of IgA and IgM compared to germ-free piglets due to the intestinal gut stimulation of the intestinal immune system (Laycock et al., 2012). That stimulation of the intestinal immune system by the bacterial colonisation of the gut was observed also by Inman et al. (2010), which observed a higher number of dendritic cells in the intestinal mucosa of piglets reared in high-hygiene (isolator plus milk formula) compared to piglets earing under low- hygiene condition (farm plus sow's milk). While, considering the post-weaning piglets, Mach et al., (2015) show that piglets included i the microbial enterotype constituted by *Prevotella* had a higher level of blood IgA compared to the piglet belonging to the Ruminococcaceae enterotype, thus further research in this field will be desirable.

In return, the different level of immune system maturation is able to modulate the profile of commensal and pathogenic bacteria in the intestine (Sharma et al., 2010).

Table 3. Host receptors, PRRs location on the host intestinal cells, specific MAMPs of recognition and respective bacteria.

PRRs family	PRRs type	location in IEC	MAMPs	Bacteria
TLRs	TLR1	Surface membrane	Triacyl lipopeptides	Mycobacteria
	TLR2	Surface membrane	Peptidoglycan and different lipoteichoic acid in association with TLR1 and TLR6	Gram-positive
	TLR4	Basolateral membrane, endosomal membrane	LPS	Gram-negative
	TLR5	Basolateral membrane	Flagellin	Bacteria
	TLR6	Surface membrane	Diacyl lipopeptides	Mycobacteria
	TLR9	Endosomal membrane	cytosine-phosphate-guanosine oligodeoxynucleotides (CpG-ODN)	Bacteria
NODs	NOD1	Cytoplasm	Meso-lanthionine and meso-diaminopimelic acid	Gram-negative
	NOD2	Cytoplasm	Muramyl dipeptide	Gram-positive and Gram-negative

Adaptation from (M. Fukata and M. Arditi, 2013; Stokes, 2017)

2.2. Food processing and nutrients and fluids uptake

Diet is one of the main factor shaping the microbial composition as already explained in chapter 1.3. Indeed, diet is the primary source of nutrients both for the host and for the microbiota. Microbiota play a key role in the digestion of dietary compound and it interacts with the host giving an important contribution in the host metabolism. Indeed, several studies have associated the variation in gut microbial composition with some metabolic disease such as 2 diabetes (T2D) (Larsen et al., 2010) and obesity

(Pedersen et al., 2013; Turnbaugh et al., 2009). The first evidence of this association has been observed in leptin deficient mice, characterized by obesity and an excess of appetite, which microbiota was composed by fewer Bacteroidetes and more Firmicutes than control mice (Ley et al., 2005). Furthermore, faecal transplantation experiment showed that germ-free (GF) mice changed their fat deposition and their total body mass according to the type of received microbiota. The different microbiota modulate the microbial and host cross-talk through the production of short-chain fatty acids (SCFA), the metabolism of branched-chain amino acids (BCAA), and the transformation of bile acid species (Ridaura et al., 2013). The cross-talk between host and microbiota for nutrients absorption and digestion varies according to the GI tract and the age of the host. In the small intestine, bacteria may compete with the host for the nutrients uptakes, while in the large intestine the microbes have a beneficial role as they contribute to the transformation of non-digestible dietary components. For this reason, the following sections present a brief description of piglet absorption and digestion variability according to the GI tract in the period around weaning.

2.2.1 Piglet intestinal absorption and enzyme function

The majority part of the nutrient absorption takes place in the small intestine. The pig small intestine is composed of three section: duodenum, jejunum and ileum. It measures 3.5 meters in length in new-born piglets and up to 20 meters in adult pigs. The nutrients transport across the small-intestinal epithelium can be divided into paracellular and transcellular. The paracellular transport is represented by the diffusion of nutrients through the intestinal cells thanks to the tight junctions and pores between the IECs. The transcellular transport requires the uptakes of the molecules and it can be carrier-mediated (active) or carrier-unmediated (passive) in the enterocytes or by endocytosis for the macromolecules (i.e. maternal Igs) in the PP (Wijtten et al., 2011). In order to correctly allow the absorption of nutrients, in particular in the small intestine, the feed needs to be digested by the digestive enzymes. In the small intestine, the enzymes are synthesized by the mature enterocytes, located in the apical part of the intestinal membrane in the microvilli (Cheng and Leblond, 1974). For this reason, the general enzyme activity is strictly correlated to the villus height to crypt depth ratio and it has been recognised to reduce immediately after weaning (Lallès et al., 2004). The amount and type of digestive enzyme in pigs are age-dependent. In details, the lactase activity in brush border is generally reduced after weaning (Motohashi et al., 1997), while for the other enzymes involved in disaccharides digestion contrasting results are reported. Indeed, Kelly et al. (1991) observed an increase of sucrase, maltase and glycoamylase activity one week after weaning, while a reduction of sucrase and maltase activity was observed by Miller et al. (1986) and Hedemann and Jensen (2004). The peptidase activity around weaning is also compromised, probably for

the low feed intake associated to the weaning (Hedemann et al., 2003). Indeed, after the first 5 days post-weaning, when piglet rises the feed intake, enzyme activity is generally raising (Pluske et al., 1997).

Despite the main part of nutrients are absorbed in the small intestine, a lower percentage of nutrients absorption occurs also in the proximal part of the large intestine of new-born pig as rudimentary villi have been described (Xu, 1996). Furthermore, in the large intestine, where the number of intestinal bacteria is higher respect to the small intestine, the absorption of bacteria products take place.

2.2.2. Microbial products and host metabolism

The anaerobic bacteria are mainly responsible for the SCFAs production that originated mostly by the dietary fibres fermentation. As pigs lack the enzymes to degrade the bulk of dietary fibres, anaerobic bacteria hydrolase no digestible carbohydrates into oligosaccharides and then monosaccharides. This metabolic process occurs mainly in the large intestine where the environmental (higher pH and low level of oxygen) and the absences of an organized immune surveillance allow a higher development of bacteria (Besten et al., 2013). Acetate, propionate, and butyrate are the main SCFAs that are produced by bacteria and used for energy metabolism by the host. Acetate is either generated by hydrolysis of acetyl-CoA or from CO2 via the Wood-Ljungdahl pathway (Macy and Probst, 1979; Pryde et al., 2002). Propionate can be formed from phosphoenolpyruvate (PEP) via the primitive electron transfer chain or by the reduction of lactate to propionate, through the acrylate pathway; butyrate is formed by condensation of two molecules of acetyl-CoA and a following reduction to butyryl-CoA (Miller and Wolin, 1996). When SCFAs are in the intestinal lumen, they can be absorbed by the host. The level of bacteria SCFAs showed the ability to regulate the host absorption, through the presence of GpR41, a G protein that binds the bacteria SCFAs. Furthermore, GpR41 can stimulate the peptide tyrosine tyrosine (PYY), a hormone able to stimulate the intestinal motility and SCFAs absorption (Samuel et al., 2008). The absorbed SCFAs are then available for the host energy metabolism. Acetate can enter in the tricarboxylic acid (TCA) cycle or can be used for the synthesis of cholesterol, ketone bodies, and long-chain fatty acids; propionate take part in gluconeogenesis in the liver; and butyrate is involved in the mitochondrial fatty acid oxidation by which the resulting acetyl-CoA can be used in the same way of acetate (Besten et al., 2013). The intestinal bacteria can thus regulate the host energy metabolism, however, the host in exchange of SCFAs release bicarbonate. This exchange can be considered a mutualism response between host and microbes, indeed the host takes advantage to SCFAs and the bacteria need the bicarbonate to regulate the pH level to survive as the SCFAs production tends to decrease the pH level (Besten et al., 2013).

Gut microbiota interact with the host also for the protein utilization. Bacteria can contribute to the protein deconstruction of amino acid through the protease enzyme. Once that the amino acids (AA) are available,

in the small intestine, where the AA absorption occurs, the bacteria can compete with the host. Indeed by measuring the portal balance, it was shown that 30–50% of the dietary essential AA were not recovered in the portal blood and less than 20% of the AA were utilized to synthesize the mucosal protein (Stoll et al., 1998), thus the rest part of the AA could be metabolized by intestinal bacteria. Indeed, it is known that cells of the piglet intestinal mucosa are able to degrade non-essential AA and BCAA, while no observation of lysine, tryptophan, threonine, histidine, phenylalanine ormethionine catabolism in the intestinal mucosa has been established (Wu, 1998; Torrallardona et al., 2003). Though Riedijk et al. (2007) showed that enterally administered methionine was degraded in the piglet intestine and van Goudoever et al. (2000) observed in growing pigs fed a high-protein diet that the intestinal oxidation of enteral lysine contributed to total body lysine oxidation with a quote of 30%, the clear evidenced that these AA are oxidated by mucosal cells is still lacking (Chen et al., 2009). Furthermore, bacteria particularly participate in the metabolism of lysine, threonine, arginine, glutamate and glutamine in the small intestine (Dai et al., 2010, 2012). On the other side, in the large intestine, where the host amino acids absorption cannot occur, and where the number of microbes is higher, the amino acids are metabolized by bacteria without a competition with the host (Neis et al., 2015).The amino acids can be directly incorporated into bacterial cells as protein building blocks, or become catabolized to produce SCFA and branched-chain fatty acids (BCFA; valerate, isobutyrate, and isovalerate) or ammonia (Neis et al., 2015).

Gut microbiota are also involved in the lipid and cholesterol metabolism as study in mice showed that altered microbiota can modulate the bile acid production and thus regulate the cholesterol metabolism (Gérard, 2013). Furthermore, association studies have linked the gut microbial composition to lipid-metabolism disease such as diabetes (Wen et al., 2008). Indeed, the intestinal bacteria are able to use the bile acid produced by the host in the liver and transform them in secondary bile acid involved in the host lipid-metabolism (Ridlon et al., 2006). On the other side, the level of available bile acid produced by the host is able to modify the gut microbiota composition by a selection of BA-tolerant bacteria (Ding et al., 1993).

The gut microbiota and in detail specific bacteria, are also recognised for their role to produce some vitamins such as K, B12, Biotin, Folate and Thiamine. Indeed germ-free animals required a vitamin K and certain B vitamins supplementation (Hirayama et al., 2007), while in conventionally colonized animals, these vitamins can be produced by several intestinal genera, mainly belonging to *Bacteroides* and *Eubacterium* (Nicholson et al., 2012).

2.3. Gut-Bain axis

As described above, the microbiota is involved in the activation and modulation of host digestion and metabolism as well as in his intestinal immune response and intestinal integrity. Those GIT functions are strictly linked with the host brain functions, therefore, the potential direct cross-talk between microbiota and host brain has taken interest in studying. Nowadays, it is indeed recognizing that the intestinal microbiota takes part of the gut-brain axis for its capacity to produce some particular metabolites and hormones. The core neuroendocrine pathway mainly involves the hypothalamic-pituitary-adrenal (HPA) axis (Grenham et al., 2011), however, several mechanisms, summarised in Table 4, by which the gut-brain interact are now being unravelled (Cryan and Dinan, 2012). These mechanisms involve the immune response activation already described in the previous section, the signalling of vagus nerve, the bacteria production of neurometabolite such as gamma-aminobutyric acid (GABA), serotonin, noradrenalin and acetylcoline, the modification of the tryptophan metabolism and the specific bacteria cell wall sugar composition.

In detail the HPA axis is one of the most important parts of the neuroendocrine system. It is involved in the modulation of stress responses and some physiological processes such as digestion and immune regulation. A study in germ-free (GF) mice showed that, the gut microbiota population in GF mice respect to specific pathogen free (SPF) microbiota mice can produce higher corticosterone and adrenocorticotropic hormone that are included in the hypothalamic–pituitary–adrenal (HPA) axis and Is responsible for a higher level of stress in GF mice (Sudo et al., 2004).

The vagus nerve is implicated in the intestinal motility and gastric juice production, however, a study of Wang et al., (2002) showed that mice exposed to subdiagphragmatic vagotomy had lower blood level of CD8+ T cells and of CD4+ and CD8+ T cells and that, when mice were infected with *Salmonella*, they were less resistant to the infection. This was associated to the lower stimulation of CD4+ and CD8+ which are involved in the pathogen's response, thus this study showed that vagus nerve can also be involved in the communication of immune signals from the gut to the brain and in maintaining the immune balance of the organism.

The microbiota-neurohumoral relationship starts since from the early microbial colonisation of the gut. It depends on the production of bacterial by-products which can stimulate the enteroendocrine cells (EECs) of the host. The EECs can than produce neuropeptides including peptide YY, neuropeptide Y (NPY), cholecystokinin, glucagon-like peptide-1 and -2, and substance P, which can regulate the brain functions (Cani and Knauf, 2016). How those neuropeptides can interact with the brain is not completely understood, anyway, the hypothesis is that they can interact with the immune cells of the lamina propria

or they can activate local receptors or the neuronal innervations (Foster et al., 2017). Among the bacteria production compounds, serotonin is one of the key compounds involved in the gut-brain axis. Different type of serotonin receptors including 5-HT1, 5-HT2, 5-HT3, 5-HT4, have been identified and in the gut are expressed the 5-HT3, and 5-HT4 which are involved in the GI secretion of Cl^- and in the intestinal motility by acting in the smooth muscle contraction (Costedio et al., 2007). In the brain, serotonin is mainly involved in mood and cognition regulation. In addition, serotonin is also strictly linked with the tryptophan pathway, as tryptophan is a serotonin precursor. For instance, using a GF mice model, it has been observed a positive correlation between the increase of hippocampal levels of serotonin and a respective increase of tryptophan concentrations in blood of GF mice compared to conventional mice (Clarke et al., 2012). This study demonstrated the relation of these compounds and their role as mediators between the gut microbiota and the brain function. Furthermore, a study carried out in caesarean pigs demonstrated a negative correlation between the level of faecal *Ruminococcus,* the level of serum serotonin and cortisol, and the amount of celebral N-acetylaspartate (NAA), which is considered a marker of neuronal health (Mudd et al., 2017).

In addition, a new possible pathway that involves the nervous cannabinoid receptors, with particular reference to the activation of CB1 and CB2 receptors, has also recognised as a possible way of interaction between gut microbiota and host brain (Magno et al., 2015). Indeed it is shown that the number of gut *Lactobacillus* sp. is positively correlated whit the activation of these receptors which are able to promote the activation of NF-kB pathway involved in the stimulation of cytokines production (Aguilera and Martínez, 2015).

Table 4. Mechanisms involved microbial-host cross-talk involved in the gut-brain axis and their implications.

Mechanism	Implication	Reference
Immune activation	Pro-inflammatory and anti-inflammatory cytokines that directly affect brain function	(Dantzer et al., 2008)
Vagus nerve	Gut motility, anti-inflammatory capacity	(Foster et al., 2017)
Microbial neurometabolites	GABA, serotonine, noradrenalin, acetylcholine	(Lyte, 2011; Barrett et al., 2000; Costedio et al., 2007; Clarke et al., 2012; Cani and Knauf, 2016)
Tryptophan metabolism	Kynurenine-tryptophan inflammatory pathway	(Clarke et al., 2009)
Bacterial cell wall sugars	Exocellular polysaccharide protect bacteria by acid and bile	(Fanning et al., 2012)
Nervous Cannabinoid Signaling	Activation by CB1 and CB2 receptors of the	(Magno et al., 2015)

Table adapted by Cryan and Dinan, 2012 and Foster et al., 2017

On the other hand, the host stress can impact on the gut microbiota composition. Studies show that the stress compound like neuroendocrine hormones related to the catecholamine family can reduce the level of *Bacterodies* given an advantage to *Clostriudium* development (Lyte, 2011). Social stress, that increases the circulating cytokines, has reported to reduce the *Coprococcus*, *Pseudobutyrivibrio*, and *Dorea* by increasing the level of IL6 and MCP-1 (Bailey et al., 2011). Only a few studies in pigs are available, anyway considering the weaning one of the crucial stress events of piglets, a reduction of beneficial bacteria such as *Lactobacillus* spp. and an increase of *Peptostreptococcus anaerobius*, *Moraxella cuniculi*, *S. suis* and *Porphyromonas catoniae* are observed (Su et al., 2008).

3.THE ROLE OF GENETICS IN MODULATION OF GUT EUBIOSIS

The environmental factors can strongly influence the microbiota composition, but recently the host genetics shows to play an important role in shaping the microbiota composition (Spor et al., 2011), to such an extent that Nibali et al., (2014) introduced the term "genetic dysbiosys" to underline the crucial role of genetics in driving the microbial development and the occurrence of disease. This term started by the observation of that the microbiota composition is associated to metabolic diseases and that those metabolic diseases are also influenced by genetics variants and that thus microbiota and host genetics had a shared common effect. A mice study has then demonstrated a correlation between the host-genetics the microbiota composition and a mutual effect on the development of metabolic disease such as obesity (Ussar et al., 2015). Since those observations, several studies started to disclose the potential role of genetics on gut eubiosis using different genetics studying approach including the use of differential genetics lines or breeds, the genetic loci approach, the Genome Wide Association (GWA) approach, the candidate gene approach or population-based twin approach.

The work of Goodrich et al., (2014) conducted on human twin pairs individual has shown that several bacteria family such as Lachnospiraceae, Ruminococcaceae and Christensenellaceae are hereditable (until 0.39 of estimated heritability) in human gut. The previous study using the linked genetic loci have associated specific Quantitative Trait Loci (QTLs) to abundances of gut bacteria in mice (Benson et al., 2010; McKnite et al., 2012). Research in human have shown that host mitochondrial DNA haplogroups are associated with microbiome communities such as Prevotella and Bacteroides (Ma et al., 2014), and applying the genome wide association (GWA) study, new specific host genetic mutations have been associated to different abundance of bacterial taxa (Davenport et al., 2015). Furthermore, some additional studies observed the effect of particular candidate or functional genes in shaping the gut microbiota as reported for a mutation on Nucleotide Binding Oligomerization Domain Containing 2 (*NOD2)* gene or for or a mutation on Fucosyltransferase- 2 (*FUT2)* gene. In details, *NOD2* gene is responsible for the degree of NOD2 receptors in the IECs of the host. A mutation on *NOD2* gene, giving less NOD2 receptors (NOD2-deficient mice: *NOD2*-/-), showed that *NOD2*-/- mice have higher loading of commensal bacteria, different commensal microbial composition and a reduced capacity to contract pathogens infection (Petnicki-Ocwieja et al., 2010), and accordantly to these, also in human a variation in *NOD2* genotype has been associated with a higher abundance of Enterobacteriacae and to the IBD disease occurrence (Knights et al., 2014). The fucosyltransferase- 2 gene is involved in the creation of a precursor of the H antigen, which is required for the final synthesis of soluble A and B antigens of ABO blood groups. The biological connection between *FUT2* gene and ABO blood groups, coupled with the recent finding that differences on ABO blood groups can influence the antigens expression, which can act as receptor or co-

receptor for intestinal bacteria in the intestinal mucus layer, increased the interests on these two genes in modulating the intestinal bacteria composition (Cooling, 2015). However, contrast results have been reported. A first study carried out in mice, observed an influence of *FUT2* gene in determining the mice susceptibility to *Helicobacter pylori* infection through the modification of the gastric glycosylation profile (Magalhães et al., 2009). the first study in humans, carried out by Wacklin et al. (2014), showed that the secretory status, determined by *FUT2* gene, was able to affect the faecal microbia composition of adult human in terms of species richness and taxa composition, while a subsequential study carried out by Davenport et al. (2016) failed to verify that human ABO profile or secretor status were significantly associated with a different faecal microbial community in terms of structure, diversity, and the relative abundances of individual taxa. In light of what has been observed, further research will be required to disclose the possible role of genetic variance in influencing the gut microbial community and the host physiological status.

Considering the livestock animals, to date, very few studies have been undertaken to explore the genetic effect in GIT microbial composition using the heritability and gene loci approach, anyway, a study of Roehe et al. (2016) conducted on two breeds of bovine showed that genetic can influence ruminal microbiota and in detail the bacteria involved in the methane production, opening new prospects for the breeding selection schemes in animals. In pigs, this field is not completely explored as well, but the difference in microbial composition has been ascribed to different breeds. A similar microbial composition is observed in faeces of adult pigs of Landrace and Yorkshire breeds compared to Duroc and Hampshire breeds, the differences between the two groups have been attributed to the higher butyric, isobutyric, valeric, and isovaleric acid bacteria species present in Landrace compared to a higher propionic acid bacteria producer observed in Hampshire (Xiao et al., 2017). Faeces analysis of fifteen weeks old pigs belonging to Landrace breed also show that this breed has a higher microbial variability in the faces compare to Yorkshire and Duroc breeds, while Duroc breed is recognized to be a unique cluster for its microbial composition (beta diversity index) respect to the microbial composition of Landrace and Yorkshire breeds. That could be partially explained by the fact that Duroc breed is genotypically closer to the wild type pigs compared to Landrace and Yorkshire (Pajarillo et al., 2014). In the same way, Kanengoni et al., (2015) found differences between common commercial breed such as Large White × Landrace cross-breed and the South African Windsnyer-type indigenous adult pigs. The indigenous pigs showed a more abundant level of *Bacteroides*, *Succiniclasticum*, *Peptococcus* and *Akkermansia* then the commercial cross-breed and authors linked these difference to the better capacity of indigenous pigs to digest higher fibrous diets. Considering the Chinese breeds, faecal samples of adult pigs reared in the same farm and feed the same diet showed that the microbiota composition of commercial breed such as

Large White, and derived cross-hybrid population, is different to the one of the native Chinese Bama minipigs and the BaRing pigs (Xiao et al., 2016). However, these studies have been carried out on mature and adult pig, then a lack of information in the post-weaning period is present, indeed only a study of Bian et al. (2016) explored the genetic influence on microbiota composition in young piglets by using the cross-fostering model. The results showed that genetic impacts the faecal microbial composition without a continuous effect among age of animals, indeed, it was significant only on days 14 and 49of age, but not immediately after birth (1,3 and 7) and neither immediately after weaning at day 28 of age.

Applying the functional or candidate genetic approach few genes have been highlighted for their potential role in influencing the microbial eubiosis and host-microbial cross-talk. This genetic approach in pigs has mainly be applied to some important intestinal disease such as the PWD, and specifically, the colibacillosis caused by ETEC F4 and F18 showed to be genetically affected. The putative candidate genetic markers for ETEC F4ac and F18 are described in the following paragraphs.

3.1. Candidate genes for piglet ETEC F4ac infection

The ETEC F4ac is one of the main cause of PWD in piglets and it remains until now a critical problem especially because of the need to reduce the use of antibiotic and ZnO. Since no effective vaccines are available the potential strategy to select genetically resistances pigs could represent an additional potential strategy to reduce the occurrence of the infection. For this reason several putative candidate genes for controlling the ETEC F4ac infection have been identified during the last year including Mucin4 (*MUC4*), Mucin 13 (*MUC13*), Mucin 20 (*MUC20*), transferrin receptor (*TFRC*), tyrosine kinase, non-receptor, 2 (*ACK1*), UDP-GlcNAc:BetaGal Beta-1,3-N-Acetylglucosaminyltransferase 5 (*B3GNT5*). All these genes were have been mapped on the locus responsible for ETEC F4ac susceptibility locate on pig chromosome 13 in the q41 region (Jørgensen et al., 2003; Python et al., 2002).

MUC4 gene encodes for a membrane-bound-O-glycoprotein that belong to the mucin family and constitutes the mucus layer. A single nucleotide polymorphism (DQ848681:g.8227C>G) of *MUC4* gene had been associated with ETEC F4ab/ac strains diarrhoea with the dominant G allele representing F4ab/ac ETEC susceptibility and the recessive C allele representing F4ab/ac ETEC resistance (Jørgensen et al., 2003). This genetic marker has been widely utilized in different research studies and is considered one of the most associate genetic markers for ETEC F4ac.

MUC13 gene encodes for a transmembrane mucin expressed predominantly in the epithelial surface of piglet jejunum and has been intensively studied as a candidate gene for ETEC F4ac susceptibility,

therefore several putative mutations have been identified. Ren et al. (2012) suggested that 2 SNPs on *MUC13* gene (MUC13A and MUC13B). MUC13B showed distinct tandem repeats in exon 2 that encodes for a heavily O-glycosylated region in the protein, that mutation was observed as the most promising by the authors as it could act as a putative binding site for ETEC F4ab/F4ac. An additional SNP A157G on *MUC13* has also been patented as putative genetic marker for selecting pigs resistant to ETEC F4ac (Huang et al., 2006) and recently a new region located between chr13: 144,810,100–144,993,222 of *MUC13* gene have been associated to the ETEC F4ac adhesion on the intestinal villi (Goetstouwers et al., 2014).

The porcine *MUC20* gene maps in the region associated to the ETEC F4ab/ac receptor (SSC13q41) and was proposed as potential candidate gene for the ETEC F4ab/ac receptor. Two SNPs: g.191C/T in intron 5 and c.1600C/T in exon 6 were identified and associated to the *in vitro* ETEC F4ab/ac adhesion and among them the g.191C/T was significantly associated with the ETEC F4ac adhesion phenotype, however, *in vivo* trials for its validation have not been performed until now (Ji et al., 2011).

TFRC gene encodes for a cell surface receptor necessary for cellular iron uptake by endocytosis and it could biologically influence the *E. coli* as this bacterial survival and development are influenced by iron availability. Three SNPs were identified: g.591 A/G and g.632 A/G located in an exon and SNP 291 C/T located in an intron. Among these SNPs, 291 C/T polymorphism showed the highest association with F4ac receptor locus (Wang et al., 2007), however, this genetic marker has not been used by other researchers thus we lack reproducibility. The *ACK1* gene is closely linked to *TFRC* as it can influence *TFRC* expression but not further studies have been performed on *ACK1* association with *ETEC* F4ac infection.

B3GNT5 gene located on SSC13q23-q41 encodes for the enzyme that transfers N-acetylglucosamine to glycolipid substrates. As N-acetylglucosamine is involved in the adhesion of ETEC F4ac in the intestinal epithelium, *B3GNT5* has been studied as candidate gene for the infection. Ouyang et al. (2012) identified 4 SNPs on *B3GNT5*: g1242 A/G; g.1389 G/T; g.1476 G/A and g.1629 T/C and associated the [A;T;G;T] and [A;G;G;T] haplotypes with susceptibility/resistance to ETEC F4ac, however the authors concluded that the association was weaker respect to the one identified for the other candidate genes such as *MUC13* and *MUC4* as the significant association found for *B3GNT5* was numerically lowed and not verified in a different population.

3.2. Candidate genes for piglet ETEC F18ac/ab infection

To date, genetic research for ETEC F18 susceptibility have been performed with a lower intensively compared to the genetics research for ETEC F4ac susceptibility, however, two candidate genes have been identified: Alpha (1,2)-fucosyltransferase (*FUT1*) and Bactericidal/permeability-increasing protein (*BPI*).

FUT1 gene has been initially highlighted by Vogeli et al. (1996), they showed that the susceptibility for ETEC F18 infection was closely linked to the blood group inhibitor S located in the porcine chromosome 6. Following this research and using the candidate gene approach and linkage analysis Meijerink et al., (1997) suggested *FUT1* as the candidate gene for the regulation of the expression of the putative ETEC F18 receptor, already described in the chapter 1.2.2. Furthermore, the study showed that a single point mutation located at 307 pb on *FUT1* gene was able to influence the ETEC F18 pig susceptibility. The mutation results in three genetic variance AA, AG and AG and, pigs with GG or AG genotypes were considered susceptible to ETEC F18 infection while AA animals were considered resistant (Meijerink et al., 1997; Vogeli et al., 1997). The *FUT1* 307 A/G mutation causes the Ala→Thr substitution at amino acid position 103 (Meijerink et al., 1997) resulting in a variation of properties or the quantity of the mature FUT1 enzyme and that was recognized as the mechanism by which *FUT1*307 mutation can influence the differential expression of the F18 receptor (Snoeck et al., 2004). Indeed, levels of FUT enzyme were observed significantly lower in F18 ETEC-resistant animals than in susceptible animals (Meijerink et al., 2000). FUT enzyme is involved in the formation of blood-group antigen sugar structures as it catalyses the addition of terminal alpha (1,2) fucose residues and concurs to produce the H type 1 carbohydrate that is also expressed on the surface of epithelial cells and in mucosal secretions (Henry et al., 1996). For this reason, pigs with opposite *FUT1* genotypes may present a different fucosylation patter of carbohydrate structures expressed on cell surfaces of the gut as observed by Coddens et al (2008) and Hesselager et al. (2016).

Additional studies using transcriptomic and proteomics approach in the small intestine of *FUT1* resistant and susceptible pigs tried to clarify the pathway and mechanism by which *FUT1* gene can regulate the host susceptibility. These study confirmed the role of *FUT1* in the glycosphingolipid biosynthesis pathway and observed a correlation of *FUT1* whit the expression of *A0* and *ST3GAL1* genes which are both involved in the production of sugar expressed in the intestinal epithelium surface (Bao et al., 2012a). The proteomics approach applied to disclose the differences in the duodenum profile of healthy resistant and susceptible pig showed that weaners with the susceptible genotype have lower transferrin (TF) expression and higher expression of heat stress proteins (HSPs), two proteins mainly involved in immune responses

and in case of TF also in the bacteria growth, as transferrin is implicated in the iron chelatesi and iron is mainly important for *E. coli* bacteria metabolism (Wu et al., 2015).

Furthermore, the *FUT1* gene expression seems to vary according to the age in duodenum and jejunum, as the expression decreased from day 8 to day 18 and then increased continuously to days 30 and 35, partially explaining why the ETEC infection occurs in the post-weaning period and not in the neonatal piglets (Bao et al., 2012b).

BPI gene is located at 7q21-23, it consists of 15 exons and 14 introns and encodes for the lipopolysaccharide binding protein (LBPI). The LBPI protein can be produced by neutrophils and is involved in several immunological pathways as it has an important affinity for LPS and other endotoxins (Levy et al., 2000). Mutation on the exon 10 identified by Christopher et al., 2004 and already patented as candidate markers for *Salmonella* infection in pigs, has also been correlated with the resistance to ETEC F18 infection by Liu et al. (2013). After the HpaII digestion, the gene produced AA, AG and GG genotypes where the AA genotype is associated with the ETEC F18 resistant animals with an error of 10% and the other two genotype with a lower capacity to resist to ETEC F18 infection: AG with a percentage 57.1% of resistant animals and GG only with the 17.4%. This animal capacity to resist to ETEC F18 have been directly associated by the authors to the higher expression of the *BPI* gene in the intestinal tissue and thus to the gene functionality.

4. IN VIVO ANIMAL MODEL TO INTEGRATE THE GENETIC AND FEEDING RESEARCH

Genetic, as described in the previous chapter, has taken a large interest in the last years for its role in shaping the intestinal microbial community structure as well as the abundance of specific taxa. Furthermore, in piglet, genetic markers have shown to play a key role in influencing the animal susceptibility to specific pathogens. Diet, is recognised as one of the main factors affecting the intestinal microbial eubiosis and different feeding strategy, particular during the weaning phase, have been studied to improve the piglet capacity to face the complex situation of weaning. For these reasons, both host-genetics breeding schemes and feeding strategies are now considered as a potential strategy to improve piglets' resistance, however, to date, the major part of research on piglet post-weaning phase have explored the effect of genetic and feeding separately.

In this chapter, is reported an overview of the pigs *in vivo* studies in which the feeding strategies and genetics approach have been applied to improve the piglet health, his immune response and his growth performance in the weaning period, with a special attention in the experimental model design. In this context, as the main problem of piglet at weaning are the colibacillosis infection due to ETEC F4ac and F18, the overview mainly focuses on this infection model, the relative feeding strategies tested to contrast these infections and the inclusion in the model of the genetic markers for the respective ETEC resistances (*MUC4* for ETEC F4 and *FUT1* for ETEC F18).

According to the genotype control for ETEC F4ac and F18 susceptibility and the phenotypically control of ETEC F4ac and F18 adhesion on jejunum villi through the *in vitro* test, a score from 1 to 4 for the experimental model has been assigned as following: 1= no genetic marker control and no phenotypically control; 2 = genetic marker control and no phenotypically control; 3 = phenotypical control and no genetic marker control; 4= genetic marker control coupled with the phenotypical control. The complete list of the identified studies is presented in Table 5a-f, summarizing the presence/absences of genetic markers control and *in vitro* test verification, the model score, feeding intervention, dose of the additive, period of administration, challenge time, and animal observed parameters.

As shown in Table 5a-f, it can be noted that among the selected studies, the majority part of them focus on ETEC F4ac infection. Several feeding supplementations have been tested including the use of some AA involved in the immune response and host microbial cross talk such as tryptophan and threonine, some probiotics including *Lactobacillus sobrius*, *Lactobacillus rhamnosus*, *Bacillus licheniformis* and *Saccaromyces cervisae*, organic fatty acid, nucleotide, spray-dried plasma, however, the experimental

model as well as the response parameters vary among the studies. Based on the model score classification, five studies reach the level 4, three studies were included in class 2 and five in class 1, while the majority part of the studies belongs to the level 3 (7 studies). Considering the studies ranked the model score one, it can observed that despite Li et al., (2015), all the other studies included the challenge and the not-challenge groups to evaluate the effect of the feeding additive after the ETEC infection, however, no specific control for ETEC F4 infection have been included indeed the verification of the infection was mainly based on the faecal score after ETEC challenge, the count of *E. coli* in faeces or in the intestinal content (Liu et al., 2010) or parameters for intestinal integrity and permeability (Pan et al., 2017).

However, as described above and based on the confirmation of Geenen et al. (2007) and Roubos-van den Hil et al. (2017), the inclusion of genetic markers as a considerable factor in the experimental model could add important information regarding the piglet response thus, it could be considered a control point for reducing the variability of the model and resulting in a decrease in number of needed animals for the study. In the collection presented here, three studies were based on the genetic control of the animals and were included in the group of study with model score 2. These studies applied the genetic information in the *in vivo* experiment with two different aims. Sargeant et al. (2010) controlled the *MUC4* genotype of piglets after the animal *in vivo* trial, to include the genetic information as factors in the statistical model to explain the intestinal transcriptomic profile and the animal performances responses; while Yang et al. (2016) and Zhang et al. (2017) used the *MUC4* genotyping to select and include in the *in vivo* trial only the animals with the resistant genotypes for *E. coli* F4ac in order to reduce the animals variability to face the ETEC infection and evaluate the effective use of probiotics.

The studies with model scores three did not take into account the genetic susceptibility to the ETEC F4ac infection, but controlled the possible animal variability to ETEC infection in the post *in vivo* trial through the *in vitro* adhesion test. In case of ETEC F4ac model, this test is mainly based on the protocol of Van den Broeck et al. (1999). It consists of the collection of villi from the jejunum through repetitive mucosal washing and a gentle scraping of the villi. Once that the villi are collected, they are transferred into PBS supplemented with 10 g/L of D-mannose, incubated with the bacterial solution and sub sequentially examined by phase-contrast microscopy to count the number of adhering bacteria. This *in vitro* adhesion test allows to classifing the animal based on the quantity of adhering bacteria and therefore it gives the phenotypical information about piglet susceptibility to ETEC F4ac, resulting in a reliability point for the study. Among the studies included in the group with model score 3, additional specific parameters were used by the authors to effectively confirmed that the specific pathogen infection has been carried out in the infected animals. In case of ETEC F4ac, those specific parameters were recognised as the level of specific immunoglobulin associated to the ETEC F4ac infection such as F4IgA and F4IgG, in blood saliva

and/or faeces (Bosi et al., 2007; Konstantinov et al., 2008) or the degree of ETEC F4ac excretion continuously after the infection for at least one week (Virdi et al., 2013).

Considering the studies included in the model score 4, it is interesting to note that, both tryptophan and threonine supplementation had improved the growth performances of piglets upon the challenge condition and that in both the studies an interaction between the AA supplementation and the *MUC4* genotype had influenced the piglets responses in terms of intestinal integrity and immune responses (Trevisi et al., 2015; Trevisi et al., 2009). Furthermore, this interplay between AA supplementation and ETEC F4ac susceptibility tended to influence feed efficiency response also under normal healthy condition, particularly in case of tryptophan dose in first week after weaning (Trevisi et al., 2010) as well as the mucosal expression profile in the jejunum (Trevisi et al., 2012). Indeed in the ETEC F4ac susceptible piglets, the expression of genes related to innate immune response (*REG3G*, *SPTPD* and *LBP*) were higher in the jejunum of piglet fed the lower Trp:Lys ratios (0.17) then the piglet fed the higher Trp:Lys ratios (0.22). The authors speculate that this differenced in the immune response associated to Trp:Lys ratios and ETEC receptor susceptibility could be partially ascribed to the gut microbiota composition, thus in the subsequential study they analysed the jejunum microbial composition through the e 16S rRNA gene-targeted denaturing gradient gel electrophoresis (DGGE) fingerprinting analysis and they found a decrease of microbial variability in piglet with higher ETEC F4ac susceptibility (Messori et al., 2013).

With regard to the model including the genetic marker and the feeding strategies in challenge experiment with the ETEC F18, seven research studies have been found so far. Among them, three studies had the main objective of studying the effectiveness of a putative vaccine or type of immunization to face the ETEC F18 infection. One of these studies was classified with model score 4, one with score 3 and one with score 1. Focusing on the *in vivo* model that tested the feeding strategies, two studies have been found and include in the group with moel score two as they were taking into account the piglet genetic susceptibility to ETEC F18 infection. However, the experimental protocol of these studies was set up in order to use piglets as a model for human with the aim to test feeding additive to contrast the colitis in human. Thus the model included new-born piglets with the *FUT1* susceptible genotype which were delivered at term by cesarean section, they were initially feed with parenteral nutrition and sow's plasma and were inoculated with maternal faeces on day 1 after birth. From day 2 they received enteral feeding with boluses of milk replacer and from day three the experimental feeding additive. Cilieborg et al. (2017) studied the effect of feed new-born piglet genetically susceptible to ETEC F18 with a1,2-Fucosyllactose (10 g/L 20-FL) upon ETEC F18 infection, while Andersen et al. (2017) tested the protective effect of a supplementation with *Lactobacillus paracasei* (2·6×108 CFU/kg per day) or *Pediococcus pentosaceus* ($1{\cdot}3\times10^{10}$CFU/kg per d) to face the ETEC F18 infection. The results showed that neither a1,2-

Fucosyllactose nor probiotics supply were sufficient to prevent the piglet diahaerrea. However, Cilieborg et al. (2017) found a negative correlation of *E. coli* abundance with endogenous fucose in the 1,2-Fucosyllactose group. Furthermore, the weight loss of ,2-Fucosyllactose group was lower than the infected group and the intestinal integrity was less compromised in term of villi height in small distal intestine.

Considering the studies that actually tested a practical feeding strategy to enface the post-weaning colibacillosis problem, the collection includes two studies, one with model score 1 carried out by Nollet et al., (1999) and one carried out by Sugiharto et al. (2015) with model score 4. Sugiharto et al. (2015) tested the effect of feeding weaning piglets with bovine colostrum or with conventional milk replacement instead of sow milk. Bovine colostrum showed a positive effect in reducing piglets diarrhoea, *E. coli* excretion and mucosal immune responses, however, in this study the animals were not challenged with ETEC F18. Overall, further research for genetic and feeding strategies to contrast ETEC F18 infection are actually desirable.

Considering together the results obtained in the presented collection, we can conclude that both feeding strategies and genetics susceptibility should be considered as factors that can influence the piglet responses to the ETEC infection. Therefore, both these factors should be included in the experimental model in order to maximise the reliability of the experiment and control individual variability of the animals, with the final purpose to reduce the number of animals needed for the experiment and increase the ethical acceptability of animal research.

Furthermore, a genetic-feeding integrate approach could enrich the knowledge regarding the specific feeding requirement of animals according to their genetic background, and could potentially improve the scientific research that aims to develop a more precision farming approach in pig production.

Table 5a. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	Adhesion test	Model score	Feeding intervention and additive dose	Period of administration and animal scarification	Challenge	Observed Parameters	Reference
NA	NA	1	Nucleotide in three doses: 150 mg/kg nucleotide (R150), 220 mg/kg (R220); 275 mg/kg (R275)	From weaning to 42 days post-weaning	ETEC F4 at day 14 post-weaning	Growth; Faecal score, blood total IgA, red blood cell (RBC), white blood cell (WBC) lymphocyte, TNF-α and cortisol after challenge; E. coli and Lactobacillus number in faeces after challenge	(Li et al., 2015)
NA	NA	1	*Lactobacillus rhamnosus* ACTT 7469; two doses $10*^{10}$ CFU/d (LGG) or $10*^{12}$ CFU/d (HGG)	From weaning for 8 days; scarification at day 15 post-weaning	ETEC F4 at day 8 after weaning	Faecal score, jejunum and faecal microbiota, blood samples, jejunum and ileum trascriptomics profile, immunosctocheimistry control of TLR4 in jejunum	(Li et al., 2012)
NA	NA	1	*Bacillus licheniformis* (1.5×10^{10} cfu/g) and *Saccharomyces cerevisiae* (0.3×10^{10} cfu/g)	From weaning for 12-days. Sactification at day 12	ETEC F4 at day 9 after weaning	Performance; Diarrea scores, Intestinal SIgA; intestinal permeability; Small Intestinal Morphology; cecal microbial composition	(Pan et al., 2017)
NA	NA	1	Dietary locust bean gum (LBG 5 g/kg) and *Saccharomyces cerevisiae Sc47* (SC 1 g/kg)	From weaning for 16 days. Scarification at day 16.	ETEC F4 at day 14 after weaning	Blood C-reactive protein; bile sIgA; TLR2 and 4 TLR4 in ileum and MLN	(Badia et al., 2012)
NA	NA	1	Chito-oligosaccharide (160 mg/Kg)	From weaning to 7 d post-weaning, scarification d 14 post-weaning	ETEC F4 at day 7 after weaning	Faecal score formd 7 to d 14, IL-1β, IL-10, and IGF-I in blood; ileum IgA positive cells; E. coli number in small intestine, small intestinal hysological paramethers	(Liu et al., 2010)

Table 5b. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	Adhesion test	Model score	Feeding intervention and additive dose	Period of administration and animal scarification	Challenge	Observed Parameters	Reference
MUC4 -/- +- and +/+	NA	2	ZnO (3.1 g/Kg)	From weaning for 10days.	ETEC F4 at day 3 after weaning	Jejunum trascriptomics profile; piglet performance	(Sargeant et al., 2010)
MUC4 -/-	NA	2	Mixture of Bacillus Probiotics; two doses: $7.8*10^8$ CFU/day (MDBE) and $3.9* 10^9$ CFU/day (HDBE)	From weaning for 8 days. Scarification day 15 after weaning	ETEC F4 at day 8 after weaning	Gut Microbiota, Goblet Cell Function, intestinal gene expression	(Zhang, W. et al., 2017)
MUC4 -/-	NA	2	Mixture of g *B. licheniformis* (DSM 5749) 3.9 × 107 CFU/mL (LB) and *B. subtilis* (DSM 5750) and7.8 × 107 CFU/mL (HB)	From weaning for 8 days. Scarification day 15 after weaning	ETEC F4 at day 8 after weaning	T-cell responses, IL-7Rα and tight junction protein	(Yang et al., 2016)
NA	Yes	3	ZnO or Zn-glutamate in two doses (two doses 2000 and 2500 mg)	From weaning for 9 days, Scarification day 9 after weaning	ETEC F4 at day 1 after weaning	IgA-F4ac in saliva and blood; E.coli count in feces; ZnO level in liver, brain , muscle; villus hight in small intestine	(Bosi et al., 2003)
NA	Yes	3	Spray-dried plasma (6%)	From weaning for 15 days. Scarification day 15 after weaning	ETEC F4 at day 4 after weaning	IgA Saliva and blood, jejunum villi hight and cytokine expression	(Bosi et al., 2004)

Table 5c. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	Adhesion test	Model score	Feeding intervention and additive dose	Period of administration and animal scarification	Challenge	Observed Parameters	Reference
NA	Yes	3	Free calcium formate (FF 12 g/kg) or calcium formate in fat-protected form (PF 12 g/kg)	From weaning for 8 days. Scarification day 8 after weaning	ETEC F4 at day 2 after weaning	Faecal scores; Faecal *E. coli* number; totoal IgA, specifi F4 IgA in saliva and blood; Hystochminstrty paramether in small intestine	(Bosi et al., 2007)
NA	Yes	3	*Lactobacillus sobrius* (10^{10} CFU/Kg)	Two oral doses at day 1 and 2 after weaning and then in the feed until day 14 after weaning. Scarification day 14 after weaning	ETEC F4 at day 7 after weaning	Faecal scores*; E. coli and Lactobacillis sobrius* count in feces; totoal IgA, specifi F4 IgA in saliva and blood; Hystochminstrty paramether in small intestine	(Konstantinov et al., 2008)
NA	Yes	3	Tryptophan 0.34 g/kg of total	From weaning for 9 or 23 day. Scarification: 50% animals at day 9 after weaning; 50% day 23 after weaning	ETEC F4 at day 5 after weaning	Growth; faecal score, histological measurements of small intestine; ileal count of total *E. coli* and ETEC F4;	(P. Trevisi et al., 2009)
NA	Yes	3	Arabinoxylan oligosaccharides (80.5% AXOS on a dry matter basis)	From weaning for 14 days. Scarification day 14 after weaning	ETEC F4 at day 13 after weaning	Jejunal transcriptomics profile	(Niewold et al., 2012)
NA	Yes	3	Anti-F4+ETEC antibodies in seeds: 21 mg/d of IgA per pig (VHH- IgA) or 80 mg/d of IgG per pig (VHH-IgG)	From one week before until 11 days after weaning. Scarification day 11 after weaning	ETEC F4 the day of weaning	Serum IgA, IgM, IgG; Faecal ETEC F4ac excretion	(Virdi et al., 2013)

Table 5d. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	**Adhesion test**	**Model score**	**Feeding intervention and additive dose**	**Period of administration and animal scarification**	**Challenge**	**Observed Parameters**	**Reference**
MUC4 -/- and +/+	Yes	4	0.17 or 0.22 ileal digestible Trp:Lys ratio	From weaning for 21 days. Scarification day 21 after weaning	NA	Jejunum trascriptomics profile	(Trevisi et al., 2012)
MUC4 -/- and ++	Yes	4	0.17 or 0.22 ileal digestible Trp:Lys ratio	From weaning for 21 days. Scarification day 21 after weaning	NA	Growth and feed intake	(Trevisi et al., 2010)
MUC4 -/- and ++	Yes	4	0.17 or 0.22 ileal digestible Trp:Lys ratio	From weaning for 21 days. Scarification day 21 after weaning	NA	Jejunal microbial composition; ETEC adhesion; Jejunual gene expression	(Messori et al., 2013)
MUC4 -/- and +/+	Yes	4	Threonine: 8.5 (LThr) and 9.0 (HThr) g Thr/kg	From weaning for 12/13 days. Scarification day 12/13 after weaning	ETEC F4, 7 days after weaning	Growth; jejunum and colon morphometry; Faecal scores; total IgA and IgM in blood and jejunum; anti-F4 IgA and IgM in blood	(Trevisi et al., 2015b)
MUC4 +/+	Yes	4	*Saccaromyces cervisae* ($5*10^{10}$ CFU/kg)	From weaning for 21 day (PR) or from day 7 after weaning until 21 days (CM). Scarification day 21 after weaning	ETEC F4 7 days after weaning	Growth, Blood metabolomics profile; Blood IgA; faecal scores	(Trevisi et al., 2015a)

Table 5e. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	Adhesion test	Model score	Feeding intervention and additive dose	Period of administration and animal scarification	Challenge	Observed Parameters	Reference
NA	NA	1	Non-immune plasma powder in two doses: 45g/Kg (LP) or 90 g/kg	Form weaning for 14 days. Scarification day 14 after weaning	ETEC F18, 2 days after weaning	Clinical sign, faecal score, faecal *E. coli* enumeration	(Nollet et al., 1999)
NA	NA	1	Tobacco seed containing Vt2e-B gene and three the FedAsubunit of F18 fimbriae gene. Doses: 20 g of milled transgenic tobacco seeds (T1); 10 g of milledVT2eBTS (T2); 25 g VT2eBTS (T3)	Immediately after weaning 0 and at day 1,2 and 14 days' post primary administration. Scarification day 51 after weaning	ETEC F18, 21 days after weaning	Clinical signs, blood and faecal Igs, faecal score, faecal haemolytic bacteria	(Rossi et al., 2014)
FUT1 +/+	NA	2	a1,2-Fucosyllactose 10g/L plus maternal faecal suspension	From day 3 after birth for 5 days. Scarification day 8 after birth	ETEC F18, 3 days after birth	Body weight; Faecal score; mucosal damage and villus atrophy or degradation in small intestine; colonical microbial composition; small intestine fucosilation	(Cilieborg et al., 2017)
FUT1 +/+	NA	2	$2{\cdot}6\times10^{8}$ CFU/kg per d *Lactobacillus paracasei* (LAP) or $1{\cdot}3\times10^{10}$CFU/kg *Pediococcus pentosac* (PEP) plus maternal faecal suspension	From day 2 after birth for 3 days. Scarification day 5 after birth	ETEC F18, from day 2 to day 5	Growth; faecal score; lactulose and mannitol in urine; small intestinal microbiota; SCFA concentration in colon; brush border enzymes	(Andersen et al., 2017)

Table 5f. List of studies focusing on ETEC infection that include as factors feeding strategies and piglet genetic susceptibility in the *in vivo* model. Table reportes the observed genotype, the occurrence of ETEC *in vitro* test adhesion, the feeding intervention, its respective dose and period, the challenge type, the infection day and the animal observed parameters.

Genetics	Adhesion test	Model score	Feeding intervention and additive dose	Period of administration and animal scarification	Challenge	Observed Parameters	Reference
NA	Yes	3	Egg antibody; 1:10 titer pre-mixed (T1) 1:50 titer pre-miked (T2)	From weaning for 9 days. Scarification day 9 after weaning	ETEC F18, the day of weaning	Clinical sign, faecal score, *E. coli* enumeration in jejunum	(Yokoyama et al., 1997)
FUT1 +/+	Yes	4	Oral or nasal immunization with purified F18 fimbria	One week after weaning at different time point: 0, 1 and 2 days post- primary immunization (dppi) and again 14 dppi.	ETEC F18, day 16 after weaning	Cholera toxin A, F18-specific antibodies in serum, saliva and nasal secretion. F18+ *E. coli* faecal excretion	(Verdonck et al., 2007)
FUT1 +/+	Yes	4	Bovine colostrum	From weaning for 8 days. Scarification day 9 after weaning	NA	IgG, IgA and IgM in the mucosa and plasma; faecal and digest microbiota; mucosal gene expression	(Sugiharto et al., 2015)

SCOPE OF THE THESIS

The scope of the thesis is to provide a comprehensive overview on the piglet gut eubiosis and dysbiosis, as well deepen the host and microbial cross-talk, and candidate genes related to pig colibacillosis infections with special attention on the post-weaning phase. Furthermore, the thesis presents an overview of the *in vivo* studies including genetic and feeding strategies in the experimental model to improve the piglet ability to face *Escherichia coli* infection. Since few data about the potential role of genetics in influencing pig immune response and gut homeostasis are available until now, the present thesis aims in contribute on the scientific progress by the presentation of two studies focus in disclosing the role of genetic background of piglets in modulate the gut microbiota as well as the host response in term of metabolic profile and gene expression of the gut mucosa in healthy conditions. Specifically, in chapter 1 we examined the effect of known SNPs located on candidate genes important for the gut homeostasis such as *MUC4*, *FUT1*, *LPB* and *TRL4*, on young growing healthy pigs in terms of growth performance, microbiota composition, expression of genes related to inflammation, glycomic binding pattern profile of the mid-jejunum, and on the blood serum targeted metabolomics profile. In chapter II the attention was focused in one of the more influencing genes identified in the previous study (*FUT1*) and the objective was to evaluate its influences on the blood metabolomics profile, gut immunological parameters and gastrointestinal microbiota during the suckling and post-weaning period. Furthermore, taking advantage from the acquired knowledge in healthy conditions, in chapter III an ad hoc experimental protocol, based on pig genotype (*MUC4*), has been presented to define the effectiveness of a probiotic strategy to counteract the *Escherichia coli* F4ac infection in the post-weaning phase of pigs.

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EXPERIMENTAL STUDIES

Manuscript I

Title: Effect of DNA polymorphisms for gut homeostasis on microbiota composition, intestinal gene expression and glycomic profile, blood serum metabolic profile and growth performance of healthy post-weaning pigs.

Prepared for submission to Journal of Animal Science and Biotechnology

Keywords

16S rRNA; fucose; *FUT1*; intestinal mucosa; immune response; *MUC4*; pig.

Abstract

Background. The interplay between host genetics, gut microbiota and host physiological responses has a crucial importance for animal health and performance. In swine, genetic markers for gut homeostasis have been identified, however, scarce data on their effect on the gut microbial composition and the host metabolic and immune response in healthy pigs are available. This study investigated the effect of Single Nucleotide Polymorphisms (SNPs) located on candidate gene for gut homeostasis (*MUC4, FUT1, A0, BPI* and *TLR4*) on the mid-jejunal microbiota composition, expression of genes related to inflammation and glycomic binding pattern profile, and on blood plasma targeted metabolomic profile and performance parameters of growing healthy pigs.

Results. Results showed a balanced distribution for the SNPs on *MUC4* and *FUT1* genes. No significant results were shown for *MUC4, FUT1, A0, BPI* and *TLR4* genotypes to average daily gain and feed to gain. Pigs with $MUC4^{GG}$ genotype had a higher mid-jejunal expression of *CCL20* and *REG3G* than $MUC4^{CG}$ and $MUC4^{CC}$ pigs ($P < 0.05$). Pigs with $MUC4^{CG}$ genotype had higher expression of *TFF3* than the other two groups ($P < 0.05$). *FUT1* genotype influenced the alpha and beta microbial indices. The sPLS-DA analysis of microbial data showed that $FUT1^{AA}$ group had a higher number of OTUs belonging to *Weissella, Streptococcus* and *Lactobacillus* genus, while $FUT1^{GG}$ group had a higher number of *Veillonella* and *Fusobacterium* genus. $MUC4^{CC}$ pigs had lower scores for *Ulex europaeus* agglutinin I (UEA) fucose-binding lectin on brush borders and goblet cells in the villi than $MUC4^{CG}$ ($P < 0.10$) and $MUC4^{GG}$ ($P < 0.05$). $FUT1^{AA}$ pigs had lower scores for UEA-positivity and higher scores for *Peanut agglutinin* (PNA) galactose-binding lectin both on bush borders and Goblet cells than $FUT1^{AG}$ ($P < 0.05$) and $FUT1^{GG}$ ($P < 0.01$). The sPLS-DA analysis on plasma targeted metabolomics indicated that the *FUT1* and *MUC4* genotypes influenced the metabolic profile of healthy pigs.

Conclusions. Present data are consistent with the role of *MUC4* and *FUT1* genotypes on piglet health and improved the available knowledge regarding the potential interaction between host genetics, gut microbiota composition and host physiological status in healthy condition status.

Background

The pig post-weaning period is commonly characterized by sub-optimal growth (i.e. low feed intake and loss of body weight) and a high prevalence of intestinal diseases, mainly colibacillosis diarrhoea (Schokker et al., 2015), impacting pig morbidity and mortality and increasing the use of antibiotics in piglets (Lallès et al., 2007; Gresse et al., 2017). Since the reduction of antibiotic use is a central

objective in animal livestock, the increase of robustness and the control of intestinal infection still represent a challenge to the pig industry (Lalles and Guillou 2014).

Nowadays the integrated "omics-approach" such as genome-wide association study (GWA), microbiome and metabolome analysis, has increased the information about the interplay between host-genetics, gut microbiota and host immune response (Ellinghaus et al., 2016; Jostins et al., 2012; Menni et al., 2017), thus genetic markers involved in gut homeostasis and host health status and growth performance are available.

In pigs, genetic markers have been associated with post-weaning intestinal disease (Coddens et al., 2008; Jørgensen et al., 2003; Ren et al., 2012; Wu et al., 2015) and gastrointestinal homeostasis (Bäckhed, 2011; Priori et al., 2016). For some of them, the association can be attributed to a specific functional difference in the gene (fucosyltransferase 1 – *FUT1* gene – Coddens et al. 2008; transferase A – *A0* gene - Priori et al., 2016). Furthermore, those genetic markers explained part of the variability of growth performance response (*FUT1,* Bao et al., 2011; *mucin 4* gene - *MUC4* - Fontanesi et al., 2012) and of the tryptophan and threonine nutritional requirement of weaning pig upon infective challenge condition (Trevisi et al., 2009; Trevisi et al., 2015). In our previous works, we observed that tryptophan nutritional requirement (Trevisi et al., 2010) and gut bacteria population (Messori et al., 2013) can be genetically affected also in healthy weaning pigs. Thus it can be hypothesized that genes that explain the pig susceptibility to some intestinal diseases can also impact the intestinal microbiota composition and that this should be considered important when designing strategies to improve piglet robustness and growth performances.

So far, however, the effect of genetic markers involved in gut homeostasis, in controlling the gut microbiota composition and in the pig's immune response is still few explored. Furthermore, the biological explanations by which those genetic markers can influence the gut microbial population, host immune response and robustness are not exhaustive. We, therefore, hypothesized that thanks to a continuous cross-talk between host and bacteria, other SNPs widely distributed in the main pig populations and located on candidate host-genes for gut homeostasis would play a role on the gut microbiota composition and host immune response in pigs with a healthy status.

Thus, the specific aim of the present study is to evaluate the effect of known SNPs located on several candidate genes important for the gut homeostasis, on the growth performance, the microbiota composition, the expression of genes related to inflammation, the glycomic binding pattern profile of the mid-jejunum, and on the blood plasma targeted metabolomic profile of young growing homeostasis pigs

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.

Animal and sampling

One-week post-weaning (35 days of age – d0 of trial), seventy-one piglets were selected based on the body weight (7063 ± 936 g average body weight) and the litter of origin. Piglets were moved to the experimental facility of the University of Bologna and located in individual pens with a mesh floor for six weeks (d42). Pigs were kept at a controlled temperature (30°C at the beginning and 25°C at the end of the experiment, with a 1°C decrease every 3 days), heated additionally by infrared lamps for the first 7 days. The experiment was conducted in three consecutive batches of 4, 3 and 3 litters. During the trial animals were fed two consecutive diets: a pre-starter diet from d0 to d21 and a starter diet from d22 to d42 (Table 1). Piglets were offered feed two times daily at 8:00 am and 16:30 am and water was always available during the trial; feed allowances were calculated to surpass the piglet's appetite. Feed intake was daily recorded and piglets were individually weighted every week until the end of the trail. Average daily gain (ADG), average daily feed intake (ADFI), feed/gain (F:G) were individually calculated. During the trial, from each pig, bristles with bulb were sampled to isolate the DNA for the further genotyping analysis. At the end of the trial, twenty minutes after the last meal, all the animals were deeply anaesthetized and sacrificed via an intracardiac injection of Tanax (embutramide, mebenzonium iodide, and tetracaine hydrochloride; 0.5ml/kg body weight). From each subject, one sample of scraped mucosa from jejunum at 50% of the small intestine length was collected and then was snap-frozen in liquid nitrogen and preserved at −80°C for gene expression analyses. In addition, from the same intestinal site, a second sample of tissue was isolated, emptied, and then the mucosa was gently scraped. The scraped mucosa was mixed with the jejunum content and was snap-frozen in liquid nitrogen and stored a – 20°C for further microbiota analysis.

Additionally, from a sub-group of twenty-four pigs, balanced for the most segregated genotypes (*FUT1* and *MUC4)* and litters, an additional sample of mid-jejunum tissue was collected for immunohistochemistry analysis, washed with 0.01 M phosphate buffer saline (PBS) and pinned tightly to balsa wood and fixed in 10% buffered formalin for 24 h at room temperature (RT). The specimens were then dehydrated in a graded series of ethanol and embedded in paraffin. Furthermore, a blood sample from the same sub-group of pigs was obtained by venipuncture of vena cava on a collection tube with K3 EDTA (Vacutest Kima srl, Arzergrande PD Italy) before the animal sacrifice.

Blood samples were centrifuged 3,000 × g for 10 min at 4°C to obtain the plasma. Plasma samples were stocked at -80°C until further targeted metabolomic profile analysis.

Genotyping

In addition to the previously cited gene polymorphisms (*FUT1*, *MUC4* and *A0*), two other genes were considered for polymorphisms associated with post-weaning intestinal disease and immune indices (*Bactericidal/permeability-increasing protein - BPI* - Liu et al., 2013 and Wu et al., 2015) and cytokine expression level and pulmonary lesion score (*Toll-like receptor 4 - TLR4* - Yang et al., 2012), in pigs. Genomic DNA of each pig was extracted from bristles following the procedure described by Ausubel et al., (1995). Genotyping of the *MUC4* g.8227C>G SNP, g.307 *FUT1* G>A, patented mutation 20040234980, for *BPI*, g.962 *TLR4*, g. 611 *TLR4* was carried out by Polymerase Chain Restriction Fragment Length Polymorphism (PCR-RFLP). With regard to the *AO* group genotyping, the procedure described by Nguyen et al. (2011) was used. Specific primers, annealing temperature, conditions, respective references and enzymes are listed in Table 2a,b.

Gene expression

From scraped mid-jejunum mucosa of each animal, the total RNA was isolated using the Trizol Reagent (Life Technologies, Carlsbad, CA, USA) extraction. Quantification and quality control of all RNA samples were assessed using the ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) with an optimal ratio 260/280 nm between 1.7 and 2.2 and visualized on 1.5% of agarose gel. DNA contamination was checked on all RNA samples performing a supplementary PCR-RFLP analysis for *FUT1* gene.

cDNA was synthesized using 1000 ng of RNA as template (ImProm-II Reverse Transcription System Promega Corporation, Milan, Italy). The semi-quantitative PCR (qPCR) of Interleukine-8 (*IL8*), Glutathione peroxidase (*GPX2*), Regenerating islet-derived 3 gamma (*REG3G*), Trefoil Factor 3 (*TFF3)*, C-C Motif Chemokine Ligand 2 (*CCL20)*, ST3 Beta-Galactoside Alpha-2,3-Sialyltransferase 1 (*ST3GAL*), Lipopolysaccharide Binding Protein (*LBPI*) were performed in a Light Cycler instrument (Roche, Mannheim, Germany). Primers' sequence, amplified fragments' length and qPCR conditions are reported in Table 3. Every single fragment was serially diluted in 1:10 steps in order to create a standard curve to perform the relative quantitative analysis. The amplification was carried out in 10 µL overall volume containing 2 µL of cDNA, 8 pmol of each primer, and 5 µL of SYBRs Premix Ex TaqTM II (Perfect Real Time) (Takara Bio Inc., Shiga, Japan). The expression data were normalized to a housekeeping gene Hydroxymethylbilane Synthase (*HMBS*) and were expressed as gene transcript copies per microgram of RNA.

Microbiota Analysis

Total bacterial DNA was extracted using Qiamp Stool Mini Kit (Qiagen, Hilden, Germany). The library formation and sequencing of 16S rRNA gene were performed with MiSeq® Reagent Kit V3-V4 on MiSeq-Illumina® platform. Generated sequences were analysed using subsampled open-reference OTU strategy with default settings in QIIME (v1.9.1). The reads of 16S rRNA gene were paired-end and demultiplexed. Subsampled open-reference OTU-picking was performed using UCLUST with 97% sequence similarity. Representative sequences were chimera checked using Chimera Slayer with default settings and taxonomy assigned against the Greengenes database V13_8 using the UCLUST method with a 90 % confidence threshold. The singletons and OTUs with relative abundance across all samples below 0.005 % were removed as recommended by Bokulich et al. (2013)

Immunohistochemistry analysis

From each selected mid-jejunum sample, transverse (5 µm thick) sections were obtained and mounted on poly-L-lysine coated slides and then processed for immunohistochemistry. The sections were dewaxed, rehydrated, heated in citrate buffer pH6 for 10 min at 700 W in the microwave for antigen retrieval, and blocked in Carbo-Free Blocking Solution (catalogue number SP-5040; Vector Laboratories, Inc., Burlingame, CA, USA) for 1 h at RT. All sections were incubated for 1h at RT using lectin-biotin conjugates (Vector Laboratories, Inc.). For lectin binding analysis, lectins were used with a concentration of 10 µg/mL as reported previously (Chae, 1997; George et al., 2007; Priori et al., 2016). *Ulex europaeus* agglutinin I (UEA; catalogue n. B-1065) and *peanut agglutinin* (PNA; catalogue n. B-1075) were diluted in lectin buffer (Rhodes and Milton, 1998). UEA lectins represented the fucose sugar specificity, while PNA is galactose-specific. After 3 washes in lectin buffer, the sections were incubated with Texas Red Streptavidin (catalogue number SA-5006; Vector Laboratories, Inc.) 5 µg/mL in lectin buffer, for 30 min at RT. The slides were then washed in PBS and coverslipped with buffered glycerol, pH 8.6. No specific staining was assessed by omitting the lectin incubation step. The morphological evaluation was performed with 20 X objective lens using a Nikon Eclipse Ni microscope equipped with the appropriate filter cubes. The images were recorded with a Nikon DS-Qi1Nc digital camera and NIS Elements software BR 4.20.01 (Nikon Instruments Europe BV, Amsterdam, Netherlands). Slight adjustments to contrast and brightness were made using Corel Photo Paint, whereas the figure panels were prepared using Corel Draw (Corel Photo Paint and Corel Draw, Ottawa, ON, Canada).

For each slide, 20 villi and 20 crypts were randomly observed. The positivity for UEA and PNA was assessed by the same operator in correspondence with the surface of the villi (microvilli brush border) (points from 0 to 3) and for labelled cells frequency in the villi and crypts (points from 1 to 4).

In detail, the intensity of fucose (UEA) and galactose (PNA) immunoreactivity in the surface of villi was scored as follows: 0 = absence of immunoreactivity, 1 = positivity of immunoreactivity distributed throughout the surface of the villi, 3 = very marked positivity of immunoreactivity and greater thickness than the score 1. The number of immunoreactive (IR) goblet cells in the villi and crypts was evaluated and scored as follows: 1 = absent/rare IR cells, 2 = few IR cells, 3 = some IR cells 4 = many IR cells.

Plasma targeted metabolomic analysis

Plasma metabolites were measured using the Biocrates AbsoluteIDQTM p180 Kit (Biocrates Life Science AG, Innsbruck, Austria) that allows to quantify a panel of 188 compounds, including 40 acylcarnitines, 21 amino acids, 21 biogenic amines, 90 glycerophospholipids (14 lysophosphatidylcholines (lysoPC) and 76 phosphatidylcholines (PC)), 15 sphingolipids and hexoses (sum of hexoses, including glucose). All samples were measured in a single assay. The kit was processed according to manufacturer instructions and analysed on the Serie 200 high pressure liquid chromatography system by Perkin Elmer (Waltham, MA) coupled with the API 4000 QTRAP by AB Sciex (Foster City, CA, USA). Amino acid and biogenic amine classes were analysed by liquid chromatography – tandem mass spectrometry (LC-MS/MS). Acylcarnitines, phospho- and sphingolipids and hexose were analysed by flow injection analysis (FIA) – MS/MS. Instrumental data were acquired and processed by Analyst 1.6.3, whereas data quantitation and validation were performed by MetIDQ-5.5.4-DB100-Boron-2623 software. Results were exported in micromolar unit (μM).

Statistical analysis

The genotype frequencies, allele frequencies and Hardy-Weinberg Equilibrium (HWE) in our population were assessed using SAS software (version 3.4 SAS Institute).

Except for microbiota and metabolomics data, all the data were analysed on SAS software (version 3.4 SAS Institute), using general linear models (PROC GLM). For growth performance data the model included all the genotypes and batch as fixed factors and the initial body weight (BW0) as a covariate, except for *A0* polymorphism that was tested in a separate analysis, because of the different number of available subjects. Sex and litter within the batch were tested and included in the model

when significant. For jejunal genes expression data the model firstly included all the genotypes, batch and litter within the batch as fixed factors. Sex and final BW (BW6) were tested but were not significant. Secondly, an additional restricted model including only *MUC4* genotype, that was the sole SNP statistically significant (P-value <0.01) and litter within the batch was carried out to confirm the single SNP effect avoiding disturbing factors. Immunohistochemistry data were analysed using a model which included *MUC4* and *FUT1* genotype as fixed factors. Batch and litter inside batch were deleted after they had been tested and resulted not significant, except for PNA score in brush borders, where batch was included because statistically significant.

The statistical analysis of the genes expression and microbiota data were performed considering the total number of available samples (65 animals), however, in order to confirm the results, the same analysis was carried out also on the data including the sub-group (24 animals) balanced for balanced for the most segregated genotypes (*FUT1* and *MUC4)* and litters.

Biostatistics on OTUs table were performed using the vegan package in R software (v.3.3.0). The richness and alpha diversity indices (the Chao1 index, and the Shannon index) were calculated on raw data matrix while beta diversity ordination and differential abundance analysis were carried out after rarefaction correction using Phyloseq package (McMurdie et al., 2013) in R software. Alpha diversity index values were compared with multivariate ANOVA testing litters and the genotypes analysed as explanatory variables. The genotype differences in beta diversity were explored using permutational manova (Adonis procedure) on Bray-Curtis distance matrix followed by pairwise Wilcoxon signed-rank test. In order to evaluate the discriminant OTUs belonging to each variant of *FUT1* gene, the Multivariate analysis was carried out using the MixOmics (mixOmics_6.1.3) package on R software (Lê Cao et al. 2009). Microbial data were previously normalized using the Total Sum Scaling (TSS) normalization coupled with the centred log-ratio (CLR) transformation according to Lê Cao et al. (2016), then Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) method was applied. It performs a variable (OTUs) selection with Lasso penalization method (Lê Cao et al. 2008). The optimal number of components was selected based on the averaged balanced classification error rate with centroids distance over 100 repeats of a 10-fold cross validation of a sPLS-DA model with 5 components (using "perf" function). The optimal number of selected variables for each component was then chosen based on the lowest average balanced classification error rate with centroids after tuning of the sPLS-DA model (function tune.spldsda) using the selected number of components and 10-fold cross-validation with 100 repeats. Stability frequency scores of the selected metabolites were calculated (function perf) on the final sPLS-DA model with 10-fold cross-validation and 100 repetitions. The plotVar function was then used to identify the structure of the correlation between the two sets of variables X (OTUs matrix) and Y (*FUT1* genotypes). OTUs showing a

correlation > 0.5 with *FUT1* genotypes were considered discriminative. Individual samples were presented on a score plot and were distinguished by the group with colour and 95% confidence eclipses using the plotIndiv function.

For targeted metabolomic analysis, data were imported into the MetaboAnalyst 2.0 software (http://www.metaboanalyst. ca; Xia et al. 2012). Metabolites with more of 20% of missing value were removed and the remained missing values were calculated using the Kpp algorithm of K-Nearest Neighbours approach. Data were then normalized by each metabolite median, log-transformed and mean centred before the statistical analyses. An ANOVA model for each genotype was then carried out for all the metabolites and *P* values were corrected for False Discovery Rate (FDR). An adjusted *P* value < 0.05 was considered as significant. Furthermore, the Multilevel approach using the sPLS-DA was applied in order to describe the *FUT1* and *MUC4* genotype variants influence on the targeted metabolome profile and to obtain the most discriminate metabolite among the different genotypes.

Results

Distribution of the genotypes in the pig set

Table 4 shows the frequencies of the alleles and the gene distribution of *MUC4, FUT1, A0, BPI* and *TLR4* tested polymorphisms in our population. For *MUC4* gene, 38 samples had $MUC4^{CC}$ genotype corresponding to resistant homozygotes for the ETEC F4ac adhesion (undigested fragment of 367 bp), 26 had the $MUC4^{CG}$ genotype and 7 had the $MUC4^{GG}$ genotype, both considered the sensitive genotype for the ETEC F4ac infection (fragments 216 + 151 bp). Allelic frequency was 0.72 for allele C and 0.28 allele G. The population was not in HWE. As regards *FUT1* mutation G>A on 71 pigs, 33 $FUT1^{GG}$, 31 $FUT1^{AG}$ and 7 $FUT1^{AA}$ genotypes were observed; where the $FUT1^{AA}$ mutated-type is the resistant homozygote for ETEC F18 (fragments length 241+ 93 + 87bp; 328 + 241 +93 + 87bp; 328 + 93 bp). An allelic frequency of 0.32 for allele A and 0.68 for allele G were observed. The χ^2 test showed that the samples were in HWE and, according to Wright's fixation index (Fis), there was an excess of heterozygous (Fis < of zero). A total of 66 animals were genotyped by multiplex-PCR for *A0* blood group. 49 pigs with $A0^{00}$ genotype and 17 pigs with $A0^{A0}$ genotype, while no $A0^{AA}$ homozygotes were found. The allelic frequency of allele 0 was higher than allele A (0.87 and 0.13 respectively) and the population was not in HWE. The results for *BPI* showed 10 pigs with BPI^{AA} genotype; 24 pigs BPI^{AG} genotype; 45 pigs with BPI^{GG} genotype. The allelic frequency was 0.20 for allele A and 0.80 for allele G. The χ^2 test showed that the samples were in HWE and Wright's fixation index (Fis) indicated an excess of heterozygous (Fis < of zero). Since only two animals had the BPI^{AA}

genotype, they were included in the *BPI*^{AG} genotype group for the statistical analysis. For the *TLR4* 962 SNP, corresponding to a G>A mutation, no variability was found and all the animal had the *TLR4* 962^{GG} genotype. The *TLR4* 962 genotype distribution was 54 *TLR4* 962^{GG} type and 17 *TLR4* 962^{AG} type, corresponding an allelic frequency of 0.88 for allele G and 0.12 for allele A. The population was not in HWE.

Genotype influence on animal performance parameters

Results of genotype influence on the performance parameters are shown in Table 5a,b. No significant differences were seen for *MUC4*, *A0*, *TLR4* genes to BW, ADG and F:G. *BPI* mutation affected the BW at day 35 and the group of animals with *BPI*^{GG} genotype had a higher value than *BPI*^{AG} genotype ($P = 0.04$). A trend of significance was observed on BW at day 14 of age ($P = 0.08$) for *FUT1* variants; the *FUT1*^{AA} group had a higher BW than *FUT1*^{AG} and *FUT1*^{GG} groups. No further association were found for *FUT1* and *BPI* on the growth performances parameters.

Genotype influence on jejunum gene expression

The tested SNPs did not show any influence on the expression of the selected genes except for the *MUC4* polymorphism which influenced the expression of *TFF3*, *CCL20* and *REG3G*. Results of the restricted model that included *MUC4* and litter within the batch as factors are shown in the Fig. 1. Pigs of *MUC4*^{GG} genotype had a higher expression of *CCL20* and *REG3G* genes respect to *MUC4*^{CG} and *MUC4*^{CC} pigs ($P < 0.05$). For *TFF3* expression, the group of pigs with *MUC4*^{CG} genotype (0.03) showed an increased expression than the other two groups ($P < 0.05$).

Genotype influence on mid-jejunal microbiota composition

Six samples were excluded from the microbiota analysis due to a low quality of sequencing data. The 3,472,457 reads obtained after quality and abundance filtering were assigned to 483 taxa and 9 Bacteria Phyla. *Firmicutes* was the most represented phylum (80%), followed by *Actinobacteria* (8.9%) and *Proteobacteria* (7.9%). A total of 137 genera were identified in data aggregated at the genus level, most of the reads were belonging to *Lactobacillus* (39.2%) genus, followed by *Streptococcus* (19.8%), *Corynebacterium* (5.5%), *Clostridium* (4.9%) and *Weissella* (2.0%) genus. Concerning the influence of tested SNPs on alpha diversity, no significant differences were found for the Chao1 index, and the Shannon index differed only for the *FUT1*. The *FUT1*^{AG} group (3.02 ± 0.72) had higher Shannon value than *FUT1*^{GG} (2.76 ± 0.76) and *FUT1*^{AA} (2.26 ± 1.01) groups ($P = 0.01$). Significant differences only for the *FUT1* genotypes were observed by the Adonis procedure on Bray-Curtis distance (Table 6) and the pairwise Wilcoxon signed-rank post hoc test showed a difference

between the *FUT1*^{AA} genotype and the other two genotype (*FUT1*^{AA} vs *FUT1*^{AG}, $P = 0.01$; *FUT1*^{AA} vs *FUT1*^{GG}, $P = 0.02$). The sparse version of partial least squares discriminant analysis (sPLS-DA) was applied to identify specific OTUs that could potentially distinguish between the *FUT1*^{AA}, *FUT1*^{AG} and *FUT1*^{GG} genotypes. Fig. 2 showed the individual score plot obtained using the plotIndv while Table 7a,b listed the discriminant OTUs for *FUT1* genotypes. As can be seen in Fig. 2, the final model was able to make the samples clustering according to *FUT1* genotype groups and the 1and 2 latent components contribute towards 7% and 8% of explained variance. Notably, most of the discriminative OTUs contributing the differences between *FUT1* genotypes were belonging to *Lactobacillaceae, Leuconostocaceae, Streptococcaceae, Veillonellaceae* and *Fusobacteriacae* Family. The *FUT1*^{AA} group was correlated with a higher number of OTUs belonging to *Weissella, Streptococcus* and *Lactobacillus genus,* mainly belonging *to Lactobacillus mucosae* and *Lactobacillus reuteri* species, while the *FUT1*^{GG} group was correlated to *Veillonella* genus, in particular, *Veillonella* species *dispar,* and *Fusobacterium* genus.

Plasma targeted metabolome profile

The MS/MS targeted analysis performed on 23 plasma samples part of a sub-selected group of pigs provided results for 134 metabolites (TableS1). Three acylcarnitines, the sum of hexoses (including glucose), 35 amino acids and biogenic amines, 19 sphingolipids, and 81 phospholipids including lysophosphatidylphosphates and phospholipids were detected and quantified using the AbsoluteIDQ p180 kit. ANOVA indicated that asymmetric dimethylarginine (ADMA), carnitine (CO) and O-acyl-O-acyl phosphatidylcholine (PC_aa_) C36:1 differently responded to the *MUC4* genotype variants and spermidine to the *FUT1* variants. Higher levels of CO were found for *MUC4*^{CC} genotype (5.92 mM) than *MUC4*^{CG} (4.53 mM) and *MUC4*^{GG} (4.50 mM); a lower amount of PC aa C36:1 was observed in *MUC4*^{CC} (32.90 mM) than *MUC4*^{CG} (36.80 mM) and *MUC4*^{GG} variants (36.36 mM) and a higher level of ADMA was observed for *MUC4*^{GG} (2.42 mM) than *MUC4*^{CC} and *MUC4*^{CG} (1.68 mM) (adj. *P*-value <0.05). A higher plasma level of spermidine was observed in the *FUT1*^{AA} genotype (0.53 mM) than the *FUT1*^{AG} (0.24 mM) and *FUT1*^{GG} variants (0.29 mM) (adj. *P*-value <0.05). The multivariate analysis showed that metabolomic profile was partially affected by *MUC4* and *FUT1* genotypes variants as shown by the sPLS-DA score and loading plot (Fig. 3A,B). Specifically, the sPLS-DA score plot showed that *MUC4*^{CC} and *MUC4*^{GG} clusters were mainly distinguished by the PC2 (9.1% explained variance); *MUC4*^{CC} group had a higher level of plasma CO, serotonin, PC_aa_38:0, sphingomyelin (SM)OH_C22:1, SM_OH_C24:1 than *MUC4*^{GG}. The PC1 partially separated the *MUC4*^{CG} cluster from *MUC4*^{CC} and *MUC4*^{GG} clusters. The *MUC4*^{CG} group was typified by a higher value of plasma PC_aa_C42:1, PC_aa_C40:3, PC_aa_34:1, PC_aa_C38:5, PC_aa_C40:4

than *MUC4*^{CC} and *MUC4*^{GG} groups. *MUC4*^{GG} group had higher ADMA and PC_aa_C36:1 than the other two groups. Considering the *FUT1* variants, the sPLS-DA score lot showed that clusters representing *FUT1*^{AG} and *FUT1*^{GG} clustered together along PC1 (6.7% of explained variance) while the cluster representing *FUT1*^{AA} was partially separated. The loading score plot for *FUT1* genotypes showed that plasma levels of spermidine, spermine, SM_OH_C24:1, glycine, putrescine and taurine were more abundant on the *FUT1*^{AA} group than *FUT1*^{AG} and *FUT1*^{GG} groups, while the *FUT1*^{GG} group was characterized by a more abundant level of PC_ae_C36:5, PC_ae_C42:4, PC_ae_C44:5, PC_ae_C40:4 than *FUT1*^{AA} and *FUT1*^{AG} groups.

Immunohistochemistry

UEA and PNA immunoreactivity were observed on the surface of the jejunum villi (brush border microvilli), while IR goblet cells were observed both in villi and crypts. In pigs where the villi presented positive brush borders, immunoreactivity showed up as a layer that followed the entire profile of the villus: in other pigs, this layer was thin, while sometimes occurred as a thicker line especially marked (Fig. 4 and Fig. 5). On the contrary, in villi from other pigs, no labelling has been observed (Fig. 4 and Fig. 5). With regard to the IR goblet cells, these presented a typical columnar / pear shape morphology containing mucin granules intensely marked, and were distributed along the axis of the villus (Fig. 5) and/or aggregated in the crypts (Fig. 6) depending on the pig. Frequently, for the same brush borders, opposite intensity was seen when comparing the degree of staining with UEA or with PNA (Fig. 7). Conversely, the degree of intensity seen in the Goblet cells with PNA was averagely lower than with UEA (Fig. 5 and Fig. 6). The effect of *MUC4* and *FUT1* genotypes on the average scores for the staining with UEA or PNA in the brush borders and Goblet cells done on the sub-selected animals is shown in Table 8. Variations were seen in the intensity of the staining depending on the localization, the type of lectin used and the *MUC4* and *FUT1* genotypes. The *MUC4*^{CC} pigs had less scoring for UEA in jejunal brush borders and Goblet cells in villi than *MUC4*^{GG} ($P = 0.037$, for brush borders; $P = 0.031$, for Goblet cells) and *MUC4*^{CG} pigs (marginally, $P = 0.081$ for brush borders; $P = 0.016$ for Goblet cells). The degree of UEA staining for Goblet cells in the crypt was not affected by the *MUC4* genotype. The degree of PNA staining for brush borders and Goblet cells in villi and crypts was not affected by the *MUC4* genotype. The *FUT1*^{AA} genotype had lower UEA immunoreactivity score in brush border than *FUT1*^{AG} ($P = 0.001$) and *FUT1*^{GG} ($P < 0.0001$) genotypes. UEA-IR goblet cells in the villi and crypts were lower in the *FUT1*^{AA} genotype than in *FUT1*^{AG} genotype ($P = 0.012$ in the villi; $P = 0.001$ in the crypts) and in *FUT1*^{GG} genotype ($P = 0.005$ in the villi; $P < 0.0001$ in the crypts). The comparison between *FUT1*^{AG} and *FUT1*^{GG} genotypes showed that *FUT1*^{AG} had lower UEA-IR goblet cells in crypts ($P < 0.05$), and a trend of

significant lower UEA immunoreactivity score on the brush border (P <0.1). The degree of UEA-IR goblet cells in crypts in villi was not different between *FUT1*^{AG} and *FUT1*^{GG} genotypes. The *FUT1*^{AA} genotype had higher PNA immunoreactivity score in brush border than *FUT1*^{AG} (marginally, P = 0.062) and *FUT1*^{GG} (P = 0.006) genotypes. PNA-IR goblet cells in the villi and crypts were greater in the *FUT1*^{AA} genotype than in *FUT1*^{AG} genotype (P = 0.031 in the villi; P = 0.007 in the crypts) and in *FUT1*^{AG} genotype (P = 0.003 in the villi; P < 0.0001 in the crypts). No difference between *FUT1*^{AG} and *FUT1*^{GG} for PNA immunoreactivity in bush border and goblet cells was observed.

Discussion

The present study took into consideration the effect of specific polymorphisms that could affect the gut homeostasis of pigs by evaluating their effect on the host response in absence of specific infections during the post-weaning phase. For the SNPs located on *A0, BPI* and *TLR4* scarce genetic variability did not allow for evidenced effects on growth performance and on the expression of the selected gene markers of gut inflammation and homeostasis. The biased distribution of genetic variance for *A0, BPI* and *TLR4* did not allow to evaluate their association with the more complex data (blood metabolites and intestinal microbiota) Thus at the moment, we can conclude only that no proofs exist till now of their relevance for the growth and gut homeostasis of the young pig.

Thus we will discuss prevalently the effects of *MUC4* and *FUT1* SNPs. Our study showed that the *MUC4* SNP, that is strictly genetically linked to the modulation of *E. coli* F4ac (ETEC F4ac) post-weaning infection (Jørgensen et al., 2003), had an influence on the expression of key genes related to inflammation and immune response in the jejunum of healthy piglets. The animals with the susceptible genotypes (*MUC4*^{GG}) had a higher expression level of *REG3G* and *CCL20* genes than the group with the resistant genotype (*MUC4*^{CC}). In the intestine, REG3G is mainly produced in the crypts, is associated to the host C-type lectins with antimicrobial activity (Brandl et al., 2008) and the *REG3G* gene is typically upregulated in experimentally infected pigs (Soler et al., 2015). Our result agrees with the increase of *REG3G* expression seen with the increased susceptibility to ETEC F4ac, as determined by the in villus adhesion phenotype, in healthy pigs fed a diet covering the minimal tryptophan requirements (Trevisi al. 2012). This indicates that differences in the unknown causative gene controlling the susceptibility to ETEC F4ac can influence the *REG3G* expression even if the animals are not exposed to a full-blown infection. The chemokine *CCL20* expression has been highlighted for its relation to IBD in human (Skovdahl et al., 2015), as upregulation of *CCL20* expression, originating from bacteria stimulation, increases pro-inflammatory signals such as primary

cytokines (e.g., TNF-α) and TLR (Fujiie et al., 2001). In ETEC F4-susceptible pigs infected with this pathogen, the supplementation with antibiotic or probiotic prevented the rise of *CCL20* expression observed in the unsupplemented group (Trevisi et al., 2017). Thus, we can hypothesize here that apparently healthy *MUC4*^{GG} pigs had a more stimulated immune response than the *MUC4*^{CC} pigs; this highlights the possible role of the gene associated with ETEC F4ac susceptibility in the regulation of the immune response. The *TFF3* expression was found to be higher in the *MUC4*^{CG} genotype than *MUC4*^{CC} and *MUC4*^{GG} genotypes showing that, although these two genotypes are generally considered equally susceptible to the ETEC F4ac adhesion and infection (Trevisi et al., 2009), the response in terms of gut homeostasis of healthy pigs could be partially different. Our results add additional information to the results reported by Roubos-van den Hil et al., (2017), where, according to our results, different response, in terms of ETEC F4ac faecal shedding have been highlighted for *MUC4*^{GG} and *MUC4*^{CG} ETEC F4ac infected piglets. The different regulation of *TFF3* expression (and of *REG3G* and *CCL20*) according to *MUC4* SNP could be due to *MUC4* gene function. *MUC4* encodes for an integral membrane glycoprotein found on the cell surface of the gut and it may affect not only the ETEC F4ac adherences. However, the frequency of pigs with a detectable expression of *MUC4* gene did not vary in the jejunum of pigs of different genotype for the *MUC4* SNP (Trevisi et al., 2012). Conversely, it is possible that the unidentified gene responsible for ETEC F4ac intestinal adhesion is associated also to the composition of commensal bacteria, that with their metabolism and structural function, could be responsible for the *TFF3* upregulation. Results confirmed that different metabolic pathways in blood plasma vary according to the three *MUC4* genotypes. The *MUC4*^{CG} genotype had a higher level of several phosphatidylcholines with diacyl residues, which are involved in the free fatty acid metabolism, glycerophospholipid metabolism and membrane component. The *MUC4*^{CC} genotype (resistant genotype) displayed higher plasma serotonin level than the *MUC4*^{GG} genotype. Serotonin is mainly released from the gastrointestinal tract where it is synthesized by the enterochromaffin (EC) cells and it is then concentrated in platelets in the blood (Crowell, 2004). Serotonin is considered a neurotransmitter and paracrine signalling molecule involved in the brain-gut interactions. It plays a key role in relation with the irritable bowel syndrome (IBS) in human where a lower level of blood serotonin have been associated with constipation-predominant IBS while higher level to diarrhoea -predominant IBS (Crowell, 2004; Dunlop et al., 2005). In our study, no signal of severe diarrhoea had been reported, thus the level of plasma serotonin could not be directly ascribed to the intestinal disorder, anyway, it could be of interest to evaluate the relation between *MUC4* genotypes and the plasma serotonin level in further studies based on the functional role of this gene on pig health and the role of this compound.

The immunohistochemistry results are particularly interesting because to date, few researchers have exploited in the same set of animals the effect of *MUC4* and *FUT1* genetic variance on the degree of fucosylation and galactosylation in mucine-secreting cells or in the brush borders of villi, that represent glycoproteins anchored to the enterocyte apical membrane. The genetic effect on the fucosylation was not immediately expected for *MUC4* since other sugars have been indicated as potentially involved in the ETEC F4ac adherence mechanisms (Grange et al., 1998; Remaut and Greve, 2015). It is therefore interesting to observe that the degree of fucosylation seen for the goblet glands in the villi has been reduced for the subjects genetically susceptible to ETEC F4ac, possibly due to an indirect effect of the other transfer enzymes involved, or for a possible different stimulation of the bacteria present in the gut of not susceptible subjects. Indeed various bacteria may induce a variation in the degree of intestinal mucosal fucosylation (Pickard et al., 2014).

The degree of staining with galactose-specific PNA lectin was, in general, lower than the degree observed with UEA. It is possible that part of galactose was masked by sialic acid, as it was observed by King and Kelly (1991) comparing the staining of PNA before and after the treatment with neuraminidase, that cleaves the sialic acid; however, this observation was more evident in pigs of less than 4 weeks of age, younger than the pigs of our study. No effect of *MUC4* genotype on PNA staining scores was found, while we expected to receive a confirmation of the impact of *MUC4* SNP on the presence of galactose for its possible role in the ETEC F4ac adherence. Indeed, a galactose β-linked to two intestinal mucin-type sialoglycoproteins was found to be an important component for their recognition by F4ac fimbriae (Grange et al., 1998), and two galactose-lectins were able to contrast the adhesion of ETEC F4ac to intestinal villi (Trevisi et al., 2017). Thus we expected to find higher immune reactivity for PNA in the mucosa of *MUC4*^{GG} and *MUC4*^{CG} pigs than in *MUC4*^{CC} pigs, as they are the susceptible genotypes. Nevertheless, a number of additional putative receptors for the different ETEC F4ac types, with a glycosphingolipids nature such as lactosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, globotriaosylceramide, lactotetraosylceramide, and lactotetraosylceramide have been described and characterized (Coddens et al., 2011; Grange et al., 2002, 1999) The absence of differences of the scores for PNA in the brush borders and goblet cells of pigs of *MUC4* genotype, found in our study, makes questionable the association of this pathology with a galactose-related receptor for F4ac fimbriae. Conversely, our data do not exclude the sialoglycoprotein nature of the receptor (Grange et al., 1998) or n-acetyl-hexoseamine (Grange et al., 2002), while galactose may play as an enhancer of the adhesion (Grange et al., 2002).

As opposed to our expectation, no differences in the gut microbiota composition were found in the association with *MUC4* genotype using the NGS technology. Our result is partially in contrast with a previous study of Messori et al. (2013) in which a higher presence of *Clostridium bartlettii* was found

for *MUC4*^{GG} and *MUC4*^{CG} than *MUC4*^{CC} pigs; however, it is generally recognized that the characterization by the sequencing of defined regions of 16S rRNA gene could have some limitations such as an incomplete resolution and a low sensitivity (Poretsky et al., 2014) to the overall assessment of the microbial composition. Thus, taking into account the whole results of the present study we cannot conclude that *MUC4* genotype does not have any influence on the jejunum microbiota of piglets.

Interestingly, on the other hand, differences on the jejunal microbiota were found in association with *FUT1* genotype in our population of healthy animals. The statistical analysis both on the complete set of data (72 animals) and on the selected subsample set of data (24 animals- data not shown) highlighted that *FUT1* genotype affected the alpha and beta indices. The animals in *FUT1*^{AG} group had higher internal microbial variability than the other two genotypes and the Adonis procedure on Bray-Curtis distance matrix allow to revel dissimilarity between groups. Thus, to unravelling the differences between *FUT1* variance the sPLS-DA coupled with 10-fold cross-validation was applied. The *FUT1*^{AA} genotype generally associated with resistance to ETEC F18 infection was discriminated by OTUs belonging to *Lactobacillus* genus and in particular to *L. mucosae* and *L. reuteri* that are considered beneficial bacteria. Indeed, *Lactobacillus* presence in the gut is mostly associated to healthy status while reductions of the number of lactobacilli have been reported to occur in association with pathogen presences and under stress condition such as weaning (Heo et al., 2013). *L.reuteri* is considered one of the dominating species of pig gut microbiota (Oh et al., 2009) and it is commonly used as probiotic both in animals and human (Hou et al., 2015; Kelleher et al., 2002) for its propriety such as its ability to adhere to mucin and intestinal epithelial cells, competitiveness against pathogens, its immunological and antioxidant function, reducing the weaning stress syndrome and its capacity to modulated gut microflora (Hou 2015). On the other hand, the *FUT1*^{GG} genotype that is generally associated to the ETEC F18 susceptibility was discriminated by OTUs belonging to the *Veillonella* and *Fusobacteria* genus which have been highlighted as non-beneficial bacteria. Indeed a study by Gevers et al. (2015) found an increase of these genera on the microbiota composition of Crohn's disease (CD) human patients. Thus our results suggested that *FUT1* genotype is not controlling only the ETEC F18 adherence but it can also influence the commensal gut bacteria of healthy pigs pointing out that it can modulate the intestinal eubiosis. A reason of the potential relevant role of *FUT1* genotype on the microbiota modulation could be ascribed to the function of *FUT1* gene in the gastrointestinal tract; *FUT1* catalyses the addition of fucose to a terminal galactose in a α1,2-linkage, resulting in the formation of the blood group H-antigen. The mutation on (G>A) *FUT1* gene leads to a higher expression of *FUT1* and *FUT2* genes and an increase of the enzyme activity in *FUT1*^{GG} pigs. This biological mechanism results in a different level of glycosylation on the protein structure of

porcine ileum mucosa (Hesselager et al., 2016). In accordance with the immunohistochemistry observations of Coddens et al., (2007) our work showed that the *FUT1*^{AA} had a lower immunoreactivity score for the *UEA* than *FUT1*^{AG} and *FUT1*^{GG} and an opposite response for the *PNA* immunoreactivity both in brush border and goblet cell. The *FUT1* genotype effect on brush borders is mainly important because it is at this level that presumably the adhesion of ETEC F18 occurs. Our results contribute to explain the reason why the *FUT* ^{GG} is more susceptible to the ETEC F18 infection as those pigs had a higher active fucose metabolism that is required for the *E. coli* colonization (Chang et al., 2004). The reverse pattern seen for UEA and PNA immunoreactivity between the F18 susceptible (*FUT1*^{AG} and *FUT1*^{GG}) or not susceptible pigs (*FUT1*^{AA}) can be explained by the fact that in the latter genotype the genetically-determined impaired activity of the α(1,2)-fucosyltransferase could have alternatively led to the rise of the α(1,3)-galactosyltransferase that can generate the typical porcine epitope Galα1,3Gal, (Phelps et al., 2003) by the glycosylation of the Galβ1,4GlcNAc-R group.

The role that *FUT1* SNP plays on the glycosylation in the gut and on the modulation of some bacteria genus can explain also the variation in the plasma targeted metabolomic profile that we observed in our study. The *FUT1*^{GG} had a higher amount of some phosphatidylcholines than *FUT1*^{AA}, suggesting that *FUT1*^{GG} animals may have a higher activation of immune response as phosphatidylcholines are indicative compound of disturbed inflammation homeostasis for their function in contribution to both proliferative growth and programmed cell death Vorkas et al. (2015). Overall, our results for *FUT1* genotype underline the importance of this genetic marker for the gut microbiota composition and the host metabolism, however, the animals included in the study were healthy, and that may explain why we did not detect any effect on the gene expression profile for *FUT1* gene.

Regarding the SNPs influences on ADG and F:G we did not observe any significant association. For *FUT1* only a trend for body weight at d14, however, previous studies reported contrast results on growth performance (Bao et al., 2011; Huang et al., 2008) and more studies are needed to clarify the potential effect of this SNP on growth performance. For *MUC4*, our study cannot confirm the results of Fontanesi et al., (2012), but the lack of significance can be due to the different age of the animals taken into consideration in our study as well as the lower number of considered animals. The results for *BPI* in influencing the body weight of piglets were not continuous through all period of the trial, thus we cannot conclude that the SNP can influence the piglet growth performance but we cannot exclude its possible role, thus further studies to disclose *BPI* can be desirable.

Conclusion

In the present study, we showed that in healthy piglets *FUT1* and *MUC4* genotypes partially affect microbiota and fucose and galactose glycomic profiles, and gene expression for genes related to inflammation in jejunum mucosa, as well as plasma metabolic profile. Our results improved the knowledge regarding the interplay between host-genetics, gut microbiota composition and host physiological status of piglets around weaning. Furthermore, the application of piglets genotyping for *FUT1* and *MUC4* may represent a valid strategy to stratify the outcomes and reduce experimental variability, especially in trials aimed to study the gut homeostasis and the immune response. These findings could help in reducing the number of animals involved in the *in vivo* experiments.

Availability of data and materials

The datasets used and/or analysed during the current study available from the corresponding author on reasonable request. The datasets on microbiota data supporting the conclusions of this article are available in the Sequence Read Archive (SRA).

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Table 1. Ingredients and composition of piglet pre-starter diet (from d 0to d21) and starter diet (from d22 d42) expressed on a dry matter basis.

		Pre-starter diet	Starter diet
Ingredients			
Barley	%	25.00	-
Corn	%	-	56.00
Wheat	%	21.00	
Wheat bran	%	-	18.00
Cereal flakes	%	12.00	-
Milk whey	%	11.00	-
Soybean meal	%	4.80	20.00
Soybean protein concentrate	%	11.00	-
Cooked soy grains	%	8.00	-
Corn starch	%	1.60	1.50
Sunflower oil	%	1.00	0.50
L-Lysine HCl	%	0.66	0.60
L-Threonine	%	0.30	0.25
L-Tryptophan	%	0.08	0.07
DL-Methionine	%	0.30	0.24
L-Valine	%	0.15	0.16
Salt	%	0.45	0.45
Dicalcium phosphate	%	0.86	0.73
Calcium carbonate	%	1.30	1.00
Vitamin & mineral premix	%	0.50	0.50
Composition (as fed)			
Net Energy	MJ/kg	10.36	9.6
Crude Protein	g/kg	18.5	17.3
Digestible Lysine	g/kg	1.31	1.18
Calcium	g/kg	0.98	0.65
Digestible Phosphorus	g/kg	0.38	0.22

Table 2a. Primer and specific genotyping method including genotyping technique, restriction enzyme and references

Gene[1]		Primer sequence (5'->3')	Amplicon length	Accession number	SNP mutation and position	Technique	Restriction enzyme	References
MUC4	Forward	GTGCCTTGGGTGAGAGGTTA	367	DQ848681	8227 C>G	PCR-RFPL	XbaI	Jorgensen et al. (2003)
	Reverse	CACTCTGCCGTTCTCTTTCC						
FUT1	Forward	CTGCCTGAACGTCTATCAAGATC	420	AF136896	307 A>G	PCR-RFPL	CfoI	Meijerink et al. (1997)
	Reverse	CTTCAGCCAGGGCTCCTTTAAG						
AO	Forward	CGCCAGTCCTTCACCTACGAAC	1076 -> allele 0	GU256574		multiplex genotyping		Nguyen et al. (2011)
	Reverse	CGGTTCCGAATCTCTGCGTG	1076 and 340 -> allele A	GU256573				
	Forward	AATGTCCTTATGCTGGCCTGG						
	Reverse	AACAACACACTCCTGAACAACAGA						
BPI	Forward	CCCAACATGGAGATGCAGTTC	445	EF436278	1060 A>G	PCR-RFPL	HpaII	Christopher et al. (2004).
	Reverse	CAATGAATCAATGAGCACACC						

Table 2b. Primer and specific genotyping method including genotyping technique, restriction enzyme and references

Gene[1]	Primer sequence (5'->3')	Amplicon length	Accession number	SNP mutation and position	Technique	Restriction enzyme	References	
TLR4 SNP962	Forward	TTGGCAAATGCCTCTGTC	275	AJ628065	919 G>A	PCR-RFPL	CviAII (Hin1II)	Yang et al. (2012)
	Reverse	GTCCCAAATTCATTGTGAGAAC						
TLR4 SNP611	Forward	CAGGTTCTACATCAAGTGCCCCGAC	246	AJ628065	586 T>A	PCR-RFPL	HinfI	Yang et al. (2012)
	Reverse	CAGGACAGATTTGTCAAAACTTTCCAA						

[1]*MUC4*=Mucin 4, *FUT1*=Fucosyltransferase 1; *A0*= AO porcine blood group; *BPI*=Bactericidal/Permeability-Increasing Protein; *TLR4*=Toll-like Receptor 4.

Table 3. Primer and specific condition of genes expression analysis.

Gene		Primer sequence (5'->3')	Amplicon length	Accession number	Temperature of annealing
IL8	Forward	CAAGCAAAAACCCATTCTCC	123	AB057440	63°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			
HMBS	Forward	AGGATGGGCAACTCTACCTG	83	DQ845174	62 °C
	Reverse	GATGGTGGCCTGCATAGTCT			

IL8, Interleukine-8; REG3G, Regenerating islet-derived 3 gamma; GPX2, Glutathione peroxidase 2; 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 4. Porcine genotypes distribution, allele frequencies, Hardy Weinberg equilibrium (HWE) and Wright's fixation index (Fis).

	Gene[1]					
	MUC4	*FUT1*	*AO*	*BPI*	*TLR4-611*	*TLR4-962*
Genotype[2]	71	71	66	71	71	71
11	38	7	49	2	0	54
12	26	31	17	24	0	17
22	7	33	0	45	71	0
Allelic frequency						
1	0.72	0.32	0.87	0.2	0	0.88
2	0.28	0.68	0.13	0.8	1	0.12
HWE						
X2	17.57	3.7	27.57	2.36	NA	29.83
	No HWE	HWE	No HWE	HWE	NA	No HWE
DEVIATION FROM HWE						
Fis	0.1	-0.01	-0.15	-0.07	NA	-0.14

[1] *MUC4*, mucin 4 gene*; FUT1*, 1-fucosyltransferase gene; *A0*, A0 blood group gene exon 7 and 8; *BPI*, bactericidal/permeability increasing protein gene; *TLR4* -611, toll-like receptor 4 – snp 611; *TLR4*-962, toll-like receptor 4 – snp 962. [2] 11= the first genotype in alphabet order for each gene respectively; 12= the heterozygote variance for each gene respectively;22= the third genotype in alphabet order for each gene respectively

Table 5a. Genotypes influence on Body weight, Average daily gain (g/day), Feed to gain (g/day) of young healthy pigs.

Item	*MUC4*[1]					*FUT1*[2]				
	CC	CG	GG	SEM	*P*	AA	AG	GG	SEM	*P*
Body weight (g)										
day 7	7.19	7.09	7.14	0.14	0.79	7.26	7.04	7.11	0.14	0.94
day14	9.06	9.06	9.22	0.16	0.21	9.5	9.03	9.02	0.15	0.08
day 21	12.9	12.6	12.6	0.28	0.45	12.9	12.6	12.6	0.27	0.72
day 28	17	16.8	16.8	0.51	0.84	17	16.6	16.9	0.51	0.64
day 35	22.1	22.5	22.6	0.77	0.8	22.9	22.3	22.1	0.76	0.79
day 42	28.4	28.4	28.6	0.92	0.99	29.6	28.2	27.5	0.91	0.32
Average daily gain (g/day)										
weeks 0-3	275	261	263	13	0.45	275	261	263	13	0.72
week 3-6	730	745	748	35	0.86	730	745	748	35	0.2
weeks 0-6	504	505	507	22	0.99	504	505	507	22	0.27
Feed to gain (g/day)										
weeks 0-3	1.26	1.29	1.26	0.04	0.58	1.26	1.29	1.26	0.04	0.49
weeks 4-6	1.51	1.49	1.48	0.03	0.66	1.51	1.49	1.48	0.03	0.17

[1] *MUC4*, mucin 4 gene; [2]*FUT1*, 1-fucosyltransferase gene;

Table 5b. Genotypes influence on Body weight, Average daily gain (g/day), Feed to gain (g/day) of young healthy pigs.

Item	*A0*[3]				*BPI*[4]				*TLF4_962*[5]			
	A0	0	SEM	*P*	AG	GG	SEM	*P*	AG	GG	SEM	*P*
Body weight (g)												
day 7	7.05	7.23	0.12	0.15	7.14	7.14	0.11	0.93	7.07	7.21	0.12	0.31
day14	9.21	9.16	0.13	0.7	9.16	9.21	0.13	0.74	9.25	9.12	0.13	0.41
day 21	12.7	12.6	0.23	0.72	12.6	12.8	0.23	0.46	12.8	12.5	0.24	0.32
day 28	17	16.7	0.43	0.43	16.5	17.2	0.43	0.1	17.2	16.4	0.44	0.12
day 35	22.7	22.2	0.65	0.41	21.8	23.1	0.64	0.04	22.9	22	0.66	0.25
day 42	28.8	28.1	0.78	0.44	27.9	29.1	0.77	0.11	29	27.9	0.79	0.27
Average daily gain (g/day)												
weeks 0-3	268	264	11	0.72	262	270	11	0.46	273	260	11	0.32
week 3-6	754	728	30	0.41	718	764	29	0.11	756	725	30	0.39
weeks 0-6	513	498	18	0.44	492	519	18	0.12	517	494	19	0.3
Feed to gain (g/day)												
weeks 0-3	1.29	1.25	0.03	0.36	1.28	1.26	0.03	0.38	1.25	1.29	0.03	0.37
weeks 4-6	1.51	1.48	0.03	0.32	1.51	1.48	0.03	0.32	1.5	1.49	0.03	0.74

[3]*A0*, A0 blood group gene exon 7 and 8; [4]*BPI*, bactericidal/permeability increasing protein gene; [5] *TLR4*-962, toll-like receptor 4 – snp 962.

Table 6. Significance of the beta diversity clustering for genotypes explanatory variables.

Gene[1]	Adonis (P)[2]
MUC4	0.592
FUT1	0.004
A0	0.965
BPI	0.696
TLF4_962	0.316

[1] *MUC4*, mucin 4 gene*; FUT1*, 1-fucosyltransferase gene; *A0*, A0 blood group gene exon 7 and 8; *BPI*, bactericidal/permeability increasing protein gene; *TLR4*-962, toll-like receptor 4 – snp 962

[2]Resuslts (p values) from Permutational ANOVA like test with 999 permutations in Vegan package of R software using the Adonis function on Bray-Curtis distance.

Table 7a. Significant discriminant OTUs for *FUT1* genotypes obtained using the sPLS-DA methods coupled with 10-fold cross-validation and 100 repetitions

FUT1[1]	Family	Genus	Species	value.var[2]	Freq[3]	PC[4]
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.19	0.67	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.23	0.77	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.01	0.52	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.20	0.71	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.03	0.41	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.01	0.31	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.10	0.49	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.18	0.70	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.10	0.63	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.07	0.52	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.07	0.62	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.28	0.81	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__	0.22	0.75	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__mucosae	0.23	0.72	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__mucosae	0.03	0.47	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.30	0.79	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.26	0.75	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.39	0.80	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.24	0.74	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.27	0.78	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.22	0.75	2
AA	f__Lactobacillaceae	g__Lactobacillus	s__reuteri	0.25	0.77	2
AA	f__Leuconostocaceae	g__Weissella	NA	-0.49	0.55	1
AA	f__Streptococcaceae	g__Streptococcus	s__	0.04	0.38	2
GG	f__Veillonellaceae	g__	s__	0.51	0.61	1
GG	f__Veillonellaceae	g__Veillonella	s__dispar	0.44	0.48	1

Table 7b. Significant discriminant OTUs for *FUT1* genotypes obtained using the sPLS-DA methods coupled with 10-fold cross-validation and 100 repetitions

FUT1[1]	Family	Genus	Species	value.var[2]	Freq[3]	PC[4]
GG	f__Veillonellaceae	g__Veillonella	s__dispar	0.48	0.58	1
GG	f__Fusobacteriaceae	g__Fusobacterium	s__	0.07	0.30	1

[1] *FUT1*, 1-fucosyltransferase gene. [2]value.var, expresses the variance explained by the single OUT [3]Freq, express the frequencies by which the OTUs were chosen among the 100 repetitions of the cross-validation [4]PC, stand for principal component in which the discriminant OTUs were belonging

Table 8. Effect of *FUT1* and *MUC4* genotypes on the score for *Ulex europaeus agglutinin I* (*UEA*) and *Peanut agglutinin (PNA)* staining of brush border and Goblet cells in the mid-jejunal mucosa of young pigs.

Item	*MUC4*							*FUT1*						
	Mean				P-value			Mean				P-value		
	CC	CG	GG	SEM	CC vs CG	CC vs GG	CG vs GG	AA	AG	GG	SEM	AA vs AG	AA vs GG	AG vs GG
Ulex europaeus agglutinin I (UEA)														
Brush border	0.65	1.15	1.30	0.20	0.081	0.037	0.617	0.08	1.25	1.78	0.20	0.001	<0.0001	0.069
Goblet cells														
- villi	1.65	2.65	2.59	0.28	0.016	0.031	0.878	1.37	2.53	3	0.28	0.012	0.0005	0.234
- crypts	2.28	2.78	2.97	0.33	0.274	0.161	0.695	1.08	2.96	4	0.23	0.001	<0.0001	0.032
Peanut agglutinin (PNA)														
Brush border	0.82	0.92	0.72	0.23	0.779	0.779	0.467	1.32	0.71	0.43	0.21	0.062	0.006	0.289
Goblet cells														
- villi	0.85	0.72	0.8	0.29	0.752	0.901	0.862	1.57	0.57	0.22	0.29	0.031	0.003	0.389
- crypts	0.85	0.85	1.00	0.17	1.00	0.703	0.703	1.88	0.70	0.11	0.26	0.007	0.0001	0.118

[1] Mean is expressed as mean of assigned score values.

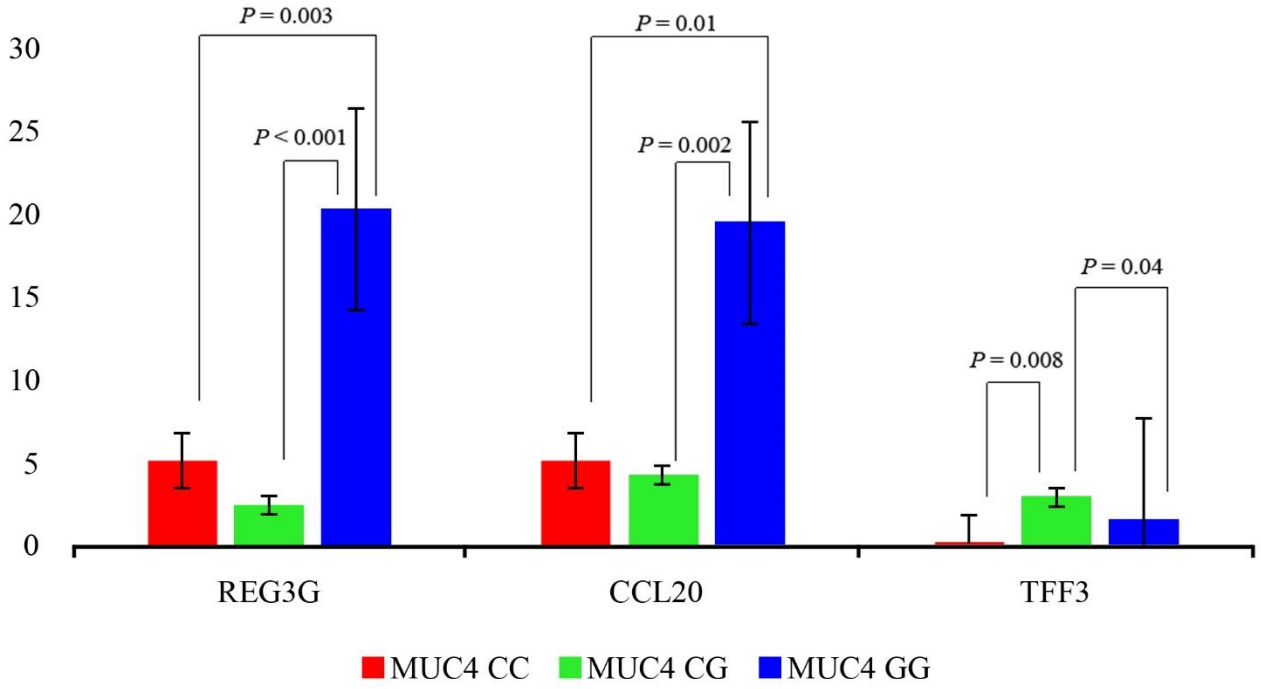


Figure 1. Effect of *MUC4* genotypes on the expression of *REG3G*, *CCL20* and *TFF3* in the mid-jejunal mucosa of healty young pigs.

Gene expression results and P values of GLM model of the comparisons between *REG3G*, *CCL20* and *TFF3* levels in *MUC4* CC, *MUC4* CG and *MUC4* GG pigs. Data are expressed as gene transcript copies per microgram of RNA multiplied by ten.

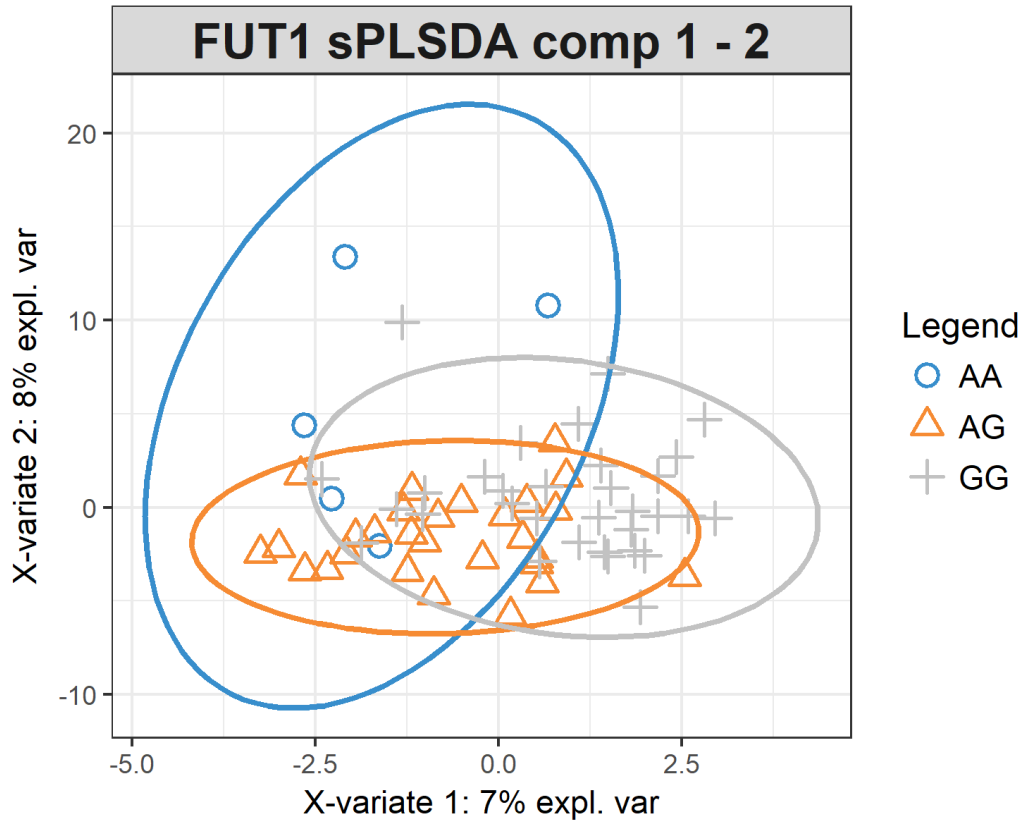
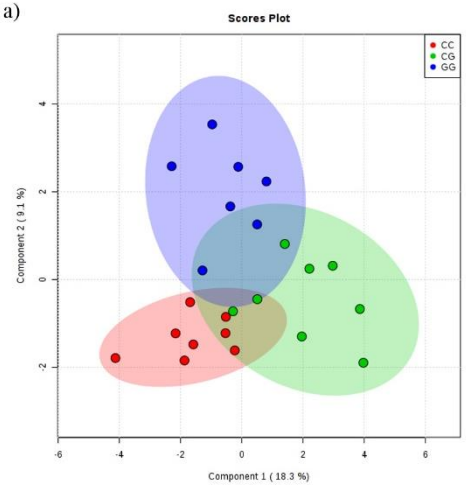


Figure 2. Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) results on jejunal microbiota composition of young healthy pigs.

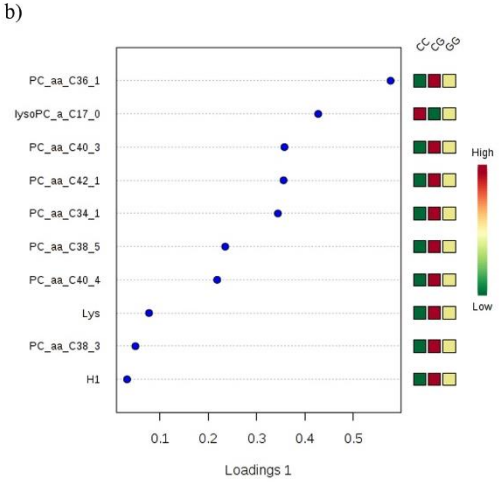
Separation of the AA (blue) AG (orange) and GG (grey) *FUT1* genotypes samples based on the optimized sPLS-DA model including three components. The first axes explain only 7% of variation in the data, while the second the 8% of variation.

A) *MUC4*

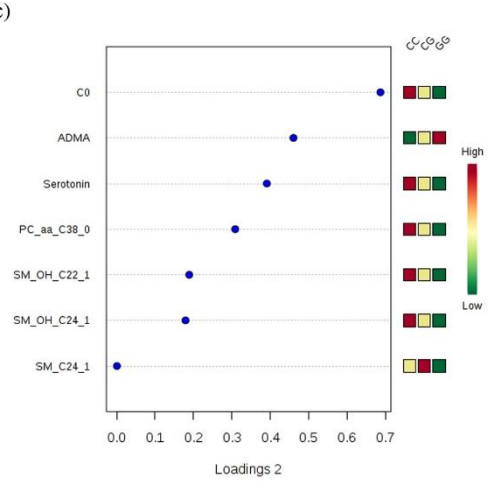
a)



b)

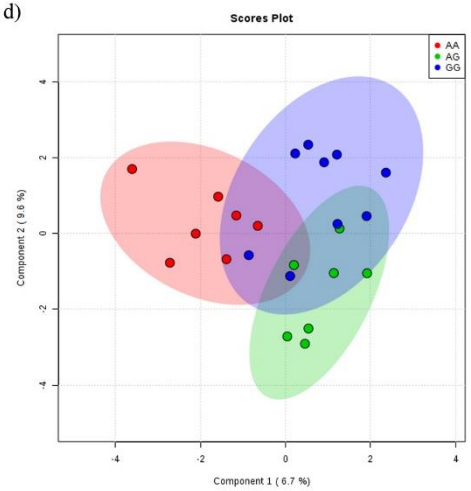


c)

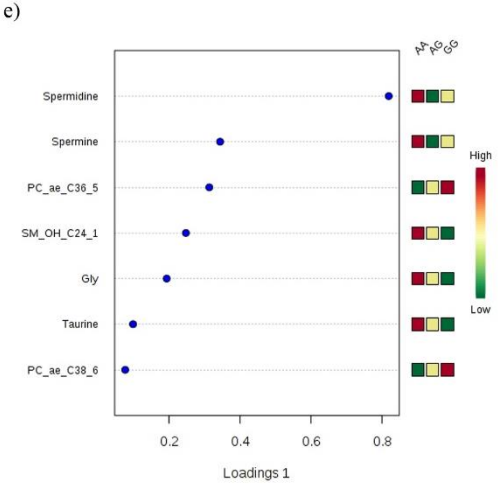


B) *FUT1*

d)



e)



f)

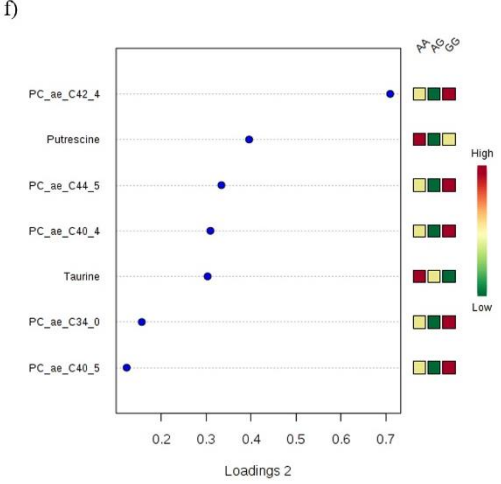


Figure 3. Score plots and loading plots of Sparse Partial Least Squares Discriminant Analysis (sPLS-DA) on serum target metabolomics profile of young healthy pigs according to *MUC4* (A) and *FUT1* (B) genotypes.

The a) and d) show the individual score plot for *MUC4* and *FUT1* variants respectively. The b) and c) represent the loading plots showing the metabolites that contribute toward the separation of sPLS-DA scores between *MUC4* variants. The d) and e) represent the loading plots showing the metabolites that contribute toward the separation of sPLS-DA scores between *FUT1* variants.

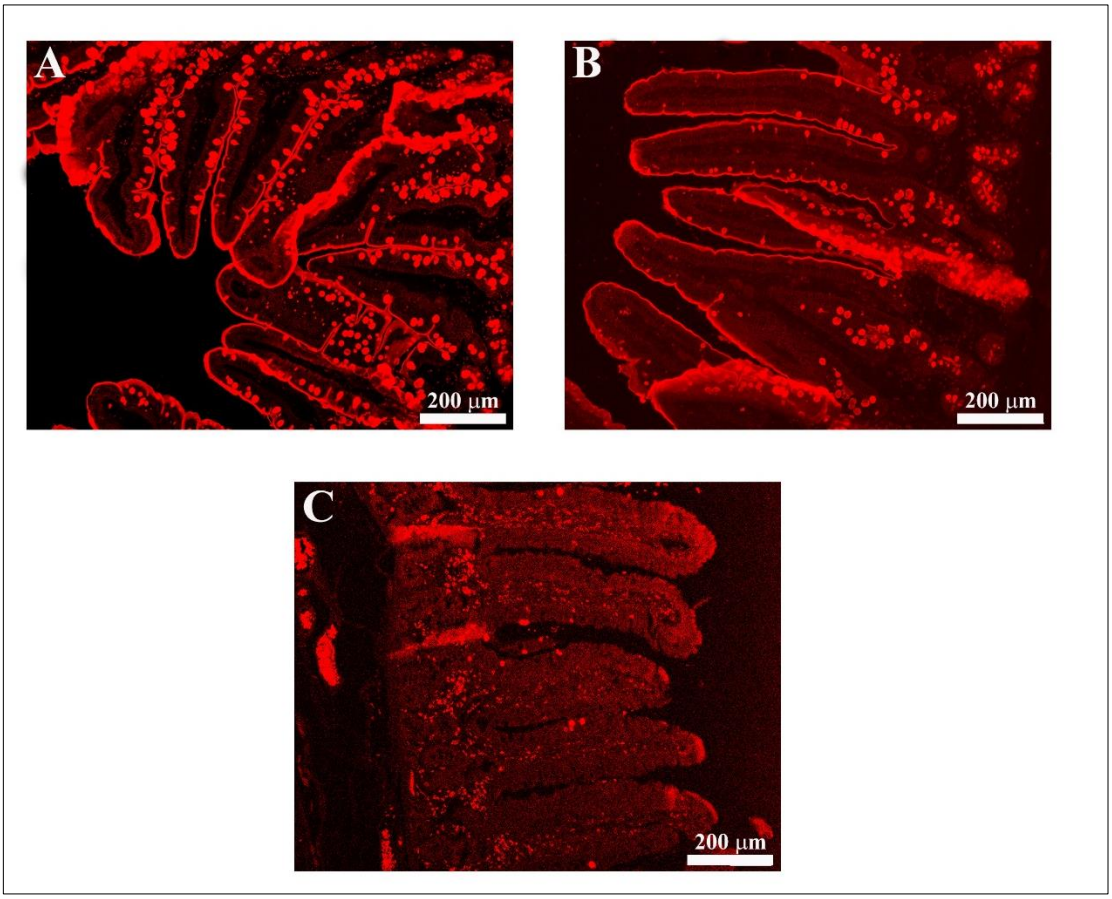


Figure 4. Representative images of pig jejunal villi obtained by immunofluorescence. The images show the different positivity obtained using *Ulex europaeus* agglutinin I (UEA) and peanut agglutinin (PNA). The image A shows the UEA high immunoreactivity of brush border in correspondence of the mucosal surface (score 2). In the villages of image B (score 1), the brush border exhibits well defined PNA positivity (less intense and thick than previous image A), while the intestinal villi of the C image were completely devoid of any marking at the brush border (score 0).

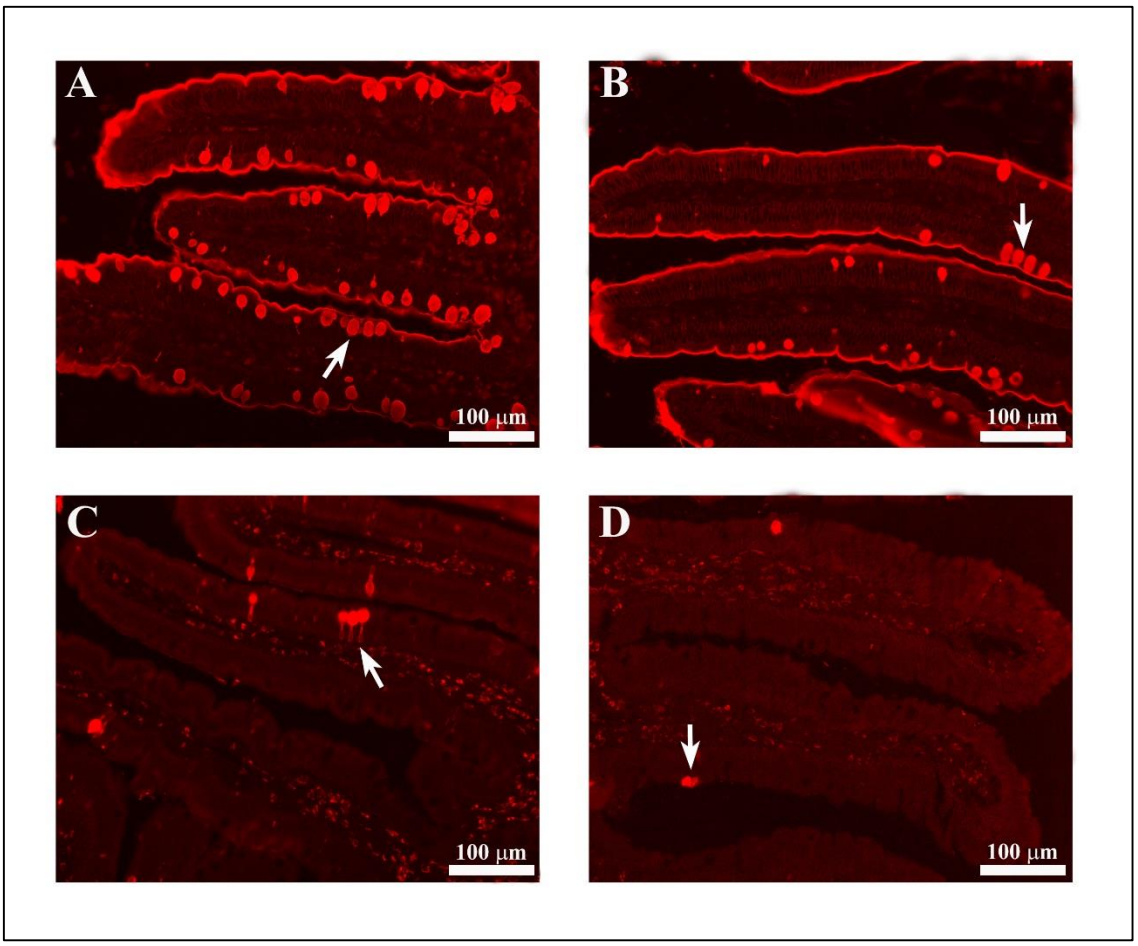


Figure 5. Images referred to the immunofluorescence method obtained using *Ulex europaeus* agglutinin I (UEA) and peanut agglutinin (PNA) in the pig jejunum sections.

The images show the different number and distribution of immunoreactive goblet cells in the villi. The number of immunoreactive goblet cells appeared high (score 4): in the image A (UEA marker), many immunoreactive goblet cells with round and / or pear shape were obserwed along the axis of the villus. The image B shows the mucosal surface of samples to which score 3 (PNA marker): some isolated or grouped immunoreactive goblet cells were observed (arrow). The image C shows the mucosal surface of samples to which score 2: the number of marked cells (slag) is reduced (UEA marker). Rare immunoreactive cells (score 1) are showed in the image D (PNA marker).

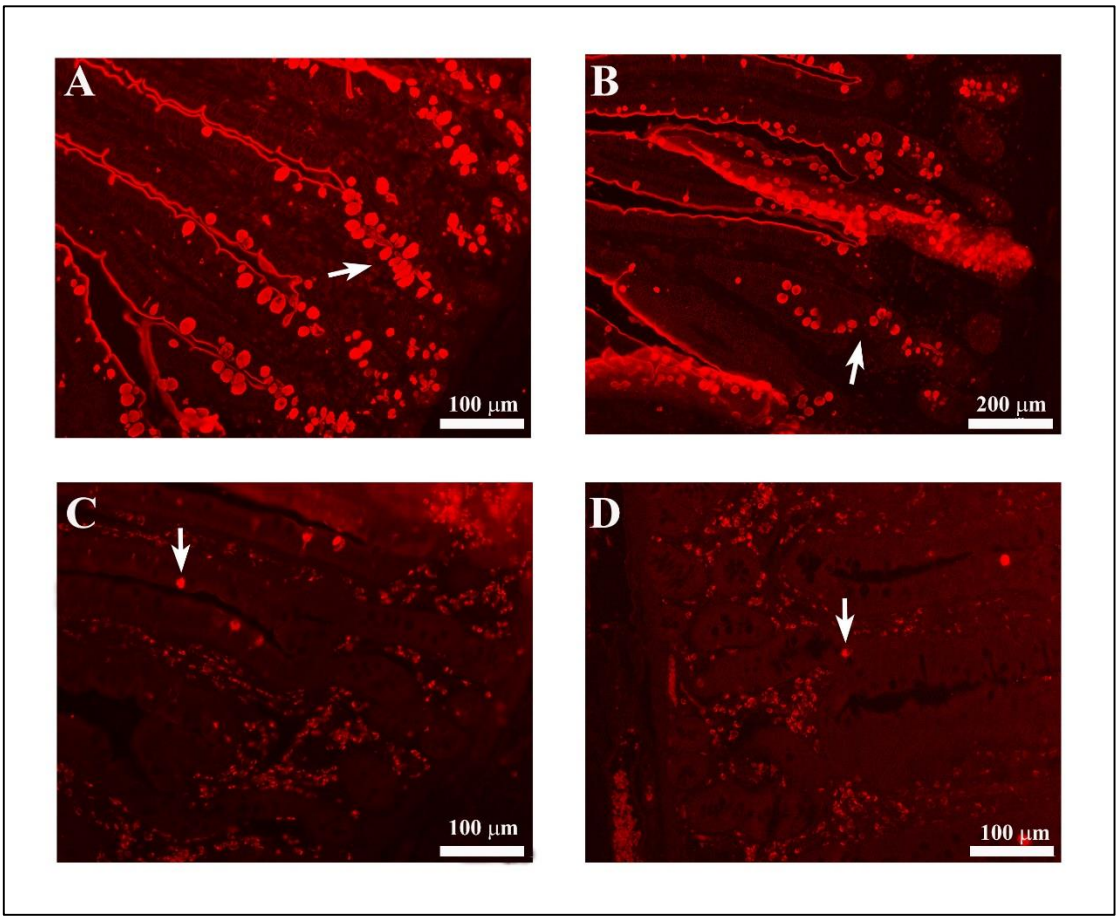


Figure 6. Representative images of swine digestive mucosa obtained with immunofluorescence using *Ulex europaeus* agglutinin I (UEA) and peanut agglutinin (PNA).

The images show the different number and distribution of immunoreactive goblet cells in the crypts. Figure A, show the score 4 : numerous aggregate goblets (arrows) in the crypts (UEA marker). Figure B shows the score 3 and Figure C the score 2 for which the number of PNA-immunoreactive goblet cells was lower than for the score 1. Figure D shows the very few immunoreactive cells (score 1).

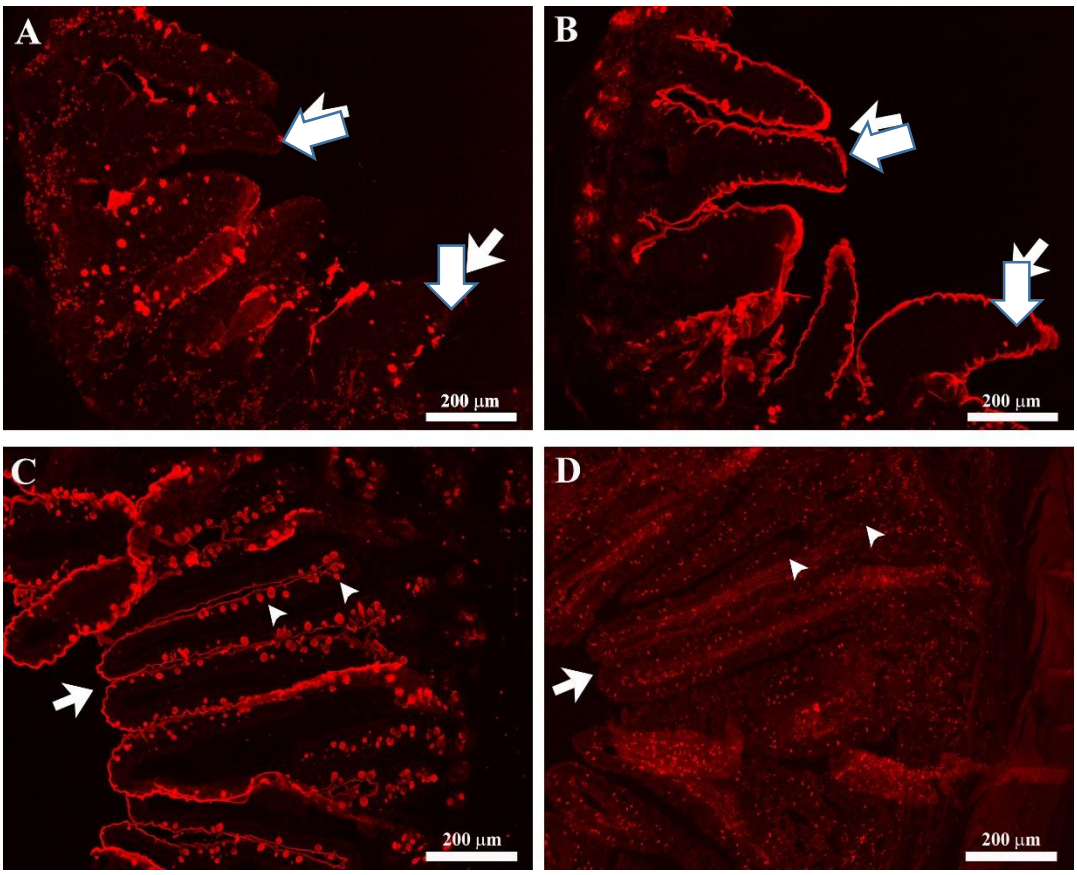


Figure 7. Images represent the same point of pig jejunal mucosa in serial sections stained with UEA (A and C) and PNA (B and D), respectively.

In the same intestinal villi (arrows), the immunoreactivity is totally opposite: the villi labeled with PNA (B) show a brush border very marked. On the contrary, in the image A (UEA marker) the mucosa surface does not show immunoreactivity. Similarity, the images C and D (arrows), show in the same point of jejunum the high UEA immunoreactivity (C, arrow) and the low PNA immunoreactivity (D arrow) of goblet cells in the villi and crypts.

Manuscript II

Effects of alpha-(1,2)-fucosyltransferase genotype variants on plasma metabolome, immune responses and gastrointestinal microbiota population of pigs pre- and post-weaning.

SUBMITTED to PlosONE

Keywords

Alpha-(1,2)-fucosyltransferase (*FUT1*), *Escherichia coli* F18, Metabolomics, Piglets, Post-weaning diarrhoea.

Abstract

In pigs, the alpha-(1,2) fucosyltransferase (*FUT1*) gene has been highlighted for its properties in controlling the intestinal expression of enterotoxigenic *E. coli* (ETEC) F18 receptors; a pathogen causing edema disease and post-weaning diarrhoea. In this study, we investigated the influence of ETEC F18 resistant ($FUT1^{AA}$;7 piglets) versus susceptible ($FUT1^{AG;}$ 10 pilgets) genotypes on: growth performance, plasma metabolic profiles, expression of candidate genes for intestinal mucosal homeostasis and immunity, number of selected bacteria and the concentration of short-chain fatty acids (SCFA) in faeces and digesta in piglets pre and post-weaning, and on the ETEC F18 adherence *ex vivo*.

The genotype had the strongest impact on the plasma metabolomic profile on day 7 and 28 (day of weaning) of age. $FUT1^{AG}$ piglets had higher level of N-methyl-2-pyrrolidinone, hippuric acid, oxindole, and 3-oxo-5-beta-chol-7-en-24-oic acid on day 7, and a higher level of guanosine on day 28 than the $FUT1^{AA}$ piglets. $FUT1^{AA}$ piglets had a higher level of betaine on day 7 and 3-methylguanine on day 28. On day 34 of age, the $FUT1^{AA}$ pigs had higher levels of S-2-hydroxyglutarate, L-phenylalanine, tauroursodeoxycholic acid and an undetermined PC/LysoPC, while Ile Glu Phe Gly peptide and genistein 5-O-glucuronide, and PC (18:0/0:0) were at higher levels in the $FUT1^{AG}$ piglets. *FUT1* genotype did not affect the growth performance and expression of candidate genes. $FUT1^{AG}$ piglets had higher number of haemolytic bacteria in faeces and in digesta than $FUT1^{AA}$ at 34 days of age. The colonic acetic acid concentration was highest in $FUT1^{AG}$ piglets. *FUT1* genotype may influence not only the expression of ETEC F18 receptors, but could potentially impact the gut homeostasis and metabotype of piglets pre and post-weaning. Further investigations on the relation between *FUT1* genotype and these aspects including the intestinal commensal microbiota will expand the knowledge on factors affecting the intestinal ecosystem.

Introduction

The gut microbiota composition and host genetics background are suggested to play key roles in the development of the inflammatory intestinal diseases (Frank et al., 2011). In pigs, the alpha-(1,2) fucosyltransferase (*FUT1*) gene has been highlighted for its properties in controlling the intestinal expression of enterotoxigenic *Escherichia. coli* (ETEC) F18 receptors; a pathogen causing edema disease and post-weaning diarrhoea. In humans, the fucosyltransferase 2 (*FUT2*) gene is responsible for H-antigen expression in the intestine, and this structure has been shown to be particularly relevant in pathogen adhesion and for the susceptibility to chronic inflammation in the intestine, e.g., inflammatory bowel disease (IBD), Crohn's disease (CD) and ulcerative colitis (UC) (McGovern et

al., 2010; Jostins et al., 2013). Indeed, genome wide associate studies have associated the mutation in the *FUT2* gene to the risk of developing CD and UC (McGovern et al., 2010; Jostins et al., 2013). In pigs, both the *FUT1* and *FUT2* genes are expressed in the intestine and the porcine *FUT1* is considered to be the orthologous to the human H gene which catalyses the addition of fucose to a terminal galactose in an alpha 1,2-linkage (Bao et al., 2012).

Sophisticated genetic tools have extensively been used to genetically select pigs with favourable production traits such as increased growth rate, carcass weight, number of piglets per litter, and improved meat quality, while only little focus has been given to animal health and robustness. However, the growing concern regarding bacterial antibiotic resistance has increased the demand for alternative therapeutic or preventive options when dealing with infectious diseases. The continued production of new knowledge in the field of disease development and molecular genetics enables to develop selection schemes that also incorporate criteria associated with innate disease resistance. *Escherichia coli* (*E. coli*) F18 is a highly pathogenic bacterium causing post-weaning diarrhoea (PWD) and edema disease (ED) in young pigs, and until now, successful and definitive tools able to control this pathogen remains to be developed (Bao et al., 2012; Meijerink et al., 2000). Breeding F18-resistant piglets has been studied as a potentially preventative strategy to limit PWD and ED (Frydendahl et al., 2003). *Escherichia coli* F18 recognizes and adheres to highly specific receptors located on the small intestinal brush border. Previous studies have suggested that the F18 receptor has a glycophospholipid structure and that its expression is genetically controlled by *FUT1* gene located near the blood group inhibitor S on chromosome 6 (Vogeli et al., 1996; Meijerink et al., 1997). Meijerink and co-authors (1997) proposed that the single point mutation G>A at base-pair 307 in the *FUT1* gene influences piglet susceptibility to ETEC F18 diarrhoea, classifying piglets with the $FUT1^{AA}$ genotype as resistant animals and piglets with the $FUT1^{AG}$ and $FUT1^{GG}$ genotypes as susceptible. The $FUT1^{AA}$ genotype leads to a lower level of *FUT1* and *FUT2* genes expression, which encode for galactoside 2-L-fucosyltransferase enzyme (FUT2), and thus reduce the enzyme activity (Meijerink eta l., 2000). In human, FUT2 enzyme catalyses the addition of terminal alpha (1,2) fucose residues, producing the H type 1 carbohydrate expressed on the surface of epithelial cells and in mucosal secretions of secretor individuals (Henry.et al., 1996). The fucosylation of carbohydrate structures expressed on cell surfaces of the gut by glycoproteins, glycolipids, and proteoglycans have been associated with biological processes such as inflammation, host–pathogen interactions, and tumor metastasis (Pickard et al., 2014; Hollingsworth et al., 2004). Recent data reported by Hesselager et al. (2016) showed that there are significant differences in the O-linked glycans of the intestinal mucosal proteins between $FUT1^{AA}$ and $FUT1^{AG}$ pigs. The $FUT1^{AG}$ leads a higher level of O-glycan structures that were fucosylated to show H-antigens (Hesselager et al. 2016). Accordingly,

a previous study in mice Hasson et al., (2005) showed that the intestinal alpha (1,2) fucosylation in FUT2-null mice, which are characterized by a null expression of *FUT2* gene and were obtained by replacing *FUT2* with the bacterial reporter gene lacZ (Domino et al., 2001) had a lower level of fucosylation in the intestinal mucosa. Additional studies in mice indicate that the intestinal alpha-(1,2) fucosylation represents a significant energy source for some bacteria, suggesting that alpha-(1,2) fucosylated glycans may contribute to the establishment and maintenance of the commensal microbial community (Nanthakumar et al., 2013).

Nevertheless, so far there exists only limited knowledge on *FUT1* polymorphism and pig- gut microbiota interactions. Both genotype and the gut microbiota are reported to be intrinsic contributors in regulating the gut mucosal maturation, the intestinal changes occurring during the weaning transition, and more generally, the overall health of the host (McKnite et al., 2012; Sommer et al., 2013; Schokker et al., 2015). In the present study we therefore developed the hypothesis that *FUT1* genotype (ETEC F18 susceptible versus resistant) would affect the host metabolism, immune response, and the gastrointestinal microbiota composition in pigs during the suckling and post-weaning period. The dual purposes of the study were to characterise the metabolomic profile and immunological parameters of the gut epithelium of $FUT1^{AA}$ and $FUT1^{AG}$ piglets, and to investigate how *FUT1* genotype influences the establishment of the gastrointestinal microbiota, using classical culture-dependent techniques, in 5 to 34 days old piglets.

Methods

The present study was conducted according to the license (J. nr 2012-15-2934-00125) obtained from the Danish Animal Experiments Inspectorate, Ministry of Food, Agriculture and Fisheries, Danish Veterinary and Food administration. The study was performed at the experimental facility at the Department of Animal Science (Foulum, Aarhus University).

Animals, study design and *FUT1* genotyping

A total of 17 piglets were included in the study. The animals were obtained by breeding two $FUT1^{AG}$ sows with the same $FUT1^{AA}$ boar ((Danish Landrace x Yorkshire breed) x Duroc), providing $FUT1^{AG}$ and $FUT1^{AA}$, but no $FUT1^{GG}$, piglets. FUT^{AG} and $FUT1^{GG}$ piglets are considered equally susceptible to ETEC F18 (Meijerink.et al., 2000). An ear tissue sample was collected shortly after birth and used for *FUT1* genotyping. In brief, the genotyping procedure included a PCR amplification of the *FUT1* gene product harbouring the mutation of interest. Subsequently, the PCR fragments were digested with the HinP1I restriction enzyme and run on a 1.5% agarose gel according to Bao et al. (2008).

The pigs were rised with their sow until the day of weaning (day 28) and had free access to water and creep feed (without antimicrobials) from day 21 of age. After weaning, piglets were housed together in pens according to litter and fed a standard post-weaning diet (without antimicrobials and zinc oxide). The temperature in the weaning unit was 23°C and piglets had free access to rooting material. All animals were individually weighed once a week from day 7 of age, and the average daily weight gain (ADWG) was calculated.

Sample collection

Blood samples were collected in EDTA-containing vacutainers (Vacuette, Greiner Bio-One GmbH, Kremsmünster, Austria) from the jugular vein on day 7, 28, and 34 after birth. The blood samples were immediately centrifuged at 3,000 × g for 10 min, and the plasma was stored at –80°C for further analyses of the metabolic profile. Faecal samples were collected directly from the rectum at 5, 7, 14, 21, 28, and 34 days of age. Subsamples were stored at -20˚C for organic acid analysis. Bacterial enumerations by plating were performed on a subsample of fresh faeces. Due to the small size of the piglets, adequate quantities of faeces for microbial analyses and of blood for metabolomics analysis could not be sampled from all piglets at all ages.

All piglets were euthanized at 34 days of age. The abdomen was incised and the gastrointestinal tract removed. Luminal contents (digesta) from the stomach, last third of distal small intestine, caecum and middle part of the colon were sampled immediately and subsamples were stored at -20˚C for short chain fatty acid (SCFA) analysis. Bacterial enumerations by plating were performed on fresh faecal subsamples. After removal of the lumen content, the epithelium was rinsed and mucosal samples were obtained from four gastrointestinal segments (stomach, ileum, caecum and mid colon). The samples were stored at -80˚C for further candidate genes expression analyses. In addition, 15 cm of distal small intestine were collected in order to investigate the presences of the specific receptors for ETEC O138:F18 adhesion on the mucosa using the Porcine Intestinal Organ Culture (PIOC) procedure.

LC-MS metabolomics determination

The LC-MS metabolomic profile of the plasma samples was analysed using a Dionex UltiMate 3000 (Dionex, Sunnyvale, CA, USA) ultra-performance liquid chromatography (UPLC) system, coupled to a MaXis impact Quadrupole Time-of-Flight (QTOF) mass spectrometer (Bruker Daltonics GmbH, Bremen, Germany) operating in positive and negative electrospray ionization mode (ESI+, ESI-). The analytical column was an Acquity UPLC HSS T3 (2.1 x 100 mm, 1.8 µm; Waters, Milford, MA, USA), kept at 30ºC. Sample preparation and the LC-MS analysis were performed as described by Ingerslev et al. (2015). In order to evaluate the performance of the analytical system, blank samples

(100% acetonitrile) and pooled plasma samples from the pigs at each time point were used as quality controls and reinjected for every six samples.

Data acquisition and control of UPLC/MS was done using oTOF control v.3.4 and HyStar v.3.2 (Bruker Daltonics GmbH, Bremen, Germany). Data pre-processing was performed by calibrating the mass spectrum using Data Analysis v.4.0 (Bruker Daltonics GmbH, Bremen, Germany) followed by the Find Molecular Features (FMF) tool. Parameters for compound detection with FMF were set to 5 for S/N threshold, 8 spectra for compound length, and a smoothing width of 2 for the peaks. Other alterations of the data included shortening of the chromatogram retention times to exclude noise and carry-over compounds, the mass range was kept between m/z 50 and 1000, and the most common adducts in positive and negative mode were subtracted.

Gene expression analysis

Total RNA was extracted from the samples of the four tissues using the NucleoSpin RNA kit (Macherey-Nagel) according to the manufacturer's protocols. The quality and quantity of RNA was evaluated using agarose gel electrophoresis and a spectrophotometer (NanoDrop ND-1000; Saveen Werner), respectively. The sample concentration was adjusted to200 ng/µl, and the RNA then reversed transcribed using the High Capacity cDNA Reverse Transcription Kit (Invitrogen) according to the manufacturer's instructions. The qPCR of glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*), Interleukin 10 (*IL10*); Tumor Necrosis Factor Alpha (*TNF-α*), cyclo-oxygenase-2 (*COX-2*), Zona Occludens 1 (*ZO-1*), and Occludin (*OCLN*) genes were run in 384-well plates by mixing 2 µl of complementary DNA with 8µl of a mix containing 5 pmol of each primer, 1pmol of the probe and 5µl TaqMan Master Mix (catalogue no. 4324018; Applied Biosystems). For the qPCR analyses of analysis of *GAPDH*, *TNF-α*, *IL10* and *COX-2* genes, were used the sequence (5'-3') of primers and probes reported by Sugiharto et al. (2015); while for the *ZO-1* and *OCLN* genes, the primers and probe from 20 x TaqMan Gene Expression Assay, catalogue no. 4351372 (Applied Biosystems), were used. The analysis was performed in duplicates for the target genes and in triplicates for the housekeeping gene (*GAPDH*) under standard amplification conditions determined for the Applied Biosystem ViiA 7 Real-Time PCR system (Life Technologies). The data was normalized to the expression of the housekeeping gene. Relative quantitation of gene expression was calculated using the 2-ΔΔCt method.

Dry matter and organic acid analysis

Dry matter content of digesta was determined by freeze-drying (ScanVac Coolsafe 55, Labogene ApS, Lynge, Denmark). The concentration of short-chain fatty acids, including lactic acid, in faeces and digesta were measured as previously described by Canibe et al. (2008).

Microbiological enumerations by plating

Faecal samples (approximately 1 g) were transferred to plastic bags and 5 or 10 ml pre-reduced salt medium was added. The content was homogenised in a Smasher paddle blender (bioMérieux Industry, USA) for 2 minutes. Digesta samples (approximately 5 g) were transferred to flasks containing 50 ml pre-reduced salt medium (Holdeman 1977). The flask content was transferred to a CO2 flushed bag and homogenized in a stomacher blender for 2 minutes. 1 ml homogenate was transferred to a Hungate tube containing 9 ml pre-reduced salt medium and 10-fold dilutions were prepared using the technique previously described by Miller and Wolin (Miller and Wolin 1974). Each sample was plated on selective and non-selective agar plates.

Enterobacteriaceae were enumerated on MacConkey agar (Merck 1.05465) after aerobic incubation for 1 day. Yeasts were enumerated on Malt, Chlortetracycline and -Amphenicol agar (Merck 1.03753 (yeast extract), 1.05397 (malt extract), 1.07224 (bacto-pepton), 1.08337 (glucose), 1.01614 (agar-agar) and Oxoid Sr0177E) after aerobic incubation for 2 days. Haemolytic bacteria were enumerated on blood agar (Oxoid Pb5039A) after aerobic incubation for 1 day. *Clostridium perfringens* were enumerated using the pour-plate technique on Tryptose Sulfite Cycloserine agar (Merck 1.11972 and 1.00888) after anaerobic incubation for 1 day. Lactic acid bacteria were enumerated on de Man, Rogosa and Sharp agar (Merck 1.10660) after anaerobic incubation for 2 days. Total anaerobic bacteria were enumerated in roll tubes containing pig colon fluid-glucose-cellobiose agar and incubated for 7 days (Miller and Wolin 1974). All plates and roll-tubes were incubated at 37˚C.

Porcine Intestinal Organ Culture (PIOC)

The PIOC procedure was included to investigate the adherence of ETEC O138:F18 to the mucosa of $FUT1^{AA}$ and $FUT1^{AG}$ piglets following the procedure previously described by Sugiharto et al. (2015b). In short, a distal small intestinal segment (15 cm) was sampled and incubated with a solution containing ETEC O138:F18 for 1 hour. The tissue was then homogenized, diluted to 10^{-6} and *E. coli* enumerated on MacConkey (Merck 1.05465) agar plates after aerobic incubation at 37˚C overnight.

Statistical analysis

Piglet performance data as weekly body weight (BW) and average daily weight gain (ADWG) were analysed using and ANOVA with a factor design including the *FUT1* genotype, litter as fixed effects and the initial body weight as covariate.

With regard to the metabolomics data, the initial step encompassed multi-level PCA (MLPCA) carried out on the full dataset (day 7, 28, 34) and separately on the individual time datasets in order to highlight possible sub-structures in the data. The data was Pareto-scaled and potential outliers were

removed after initial visual inspection of PCA plots. To evaluate metabolite differences between *FUT1* genotypes, potential confounding factors were evaluated by using a linear regression model. The linear regression model included sex, litter and weight of the animals at the three different time points (7, 28 and 34) as fixed factors. Confounding effects were removed by computing the residuals of model considering the factors described in the linear regression model. All further analyses were based on these residuals. A sparse Multilevel Partial Least Squares Discriminant Analysis (sMLPLS-DA) (Westerhuis et al., 2010) was used to investigate differences in the plasma metabolic profile between the two *FUT1* genotypes on each of the three-time point. sMLPLS-DA was applied using a dummy variable distinguishing the two genotype (AA and AG) as a response variable and the scaled subject variations as predictors. This version involves the automatically choice, using an internal 5-fold cross-validation procedure of a sparseness coefficient that controls the trade-off between the goodness-of-fit of the model and its complexity (i.e., the number of selected metabolites).

Furthermore, in order to evaluate both the stability and the effect size of the metabolites contributing with non-null coefficients to discriminant dimensions, a validation test of 1000 permutation test (permutation of predictors) coupled with a Leave One Out (LOO) procedure was applied. Metabolites having a $P < 0.1$ both for effect size (Psi) and stability (Pst), simultaneously, were considered stable and significant.

All statistical analyses on metabolomics data was performed in R v. 3.0.3 (R Development Core Team, 2008) using the "lme4" package (Bates et al., 2017) for the computation of metabolite residuals, the "spls" package (Chung 2014) (function cv.splsda and splsda) for the sMLPLS-DA analysis and the function "prcomp" for PCA.

The impact of genetic variation $FUT1^{AA}$ versus $FUT1^{AG}$ on the expression of candidate pro-inflammatory and intestinal barrier regulation genes among the different intestinal tissues was determined using a linear model in which the *FUT1* genotype, intestinal tissues, and their interaction were included as fixed effects while litter was included as random effects and pigs as repeated measurement.

The effect of *FUT1* genotype and age on bacterial composition, organic acid concentration, and pH, were analysed by fitting the data to a linear mixed model in which genotype and age/intestinal segment were included as fixed effects, while litter were included as random effects and pig as repeated measurement (by including random intercept terms) to account for multiple observations within the same litter or within the same pig. The fixed effects were tested using an F-test with Kenward-Roger approximation, where the reduced model was tested against the full model. When

age was found to have a significant effect, a post-hoc test was performed, including Bonferroni adjustment to correct for multiple comparisons (Halekoh et al., 2014). Effects were considered significant when $P < 0.05$, whereas when $P > 0.05$ but ≤ 0.10 differences were considered to indicate a trend towards a significant effect.

Statistical analysis was performed with R statistical software (R Development Core Team, 2008) using the "lm" function of "stats" package version 3.3.0 for genes expression and piglet performance data; the "lmer" function from the "lme4" package [28], the "KRmodcomp" function in the "pbkrtest" package (Halekoh et al., 2014) and the "multcomp" package (Hothorn et al., 2008) were applied for the pH, bacterial and organic acid parameters.

Metabolite Identification

Selected metabolites were identified using METLIN (http://masspec. scripps.edu/), the Human Metabolome Database (HMDB, http://www.hmdb.ca/), and LIPID MAPS database (http:// www.lipidmaps.org/) searches. Confirmation of compounds was done by comparison of retention times and MS/MS fragmentation patterns to those found in METLIN or HMDB, or to commercial standard compounds when available. Metabolites were reported as described by Sumner et al. (2007) using the four level identification system: 1. Identified compounds; 2. Putatively annotated compounds; 3. Putatively characterized compound classes; 4. Unknown compounds.

Results

All pigs remained healthy during the trial. Genetic testing revealed that ten piglets were of the sensitive genotype $FUT1^{AG}$ and seven piglets were of the resistant genotype $FUT1^{AA}$. (3 $FUT1^{AG}$ and 3 $FUT1^{AA}$ in one litter 7 $FUT1^{AG}$ and 4 $FUT1^{AA}$ for the other litter). Analyses of the data according to the described statistical model revealed that genotype had no significant effect on the BW and ADWG parameters during the entire period of the trial. The ADWG from day 7 to day 34 of age was 0.196 kg/day for the AG and 0.153 kg/day for the AA genotype (SEM=0.017 kg/day).

Plasma metabolomics profile

The score plot of the Principal Component Analysis (PCA) conducted on total plasma samples is shown in Figure 1. Clusters according to piglet age were clearly visible both during the suckling period (day 7 versus day 28) and after weaning (day 28 versus day 34). After outliers filtration (2 outlier on day 7; 1 outlier on day 28 and day 34), the datasets were composed of 12 pigs (7 $FUT1^{AG}$ and 5 $FUT1^{AA}$) and 789 metabolites on day 7, and 15 animals (10 $FUT1^{AG}$ and 5 $FUT1^{AA}$) with 727 on day 28 and 707 compounds on day 34. The litter and genotype effects in the single time datasets

are presented in the PCA score plot (Figure 2). At 7 days of age, the effects of both litter and *FUT1* genotype were evident on the metabolic profile exploring components one (PC1) and two (PC2), which explained 18.7% and 13.3% of the total variance (Figure 2A). The Figure 2B show that $FUT1^{AA}$ and $FUT1^{AG}$ clustered into two groups along the PC2 (explained 11.7% of the total variance). The PCA showed a clear separation according to litter along the PC1 (explained 15.5% of total variance) on day 28. Litter and genotype did not have an effect on the metabolic profile on day 34. Discriminant identified compounds ($P \leq 0.01$) for stability and effect size resulted from sMLPLS-DA coupled with LOO validation model in plasma metabolic profiles of $FUT1^{AG}$ and $FUT1^{AA}$ animals on day 7, 28 and 34 are listed in Table 1a-c while a complete list, including the unidentified compound, was shown on supplementary table 1 (S1). Considering the discriminant identified compound on day 7, the plasma metabolic profile of $FUT1^{AG}$ pigs was characterised by a higher level of N-Methyl-2-pyrrolidinone (Psi = 0.01; Pst = 0.01), Hippuric acid (Psi = 0.01; Pst = 0.08), Oxindole (Psi = 0.02; Pst = 0.07) and 3-Oxo-5-beta-chol-7-en-24-oic Acid (Psi = 0.02; Pst = 0.08) compared to the profile of $FUT1^{AA}$ piglets, which had a higher level of Betaine (Psi = 0.03; Pst = 0.08) (Table1). At weaning (day 28 of age), two identified compounds discriminated the *FUT1* genotypes; 3-methylguanine (Psi <0.001; Pst = 0.001) characterised the $FUT1^{AG}$ and guanosine (Psi<0.001; Pst = 0.02) the $FUT1^{AA}$ piglets. Eight identified compounds discriminated the $FUT1^{AA}$ and $FUT1^{AG}$ pigs based on their metabolome on day 34 of age. (S)-2-hydroxyglutarate (Psi = 0.02; Pst = 0.001), L-phenylalanine (Psi = 0.04; Pst = 0.07), PC 18:0/0:0 (Psi = 0.04; Pst = 0.03) and tauroursodeoxycholic acid (Psi = 0.02; Pst = 0.06 positive mode; Psi = 0.03; Pst = 0.08 negative mode) were higher in plasma of pigs with the $FUT1^{AA}$ genotype, while the $FUT1^{AG}$ piglets had higher values of an unidentified PC/ LysoPC (Psi = 0.03; Pst = 0.001), Ile Glu Phe Gly peptide (Psi = 0.04; Pst = 0.01) and genistein 5-O-glucuronide (Baicalin). (Psi = 0.02; Pst = 0.07) (Table1).

Gene expression analyses - intestinal mucosa

No significant differences between *FUT1* genotypes were observed in the intestinal expression of analysed genes (Table 2), but the *COX2* expression was higher in mucosa from the mid colon compare to the mucosa from the other gut segments (P = 0.02), and *OCLN* expression (P = 0.008) was higher in mucosa from the ileum compared to mucosa from the caecum and mid colon. A trend of significance (P <0.1) was observed for *FUT1* genotypes and tissue interaction for *ZO-1* expression.

Microbiological enumerations by plating and organic acid concentrations

Faeces. Higher faecal numbers of haemolytic bacteria at 34 days of age (P = 0.003) and a tendency to higher numbers of lactic acid bacteria (P = 0.06) were observed in $FUT1^{AG}$ piglets (Table 3). The number of haemolytic bacteria was similar between day 5 and 28, whereas a significant increase was

detected on day 34 ($P < 0.003$). An age-dependent decrease of *Clostridium perfringens* was found with numbers being lowest on day 28 ($P < 0.001$) and 34 ($P < 0.001$). The same was observed for lactic acid bacteria, with lower numbers on day 28 ($P \leq 0.03$) and day 34 ($P \leq 0.005$) compared to day 5 and 7. There was no significant effect of genotype or age on the number of Enterobacteriaceae or total anaerobic bacteria. Furthermore, no difference in the concentration of faecal organic acids between genotypes was determined, except for a tendency towards a lower concentration of butyric acid in $FUT1^{AA}$ piglets ($P < 0.1$) (Table 4). The concentration of acetic acid was higher at 34 days of age (one-week post-weaning) compared to day 5, 7, 14 and 21 of age (suckling period) ($P < 0.05$). The concentration of propionic acid was constant between day 5 and 21, but at 34 days of age, a significant increase in propionic acid was observed ($P < 0.001$). A tendency to a lower concentration of butyric acid ($P < 0.01$) with age was measured, whereas no effect of age was detected for valeric, iso-butyric and iso-valeric acid (data not shown).

Digesta. Genotype did not influence dry matter content or pH (Table S2) of gastrointestinal digesta. $FUT1^{AG}$ piglets had a higher number of Enterobacteriaceae ($P \leq 0.02$) in digesta from the distal small intestine, caecum and mid colon; of haemolytic bacteria ($P = 0.02$) in the stomach, small intestine, caecum and colon; and of total anaerobic bacteria in the distal small intestine compared to $FUT1^{AA}$ piglets ($P = 0.004$) (Table 5). The concentration of acetic acid was higher in the mid colon of $FUT1^{AG}$ piglets ($P = 0.01$) (Table 6). Digesta concentrations of propionic, butyric, lactic, valeric, and the sum of iso-butyric and iso-valeric acid were similar in both genotypes.

Discussion

In this study, a metabolomics approach was applied to evaluate the influence of *FUT1* genotype on system-biological responses in healthy piglets during the suckling and post-weaning period; bacterial counts and gene expression analysis were applied to further add information about the host's gastrointestinal homeostatic environment. The plasma metabolomics profile and faecal bacterial enumerations showed an important influence of age, which is ascribed to the piglets physiological and immunological maturation that occurs during the initial phase of life (Lallès et al., 2007; Stokes et al., 2004), and to the dietary change that occurred at weaning. In our study, we found a progressive decrease in the number of lactic acid bacteria and *Clostridium perfringens* in the period from 5 to 34 days of age. Lactic acid bacteria, especially Lactobacillus spp., are important in maintaining intestinal homeostasis as they are capable of reducing pH by lactic acid production, compete with potential pathogens, and produce bacteriocins (Hou et al., 2015; Daly et al., 2014). A significant increase in the number of haemolytic bacteria in faeces between 28 and 34 days of age indicates an important

effect of the weaning process and the switch onto a solid feed. Indeed, the gastrointestinal microbiota is strongly influenced by diet, both by the change in milk composition during suckling period (Bian et al., 2016) by the switch from the sow's milk to the solid feed, and generally an increase in microbial richness and complexity occurs between birth and the post-weaning period (Mach.et al., 2015; Slifierz et al., 2015). The observed effect of age of piglets on the metabolomics profile and bacterial population will not be discussed in depth here, as it was not the primary focus of the present study. However, it is important to note that an interaction between *FUT1* genotype and age was detected in the number of haemolytic bacteria in the faeces on day 34, suggesting that the interaction of factors can affect the number of possible pathogenic bacteria.

The presented metabolomics results shed light on the influence of *FUT1* genotype to specific architecture in the pig metabolome. The PCA score plot showed relatively consistent clusters attributable to *FUT1* genotype when considering each of the single time points separately, i.e., day 7, 28 and 34. The influence of genotype was clearest in the metabolome profile of young animals (day 7 and 28) compared to the plasma metabolic profile after weaning (day 34). In general, no particular pathway in the plasma metabolome was shown to be responsible for the differentiation of the two *FUT1* genotypes. Nevertheless, discriminant compounds were detected and validated through the LOO procedure in the metabolomics data from day 7, 28 and 34. On day 7, the $FUT1^{AG}$ piglets had higher plasma values of metabolites originating from the metabolism of phenylalanine, hippuric acid and tryptophan, oxindole. Hippuric acid, constituted by the conjugation of benzoic and acyl glycine, is synthesised in the intestine, liver, and spleen (Poon et al., 1995) and it accumulates in the blood, causes ammoniagenesis stimulation, and it is generally associated with an increased feed consumption of phenylalanin or phenolic compounds, and with the activity of the gastrointestinal microbiota (Williams et al., 2010; Wikoff et al., 2009; Armstrong et al., 1955). Since the sMLPLS-DA was performed on the metabolomics data corrected for litter and sex factors, and for BW of the animals on day 7, and that the diet (i.e. sow milk and post-weaning diet) was not different between $FUT1^{AG}$ and $FUT1^{AA}$ pigs, the higher hippuric acid amount in $FUT1^{AG}$ piglets could therefore be ascribed to a higher feed intake. However, the microbial activity in the gut is also associated with the concentration of hippuric acid in the blood (Wikoff et al. 2009), and in humans lower urinary hippurate levels in Crohn's disease patients have been associated with an altered gut microbial metabolism (Williams.et al., 2010). No significant difference in the number of various microbial groups between the faeces of two groups was found on day 7, though the analysis of digesta at this age might have provided different results. The oxindole, also named 1,3-Dihydroindol-2-one (KEEG C12312), is a putative tryptophan metabolite, which is involved in the benzoxazinoid biosynthesis, it is a secondary metabolite that have been associated to bacterial metabolism (Arora and Bae 2014). No further

information has been found regarding this compound in porcine biological systems. On the other hand, the animals with the *FUT1*^{AA} genotype showed a higher intensity in the signal of betaine, a small N-trimethylated amino acid that originates from the oxidation of choline, which functions as a methyl donor. Donation of methyl groups is important for an optimal liver function, cellular replication, and detoxification reactions (Wang et al., 2012). Furthermore, a decreased betaine level has been associated with stress due to weaning and exposure to *E. coli* (Sugiharto.et al., 2014). Our results showed that, at weaning (day 28), the main differences in the metabolic profile between *FUT1*^{AA} and *FUT1*^{AG} piglets have to be attributed to two nitrogenous bases. The higher level of guanosine in *FUT1*^{AG} piglets probably indicates that these animals presented a higher risk of suffering from inflammatory processes as it has been shown that extracellular guanosine tended to increase and accumulate in response to injury as oxygene and glucose deprivation in rat (Ciccarelli et al., 1999; Dal-Cim et al., 2013). Indeed, guanosine is related to neuroprotective and neuromodulator functions in the central nervous system (Ganzella et al., 2012) and promotes a glioprotective mechanism against the inflammatory and oxidative damage induced by Lipopolysaccharide (LPS) exposure in primary cultures of hippocampal astrocytes by the activation of heme oxygenase-1 (HO-1) pathway (Bellaver. Et al., 2015). Piglets with the *FUT1*^{AA} genotype were characterized by a higher 3-methylguanine level, a metabolite involved in the DNA repair process (Curtis et al., 2010), nevertheless, there are no studies on the role of this compound in plasma and our work does not allow us to associate it with animal health or biological processes.

Regarding the post-weaning period, the metabolome profile of plasma samples obtained at day 34 showed that the influence of *FUT1* genotype on the plasma metabolome had decreased when compared to day 7 and 28. This result may be ascribed to a range of different factors perturbing the piglet's physiological homeostasis after weaning. The effect of *FUT1* genotype on the metabolic profile discriminations was attributed to compounds such as tauroursodeoxycholic acid, a bile acid compound, and L-Phenylalanine, an amino acid precursor. Phenylalanine plays an important role in the production of amino acids, while tauroursodeoxycholic acid is involved in the digestion and intestinal absorption of hydrophobic nutrients. These two compounds were found to be more abundant in plasma of *FUT1*^{AA} piglets. On the other hand, in our study, *FUT1*^{AG} piglets have been found to have a higher level of genistein 5-o-glucuronide, a polyphenol metabolite involved in the intestinal barrier regulation (Suzuki et al., 2013). Our results on the expression of candidate genes involved in the intestinal barrier function (*ZO-1* and *OCLN*) and immunity response (*IL10, TNF-α* and *COX-2*) showed no differences between *FUT1*^{AG} and *FUT1*^{AA} piglets, however, it could be possible that the differences due to *FUT1* variants were not sufficient to demonstrate differences in these gene markers that are generally stimulated after infection, indeed our animals stayed healthy

until the end of the trial. In order to assess the microbiota metabolism along the gastrointestinal tract of the two *FUT1* groups, organic acid analysis was carried out. Acetic acid, which is the major short-chain fatty acid in the gut, was found in higher concentrations in mid-colon digesta of $FUT1^{AG}$. As the short-chain fatty acid profile reflects bacterial metabolism (Williams et al., 2005), the higher colonic concentration of acetic acid in $FUT1^{AG}$ piglets most likely reflects bacterial community differences between genotypes. Bacterial culture showed a higher number of haemolytic bacteria, both in faeces and digesta, and of Enterobacteriaceae in digesta content from $FUT1^{AG}$ piglets. *E. coli* belongs to this family and has been shown to produce higher amounts of acetic acid at high growth rates (Han et al., 1992). This alone is unlikely to explain the difference in acetic acid concentrations as we have a lack of information about the detailed commensal bacteria present in the intestinal tract of the animals, but all these results together may indicate that *FUT1* genotype can influence the microbiota balance, the microbial metabolism and thus the host physiological condition.

We were not able to detect significantly higher ETEC F18 numbers in the small intestinal tissue using an *ex vivo* porcine intestinal model. Neither we were able to detect weight gain differences although some plasma metabolomics differences between genotypes could be ascribed to the feed intake. The lack of significance between the genotypes regarding epithelial adherence of *E. coli* as obtained in the *ex vivo* challenge model, and the pig weight responses might be due to the low number of replicates in the present study.

Conclusion

The results obtained in the present study contribute to the existing knowledge on *FUT1* polymorphisms in pigs. Our investigation showed that *FUT1* genotype influenced the plasma metabolic profile and the number of haemolytic bacteria and Enterobacteriaceae in the intestinal content. Further studies are needed to identify the metabolic pathways that characterise the effect of host *FUT1* specific genotype, and to improve the knowledge on the relationship between *FUT1* genotype, the mucosal bacterial community, and the host immune response in order to elucidate the perspective of e.g. breeding strategies on gut health parameters others than diarrhoea. Moreover, our work encourages future studies that evaluate the role and contribution of Fucosyltransferase genes family into the mechanisms involved in the pathophysiology of intestinal disease and inflammation.

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Table 1a. Discriminant plasma metabolites for *FUT1*^{AA} and *FUT1*^{AG} obtained from piglets at 7, 28 and 34 days of age by the sMLPLS-DA

Metabolite	Identification level	Charge	Retention time (min)	m/z[1]	*FUT1*				Stability[2]		Effect size[3]		Direction[4]
					AG		AA						
					mean	ds	mean	ds	t	P	t	P	
Day 7 of age													
[M+Na] Betaine	1	Pos	0.63	140.9	6589	207	6794	195.3	6	0.077	-0.043	0.027	AA
N-methyl-2-pyrrolidinone	2	Pos	1.91	102.08	8167	160.9	7242	237.3	10	0.011	0.047	0.012	AG
Hippuric acid	1	Pos	3.26	180.06	13273	2988	4546	1734	6	0.076	0.04	0.014	AG
Oxindole	2	Pos	3.37	134.06	12923	2446	9476	840.5	6	0.069	0.042	0.015	AG
3-Oxo-5beta-chol-7-en-24-oic Acid	3	Pos	7.28	374.28	9412	288.2	8140	440	6	0.073	0.043	0.024	AG
Day 28 of age													
3-Methylguanine	2	pos	0.91	166.07	5824	241.9	7044	552.9	15	0.001	0.462	0	AA
Guanosine	1	pos	1.27	284.1	7980	1016	4533	1646	12	0.021	0.417	0	AG

Table 1b. Discriminant plasma metabolites for *FUT1*^{AA} and *FUT1*^{AG} obtained from piglets at 7, 28 and 34 days of age by the sMLPLS-DA

Metabolite	Identification level	Charge	Retention time (min)	m/z[1]	*FUT1*				Stability[2]		Effect size[3]		Direction[4]
					AG		AA						
					mean	ds	mean	ds	t	P	t	P	
Day 34 of age													
(S)-2-Hydroxyglutarate	2	neg	1.9	147.05	15301	796.7	17737	1058	15	0.001	-0.06	0.022	AA
L-Phenylalanine	1	neg	1.9	164.07	122786	6631	135175	8330	8	0.07	-0.042	0.037	AA
Tauroursodeoxycholic acid	2	neg	5.62	498.29	21853	26903	73051	80247	7	0.077	-0.044	0.027	AA
Tauroursodeoxycholic acid	2	pos	5.65	500.31	7702	4255	14039	15506	8	0.058	-0.042	0.023	AA
Ile Glu Phe Gly	3	pos	3.89	465.23	5903	2108	5588	1010	14	0.008	0.051	0.038	AG
Genistein 5-O-glucuronide,	3	neg	4.18	445.07	6445	2269	3886	1323	8	0.071	0.045	0.022	AG

Table 1c. Discriminant plasma metabolites for *FUT1*^{AA} and *FUT1*^{AG} obtained from piglets at 7, 28 and 34 days of age by the sMLPLS-DA

Metabolite	Identification level	Charge	Retention time (min)	m/z[1]	*FUT1*				Stability[2]		Effect size[3]		Direction[4]
					AG		AA						
					mean	ds	mean	ds	t	P	t	P	
Unknown PC/LysoPC	3	pos	8.9	560.3	1436	106.7	1750	196.9	15	0.001	-0.056	0.033	AA
PC(18:0/0:0)	3	neg	10.45	560.33	7489	7674	4109	2918	11	0.026	0.046	0.044	AG

[1] Mass to charge ratio m/z. [2]For the stability "t" represents the number of times that the metabolite was selected in the leave one out procedure (LOO) and *P* the associated probability. [3]For the effect size "t" represents the absolute value of the regression coefficient of the metabolite *P* the associated probability. [4]Direction: based on the regression coefficient it indicates metabolite concentration higher in AA and AG genotype.

Table 2. Relative expression of selected genes in mucosa from the stomach, ileum, caecum and mid colon of piglets 34 days of age one-week post-weaning)[1] belong to *FUT1*^{AG} and *FUT1*^{AA} groups.

Item	Genotype[2]				#	*P-value*		
	FUT1^{AG}		*FUT1*^{AA}					
	mean	SEM	mean	SEM		G[3]	T[4]	G[3]*T[4]
IL10						0.3	0.11	0.8
Stomach	0.1	0.02	0.15	0.03				
Ileum	0.14	0.02	0.15	0.03				
Caecum	0.14	0.02	0.18	0.02				
Mid colon	0.17	0.02	0.18	0.03				
COX 2						0.4	0.02	0.18
Stomach	0.03	0.02	0.03	0.01	a			
Ileum	0.02	0.01	0.02	0.01	a			
Caecum	0.02	0.01	0.03	0.01	a			
Mid colon	0.03	0.01	0.08	0.02	b			
TNF-α						0.22	0.14	0.41
Stomach	3.19	0.69	3.65	0.89				
Ileum	3.13	0.73	3.58	0.98				
Caecum	2.51	0.73	2.56	0.89				
Mid colon	2.99	0.69	5.27	0.89				
ZO-1						0.13	0.52	0.07
Ileum	1.58	0.15	2.01	0.2				
Caecum	1.78	0.16	1.41	0.22				
Mid colon	1.71	0.15	1.69	0.19				
OCLN						0.48	0.008	0.26
Ileum	0.73	0.07	0.72	0.09	b			
Caecum	0.45	0.07	0.57	0.09	a			
Mid colon	0.63	0.07	0.53	0.09	a			

[1] Item*: IL10*, Interleukin 10; *COX-2*, cyclo-oxygenase-2: *TNF-α*, Tumor Necrosis Factor Alfa;; *ZO-1*, Zona Occludens 1; *OCLN*, Occludin. [2] Number of piglets: *FUT1*^{AG} =10; *FUT1*^{AA} =7. [3] G = Genotype.[4] T =Tissue. #: Rows with different letters, within a gene, are significantly different (p<0.05).

Table 3. Enumeration (log cfu/g sample) of selected microbial groups in faeces from piglets at 5, 7, 14, 21, 28 and 34 days of age[1]

Item	Genotype[2]				#	*P*-value		
	FUT1^{AG}		*FUT1*^{AA}			G[3]	A[4]	G[3]*A[4]
Enterobacteriaceae						0.3	0.38	0.08
5	8.5	(7.7-9.2)	8.7	(8.0-9.5)				
7	8.3	(7.5-9.0)	8.6	(7.9-9.3)				
14	8.3	(7.5-9.1)	8.1	(7.4-8.8)				
21	8.2	(7.4-8.9)	8.1	(7.4-8.8)				
28	8.4	(7.7-9.1)	7.9	(7.2-8.6)				
34	8.9	(8.2-9.6)	8	(7.3-8.7)				
Haemolytic bacteria						0.02	<.0001	0.03
5	<6.2A (2)	(5.4-7.1)	<6.3AB (1)	(5.2-7.5)				
7	<6.2A (9)	(5.8-6.6)	<6.3A (5)	(5.8-6.8)				
14	<6.3A (3)	(5.7-6.9)	<6.3A (7)	(5.8-6.7)				
21	<6.3A (7)	(5.8-6.7)	<6.2A (6)	(5.8-6.7)				
28	<6.3A (7)	(5.9-6.7)	<6.3A (6)	(5.8-6.7)				
34	8.7aB	(8.3-9.1)	<7.5bB (3)	(7.1-7.9)				
Clostridium perfringens						0.6	<.0001	0.97
5	7.7	3.3-12.0	7.5	3.5-11.5	ab			
7	<8.0 (1)	3.2-12.7	7.8	3.5-12.1	b			
14	7.5	3.6-11.4	<7.4 (1)	3.2-11.6	ab			
21	<6.6 (1)	2.2-11.1	6.5	2.2-10.8	a			
28	5.2	0.7-9.6	<5.0 (1)	0.7-9.3	c			
34	<3.0 (6)	0-7.4	<2.8 (3)	0-7.2	d			
Lactic acid bacteria						0.06	0.0002	0.25
5	9.7	9.3-10.0	9.4	9.0-9.8	a			
7	9.6	9.3-10.0	9.4	9.0-9.7	a			
14	8.9	8.5-9.3	8.7	8.3-9.0	b			
21	9.1	8.8-9.4	8.8	8.5-9.2	a			
28	9	8.6-9.3	8.7	8.4-9.0	b			
34	8.8	8.5-9.2	8.6	8.2-8.9	b			
Total anaerobic bacteria						0.86	0.43	0.36
5	9.9	9.7-10.2	9.9	9.7-10.2				
7	9.7	9.4-9.9	9.7	9.4-9.9				
14	9.7	9.5-10.0	9.7	9.5-10.0				
21	9.7	9.5-10.0	9.8	9.5-10.0				
28	9.8	9.6-10.0	9.8	9.6-10.0				
34	9.8	9.5-10.0	9.8	9.6-10.0				

[1] Values are presented as least square means and 95% confidence intervals (in parentheses). [2] Number of piglets: *FUT1*^{AG} =7, except day 5 (n=8), day 7 (n=9) and day 14 (n=4); *FUT1*^{AA} =6, except day 5 (n=4), day 7 (n=5) and day 14 (n=7). [3] G = Genotype. [4] A = Age. #: Rows with different letters, within a microbial group, are significantly different (p<0.05). ^{A,B} Values with different superscripts within a column are significantly different (p<0.05). ^{a,b} Values with different superscripts within a row are significantly different (p<0.05). <: Indicates that at least one of the observations used to calculate the least square mean was below detection level. Numbers in brackets indicate the number of samples below detection level.

Table 4. Short-chain fatty acid concentration (mmol/kg sample) in faeces from *FUT1*^{AG} and *FUT1*^{AA} piglets at 5, 7, 14, 21, 28 and 34 days of age[1]

Item	Genotype[2]				#	*P-value*		
	FUT1^{AG}		*FUT1*^{AA}			G[3]	A[4]	G[3]*A[4]
Lactic acid[5]						0.44	<.0001	0.23
5	42.3	(32.0-52.7)	40	(29.5-49.6)	a			
7	38.1	(29.0-47.1)	35	(26.0-44.6)	a			
14	32.7	(22.1-43.4)	30	(20.4-39.4)	a			
21	38	(28.7-47.4)	35	(26.2-44.2)	a			
28	46.5	(37.1-55.9)	44	(34.7-52.7)	ab			
34	58.9	(49.9-67.9)	56	(47.2-65.0)	b			
Propionic acid						0.19	<.0001	0.13
5	12.4	(8.1-16.7)	10	(6.2-14.6)	a			
7	10.9	(7.2-14.6)	8.9	(5.1-12.8)	a			
14	12.3	(7.8-18.8)	10	(6.4-14.2)	a			
21	12.6	(8.8-16.4)	11	(6.9-14.3)	a			
28	12	(8.2-15.9)	10	(6.4-13.7)	a			
34	22.4	(18.7-26.0)	20	(16.7-24.0)	b			
Butyric acid						0.09	0.09	0.48
5	6.6	(3.5-10.7)	4.6	(2.2-8.0)				
7	5.3	(2.9-8.4)	3.5	(1.6-6.2)				
14	3	(1.0-6.1)	1.7	(0.5-3.8)				
21	2.5	(0.9-4.8)	1.3	(0.3-3.1)				
28	4	(1.9-6.9)	2.5	(1.0-4.7)				
34	5.3	(3.0-8.4)	3.6	(1.7-6.1)				
A+P+B[5]						0.23	<.0002	0.14
5	61.7	(46.5-77.0)	55	(40.6-70.1)	a			
7	56	(43.0-69.0)	50	(36.1-63.2)	a			
14	49.4	(33.5-65.2)	43	(29.2-56.8)	a			
21	54.3	(40.8-67.8)	48	(35.0-61.0)	a			
28	63.8	(50.3-77.3)	57	(44.4-70.4)	ab			
34	87.1	(74.3-100.0)	81	(67.9-93.5)	b			

[1] Values are presented as least square means and 95% confidence intervals (in parentheses). [2]. Number of piglets: *FUT1*^{AG}=10, except, day 5 (n=8), day 7 (n=9) and day 14 (n=7); *FUT1*^{AA}=6, except day 5 (n=4), day 7 (n=5) and day 14 (n=7). [3] G = *FUT1* Genotype. [4] A = Age. [5] A+P+B = acetic + propionic + butyric acid. #: Rows with different letters, within a short-chain fatty acid group, are significantly different (p<0.05).

Table 5. Enumeration (log cfu/g sample) of selected microbial groups in digesta from the gastrointestinal tract of 34 days old piglets (one week post-weaning)[1] belong to *FUT1*^{AG} and *FUT1*^{AA} groups.

Item	Genotype[2]				*P-value*		
	FUT1^{AG}		*FUT1*^{AA}		G[3]	S[4]	G[3]*S[4]
Enterobacteriaceae					0.002	<.0001	0.004
Stomach	5.6	(4.8-6.5)	5.9	(5.0-6.7)			
Distal small intestine	8.8a	(7.9-9.7)	7.5b	(6.6-8.3)			
Caecum	8.8a	(8.0-9.7)	7.8b	(7.0-8.7)			
Mid colon	8.9a	(8.0-9.8)	7.9b	(7.1-8.8)			
Haemolytic bacteria					0.02	<.0001	0.28
Stomach	5.2	(4.5-6.0)	<4.4 (1)	(3.8-5.1)			
Distal small intestine	7.9	(6.8-9.2)	<6.8 (4)	(5.8-7.9)			
Caecum	8.6	(7.4-10)	<7.3 (4)	(6.3-8.5)			
Mid colon	8.6	(7.4-10)	<7.3 (3)	(6.3-8.5)			
Clostridium perfringens					0.93	0.63	0.24
Stomach	<3.2 (1)	(2.6-3.7)	3.2	(2.6-3.7)			
Distal small intestine	<2.8 (5)	(2.3-3.4)	<2.8 (4)	(2.3-3.4)			
Caecum	<3.0 (4)	(2.5-3.6)	<3.0 (4)	(2.5-3.6)			
Mid colon	<3.0 (4)	(2.4-3.5)	<3.0 (4)	(2.4-3.5)			
Lactic acid bacteria					0.29	<.0001	0.4
Stomach	9	(8.4-9.6)	8.7	(8.1-9.3)			
Distal small intestine	8.3	(7.8-8.9)	8	(7.3-8.6)			
Caecum	8.6	(8.0-9.2)	8.3	(7.6-8.8)			
Mid colon	8.6	(8.0-9.2)	8.3	(7.7-8.9)			
Total anaerobic bacteria					0.02	<.0001	0.01
Stomach	9	(8.7-9.4)	9.3	(9.0-9.6)			
Distal small intestine	8.9a	(8.6-9.3)	8.3b	(8.0-8.6)			
Caecum	9.3	(9.0-9.7)	9.2	(8.9-9.5)			
Mid colon	9.4	(9.1-9.7)	9.4	(9.1-9.7)			

[1] Samples from the stomach, distal small intestine, caecum and mid colon were analysed. Values are presented as least square means and 95% confidence intervals (in brackets). [2] Number of piglets: *FUT1*^{AG} n=10; *FUT1*^{AA} n=7. [3] G = *FUT1* Genotype. [4] S = Intestinal segment. [a,b] Values with different superscripts within a row are significantly different (p<0.05). < : Indicates that at least one of the observations used to calculate the least square mean was below detection level, followed by numbers in brackets indicating the number of samples below detection levels.

Table 6. Organic acid concentrations in digesta (mmol/kg wet sample) from the gastrointestinal tract of 34 days old piglets (one week post-weaning)[1] belong to *FUT1*^{AG} and *FUT1*^{AA} groups.

Item	Genotype				*P-value*		
	FUT1^{AG}		*FUT1*^{AA}		G[3]	S[4]	G[3]*S[4]
Lactic acid[5]					1	<.0001	0.9
Stomach	19.6	(5.3-62.7)	19.5	(6.9-50.2)			
Distal small intestine	5.6	(0.6-20.4)	5.5	(1.1-16.2)			
Acetic acid					0.01	<.0001	0.01
Stomach	21.1	(4.2-37.9)	26.8	(11.6-41.9)			
Distal small intestine	4.2	(0.0-21.0)	5.4	(0.0-20.6)			
Caecum	47.6	(30.7-64.4)	39	(24.1-53.9)			
Mid colon	54.5a	(37.7-71.4)	39.4b	(24.4-54.4)			
Propionic acid[6]					0.02	0.0001	0.01
Stomach	8.1	(2.7-13.6)	11.7	(6.9-16.5)			
Caecum	16.7	(11.3-22.2)	13.2	(8.1-18.3)			
Mid colon	16.6	(11.1-22.0)	11.9	(6.9-16.9)			
Butyric acid[7]					0.9	<.0001	0.8
Stomach	8.3	(1.4-48.0)	8.1	(0.5-41.2)			
Caecum	2.3	(6.1-30.3)	2.2	(3.8-24.0)			
Mid colon	1.9	(6.8-29.0)	1.8	(4.6-23.6)			

[1] Samples from the stomach, distal small intestine, caecum and mid colon were analysed. Values are presented as least square means and 95% confidence interval (in parentheses). [2] Number of piglets: *FUT1*^{AG} n=10, except the distal small intestine (n=9); *FUT1*^{AA}.n=7, except the mid colon (n=6) and caecum (n=5). [3] G = *FUT1* Genotype. [4] S = Intestinal segment. [5] Samples from the caecum and mid colon had values below detection level. [6] Samples from the distal small intestine had values below detection level. No superscripts as the G*S interaction was non-significant after pairwise comparisons using Bonferroni correction. [7]Samples from the distal small intestine had values below detection level. [a,b] Values with different superscripts within a row are significantly different ($P < 0.05$).

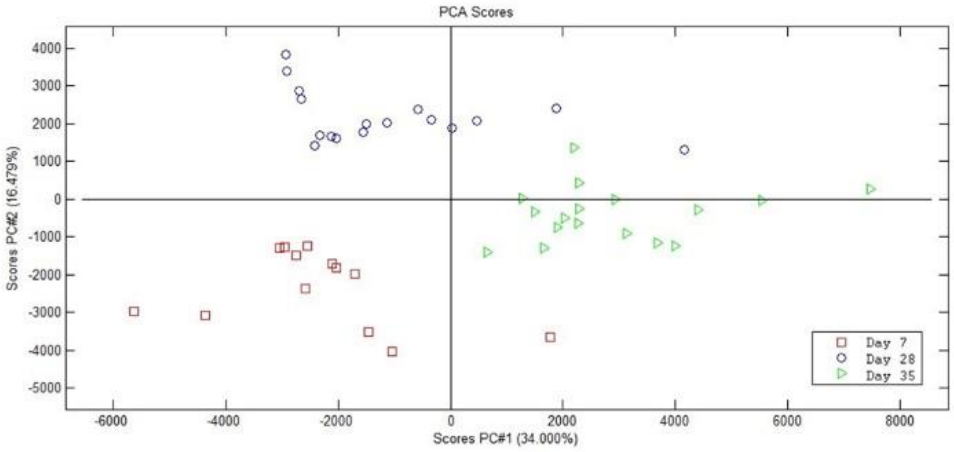

Figure 1. PCA score plot of the piglets' plasma metabolomic profiles at 7, 28 and 34 days of age. Legend: The variants accounted by the principal components are shown in the axes.

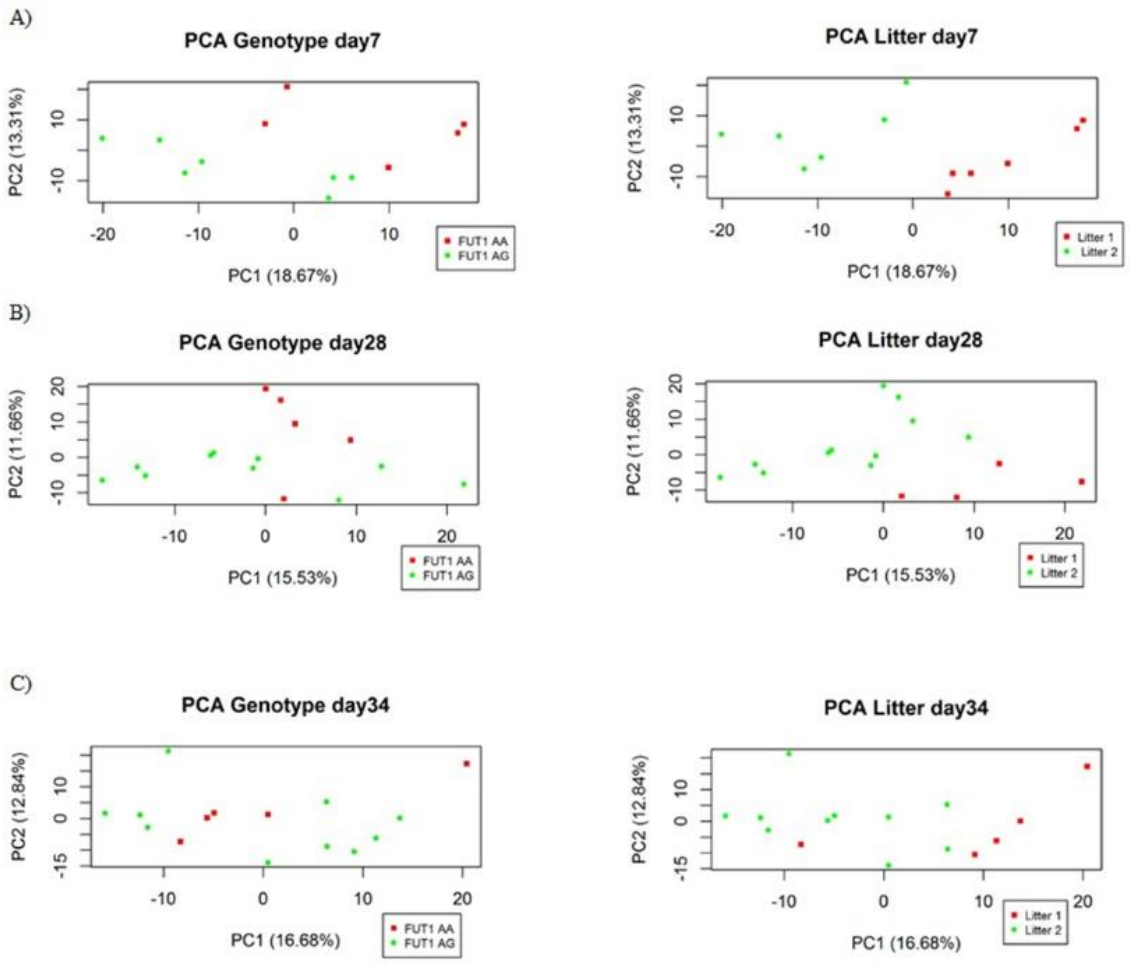

Figure 2. PCA Score plot of *FUT1* genotype and litter differences during suckling, weaning and after weaning**.** Legend: A): Score plot of plasma metabolite at day 7, during suckling period. PC1 and PC2 explain 18.67% and 13.31% respectively; B) Metabolomics score plot at day 28 (weaning), PC1= 15.53% and PC2= 11.66%. C) PCA score plot after weaning (day 34) where PC1 and PC2 explain 16.68% and 12.84% of total variance.

Manuscript III

Effect of feed supplementation with live *Saccharomyces cerevisiae* on the intestinal transcriptome profile of weaning pigs orally challenged with *Escherichia coli* F4

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Abstract

The ability of live yeasts to modulate pig intestinal cell signals in response to infection with *Escherichia coli* F4ac (ETEC) has not been studied in-depth. The aim of this trial was to evaluate the effect of *Saccharomyces cerevisiae* CNCM I-4407 (Sc), supplied at different times, on the transcriptome profile of the jejunal mucosa of pigs 24h after infection with ETEC. Twenty piglets selected to be ETEC-susceptible were weaned at 24 days of age (d0) and allotted by litter to one of following groups: control (CO), CO+colistin (AB), CO+5x1010 CFU Sc/kg feed, from d0 (PR) and CO+5x1010 CFU Sc/kg feed from d7 (CM). On d7, the pigs were orally challenged with ETEC and were slaughtered 24h later after blood sampling for haptoglobin (Hp) and C-reactive protein (CRP) determination. The jejunal mucosa was sampled 1) to confirm the presence of ETEC receptors on villi; 2) for morphometry; 3) for quantification of proliferation, apoptosis, and zonula occludens (Zo-1), 4) to carry out the microarray analysis. A functional analysis was carried out using Gene Set Enrichment Analysis. The normalized enrichment score (NES) was calculated for each gene set, and statistical significance was defined when the False Discovery Rate % was <25 and p-values of NES were <0.05. The blood concentration of CRP and Hp, and the score for ZO-1 integrity on the jejunal villi did not differ between groups. The intestinal crypts were deeper in the AB (P=0.05) and the yeast groups (P<0.05) than in the CO group. Antibiotic treatment increased the number of mitotic cells in intestinal villi as compared to the control group (P<0.05). The PR group tended to increase the mitotic cells in villi and crypts and tended to reduce the cells in apoptosis as compared to the CM group. Based on the GSEA, the transcriptome profiles of the AB and PR groups were similar. In both groups, the gene sets involved in mitosis and in mitochondria development ranked the highest while, in the CO group, the gene sets related to cell junction and anion channels were affected. In the CM group, the gene sets linked to the metabolic process, and transcription ranked the highest; a gene set linked with a negative effect on growth was also affected. C constant supplementation in the feed with the strain of yeast tested limits the early activation of the gene sets related to the impairment of the jejunal mucosa of piglets infected with ETEC.

Key words: *Escherichia coli*, health, intestine, transcriptome, Pig, Saccharomyces cerevisiae

Introduction

The yeasts of the genus *Saccharomyces* (*S.*) are used worldwide for their metabolic properties, enabling the fermentation of sugar into ethanol and CO2. Several species of this genus are considered non-pathogenic to domestic animals, and are frequently used as nutritional supplements in human foods. The beneficial properties for maintaining proper gut microbiota have been demonstrated,

primarily for S. boulardii (Justino et al., 2014). On the other hand, it is well known that colibacillosis is the most relevant bacterial infection in the pre-and post-weaning phase of pig production and *Escherichia* (*E.*) *coli* F4ac (ETEC) is one of the most diffuse causative agents. To overcome this problem without the use of antibiotics, there is a need for alternatives. Positive effects of the yeast strains in promptly counteracting the diarrhoea have been obtained in human and animal models (Kurugöl and Koturoğlu, 2005; McFarland, 2010). Furthermore, the early response of the small intestine after the administration of Saccharomyces spp. in combination with enteropathogens has been inadequately studied. An in-depth understanding of the interplay between the yeast cells and the host interface is of practical interest for refining the feeding strategies based on yeast in order to promote pig gut health.

A larger study was designed to test the dietary supplementation of *S. cerevisiae* CNCM I-4407 (Sc) at different times and doses for counteracting the infection from ETEC in weaned piglets (Trevisi et al. 2015a). Our previous results indicated the ability of live yeast to reduce the mortality of piglets, while maintaining growth performance, within two weeks' post-infection. The aim of this study was to elucidate the biological basis by which yeasts maintain the intestinal mucosa homeostasis 24 hours after infection (before the appearance of diarrhoea) as compared to the efficacy of an antibiotic effective against ETEC in *E. coli*-sensitive piglets

Materials and Methods

The procedures complied with the Italian laws pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, Italy.

Experimental design

For this study, twenty piglets were selected on the basis of the polymorphism for the Mucin 4 gene (Jensen et al., 2006) to obtain ETEC-susceptible pigs.

At 24 ± 2 days of age (d 0), the pigs were weaned, divided into four groups, balanced for litter and body weight and housed in pens with a mesh floor. The pigs were kept at a controlled temperature with free access to feed and water throughout the experimental period; feed was supplied in a dry feeder. On d 7 post-weaning, all the pigs were orally dosed with a 1.5 mL suspension containing 108 CFU of ETEC O149/mL, prepared as described by Trevisi et al. (2015b). A lyophilized live yeast strain of Sc (CNCM I-4407, Actisaf; Phileo–Lesaffre Animal Care, France) was mixed in the diet.

The piglets were assigned to one of four diets: control (CO, typical weaning diet – detailed in Trevisi et al., 2015a); CO + 1 g colistin/kg of feed (AB) from d 0; CO + 5 × 1010 CFU of Sc/kg of feed, from d 0 (PR, preventive dose); CO + 5 ×1010 CFU of Sc/kg of feed from d 7 (CM, competitive dose). Colistin (Sintofarm, Italy) treatment was used as a negative control owing to its activity against the ETEC strain used for the challenge, following a procedure frequently used for this purpose in other trials involving an ETEC challenge (Trevisi et al., 2015b). The pigs were individually penned in cages.

Experimental Procedure

Starting on d 0, each group received its experimental diet. The pigs were sacrificed 24 hours after challenge (d 8). At slaughter, the animals were deeply anesthetized with sodium thiopental (10 mg/kg body weight) and sacrificed via an intracardiac injection of Tanax (0.5 mL/kg BW).

Experimental Controls

Blood was sampled on d 8 by venipuncture of the vena cava and was centrifuged at 3,000 rpm for 10 min at 4°C; the serum was removed and stored at -80°C. At sacrifice, three samples from the jejunum at 75% of the small intestine length were collected, and formalin fixed for microscopic analysis, snap frozen in liquid nitrogen for transcriptome analyses, and gently scraped to isolate the villi for the *in vitro* adhesion test.

Haptoglobin (Hp) and C-Reactive protein (CRP) determination

In the blood serum, Hp (mg/ml) and CRP (µg/ml) were quantified using the Tridelta Phase Haptoglobin Assay and the Tridelta Phase Porcine CRP Assay Kit, (Tridelta Development limited, Ireland), respectively, following the manufacturers' instructions. For the haptoglobin determination, the blood serum was diluted 1:3 and the plates were read at 630 nm. For the CRP determination the blood serum was diluted 1:100 and the plates were read at 450 nm.

***In Vitro* Villus Adhesion Assay**

The phenotype for susceptibility to ETEC adhesion was assessed using the procedure of Van den Broeck et al. (1999). Briefly, the villi are examined by phase contrast microscopy after incubation with ETEC. The number of bacteria adhering along a 50-µm length of villous brush border is counted in 20 fields, and the pigs are considered positive when showing values ≥6; otherwise, pigs are considered negative.

Immunohistochemistry

Tissue samples of the jejunum at 75% of the small intestine length were fixed overnight in 10% neutral buffered formalin and processed for paraffin or cryostat-embedding. Sections (7µm thick) were cut, mounted on poly-L-lysine–coated slides and processed for chromogenic or fluorescence immunohistochemistry. Anti Ki67 rabbit monoclonal antibody 1:100 (cod. Ab16667, Abcam, UK) was used as a proliferation marker, the Apoptag® Plus Peroxidase in situ Apoptosis Detection Kit (cod. S7101 Millipore Corporation, USA) was used to detect the cells in apoptotic phase and anti zonula occludens 1 (Zo-1) rabbit polyclonal 1:200 (cod. 617300, Invitrogen, USA) was used as a marker for the integrity of the cell tight junctions (TJ).

For each piglet, the Ki67-immunoreactive (-IR) and the apoptotic-IR cells were counted in the epithelium, randomly selecting 10 villi and 10 crypts. The expression of ZO-1 at the apical domain, as an indicator for ZO-1 bound in the TJ complex, was analyzed along the entire villus as described in Klunker et al. (2013). Ten villi were analyzed for each subject.

Morphometric analyses

The intestinal samples were pinned tautly to balsa wood and fixed in 10% buffered formalin (pH 7.4). After embedding in paraffin wax 4 micron thick sections were dewaxed in xylene and stained with hematoxylin-eosin. For each subject, the height of 20 villi and the depth of 20 crypts were measured. The sections were examined at low magnification with a conventional microscope interfaced to a digital camera equipped with Cytometric software (Byk Gulden, Italy). The dimensions of the digitalized images were 513x463 pixels. Villous height was measured as the distance from the crypt opening to the top of the villous whereas crypt depth was measured from the base of the crypt to the level of the crypt opening.

RNA Isolation, Microarray Processing, Quality Control

Total mRNA was isolated from the jejunum tissue according to the Takara Fast Pure kit (Takara Bio, Japan) protocol. The purity and concentration of the total RNA extracted were checked spectrometrically using the Nanodrop ND 1000 (Nanodrop Technologies, USA) while RNA integrity was assessed using agarose gel electrophoresis analysis and confirmed by Agilent Bioanalyzer 2100 (Agilent Technologies, USA).

On the extracted quality-test RNA, the analysis of whole transcript expression was carried out using Affymetrix©Porcine Gene 1.1 ST array strips. Hybridized arrays were scanned on a GeneAtlas imaging station (Affymetrix, USA). Performance quality tests of the arrays including the labelling,

hybridization, scanning and background signals from a Robust Multichip Analysis were carried out on the CEL files using Affymetrix Expression ConsoleTM.

Statistical analysis

Blood serum and intestinal morphology data were analyzed with ANOVA using the general linear model (GLM) procedure of SAS (SAS Inst., Inc., Cary, NC) with a completely randomized design and four dietary treatments. Degrees of freedom for the dietary treatments were used to test the following orthogonal contrasts: CO vs. YEAST (PR, CM), PR vs. CM and CO vs. AB. $P < 0.05$ was statistically significant and $0.05 < P < 0.10$ was considered a trend.

Microarray Data and Pathway Analysis

The Affymetrix Trascripts IDs were associated to 13,406 Human gene names, based on Sus scrofa Ensembl (release 69, www.ensembl.org). An exploratory functional analysis was carried out on the processed gene expression values with Gene Set Enrichment Analysis using the C5.BP catalog of gene sets (based on Gene Ontology (GO) (http://www.broadinstitute.org/gsea/msigdb/Index.jsp). The normalized enrichment score (NES) was calculated for each gene set, and statistical significance was defined when the False Discovery Rate % was < 25 and the P-values of NES were < 0.05. Enrichment score P- values were estimated using a gene set-based permutation test procedure.

The overlap in enriched GO terms was visualized using the Enrichment Map (http://baderlab.org/Software/EnrichmentMap20) plugin for Cytoscape 2.8.0 (http://www.cytoscape.org/21), including gene sets having a P-value <0.005 and an FDR q-value <0.10. The nodes were joined when the overlap coefficient was ≥0.5.

The expression for each gene in the experimental groups was assessed using the Student's t test against the control group, and the statistically significant genes ($p<0.05$) were listed when the linear fold change for log2 values was 2.0 in the case of the contrast AB vs. CO, or 1.5 for PR or CM vs. CO.

Results

The *in vitro* adhesion test, confirmed that all the pigs were ETEC-susceptible. No signs of diarrhoea were observed up to the time of slaughter.

Inflammatory proteins in blood and Intestinal morphology

The concentrations of CRP and Hp did not show differences between the experimental groups, with average values of 322 µg/ml (SEM = 23.6) and 1.75 mg/ml (SEM = 0.32), respectively.

Table 1 shows the effect of the dietary supplementation of yeast at different times on the mucosa of the distal jejunum. No significant morphometric differences were observed between the experimental groups in villus height, villus width and crypt width, mucosal surface area and ZO-1 protein expression on the intestinal villi. Crypt depth increased with both antibiotic and yeast administration ($P=0.05$ and $P<0.05$, respectively).

In the jejunum villi, the AB group had a greater number of cells in mitosis than the CO group ($P<0.05$). The PR group tended to increase the number of mitotic cells both in the villi and the crypts as compared to the CM group ($P=0.10$ and $P=0.08$, respectively) and number of apoptotic cells tended to be lower in PR group than in CM group ($P=0.07$).

Microarray analysis

Tables 2, 3 and 4 show the gene set enriched in the mucosa of the distal jejunum after the various dietary treatments as compared to the controls.

When comparing AB vs. CO, 143 and 17 gene sets were significantly enriched for AB and CO, respectively (Table 2). In the AB group, several gene sets involved in mitosis and in mitochondrial development ranked among the highest while, in the CO group, the gene sets related with cell junction and anion channel ranked the top. The comparison of PR vs. CO showed that 209 and 10 gene sets were significantly enriched for PR and CO, respectively (Table 3). In the PR group, several gene sets involved in mitosis and cell development ranked the top. Moreover, the gene set T_CELL_ACTIVATION was enriched in PR group. In CO several gene sets for anion channel and apical junction ranked among the highest. When comparing CM vs. CO, 118 and 4 gene sets were significantly enriched for CM and CO, respectively (Table 4). In the CM group, gene sets linked to the metabolic process and transcription ranked among the highest, but the gene set which negatively regulates growth also ranked among the highest.

Figure 1 depicts the nodes of the gene sets in common or those regulated separately by PR and AB, both as compared to CO. The nodes represent gene sets. The color at the center of the node visualizes the PR treatment, the color on the ring represents the AB treatment while the edges represent the mutual overlap. The enrichment significance (P-value) is proportional to the node color intensity where red represents upregulation and blue downregulation. The node size represents the number of genes enclosed in the gene set. The AB and PR groups show three well-defined common nodes containing the gene sets involved in cell mitosis, nuclear activity and transcriptional factors. Moreover, the AB group shows an independent node, for the most part containing gene sets for mitochondrial development and a small node related to the response to the oxidative stress. A low number of nodes in the gene sets were downregulated in the comparison of groups AB or PR versus the CO group.

Figure 2 depicts the nodes of the gene sets which were in common or regulated separately by PR (the center of the nodes) and CM (the ring of the nodes), both compared to CO. A large cluster of nodes related to the nucleotide metabolic process and to the positive regulation of transcription was upgraded both in PR and CM as compared to CO. Another cluster upregulated with both yeast treatments was related mainly to constitutive genes of nucleoplasma while other sets related to nuclear structure and function were mainly upregulated in PR.

Figure 3 depicts the nodes of the gene sets which were in common or regulated separately by CM (the center of the nodes) and AB (the ring of the nodes), both compared to CO.

A few nodes overlapped regarding regulation in both CM and AB. Among these, intense upregulation was seen in these treatments for nodes related to the oxidative stress response, generally involved in the oxidoreductase activity as compared to CO. The upregulation of the genes related to the mitochondrial and nuclear structure was also seen to be associated.

Genes showing statistical significance and with linear fold change for log2 values equal or greater than 2.0 in the case of AB vs. CO, or 1.5 for PR or CM vs. CO are reported in Supplementary Tables 1, 2 and 3, and are herein listed in synthesis. When comparing AB vs. CO, 8 genes were upregulated: ectonucleotide pyrophosphatase/ phosphodiesterase 7, SEC14-like protein 2, Carbonic anhydrase 12, Deoxyribonuclease I-Like 3, solute carrier family 5 member 4 (SLC5A4), glutamic pyruvate transaminase (alanine aminotransferase) 2, Stearoyl-CoA desaturase (SCD) and Apolipoprotein C-III, and 4 genes were downregulated: N-Acetyl-Glucosaminy Transferase 3, Mucin Type (GCNT3), G-protein coupled receptor 126 (GPR126), Chemokine (C-C Motif) Ligand 20 (CCL20) and Dual oxidase 2 (DUOX2).

When comparing PR vs. CO, 12 genes were upregulated and the first 7 were: Transcription initiation factor TFIID subunit 7, Stearoyl-CoA desaturase, Cluster of Differentiation 69, Granzyme K (GZMK), Protein kinase C theta (PRKCQ), Neurolysin, Ikaros family zinc finger protein 3 (IKZF3), RAS guanyl-releasing protein 1, Cluster of Differentiation 8, and 7 genes were downregulated: Interferon-related developmental regulator 1 (IFRD1), 6-Phosphofructo-2-Kinase/Fructose-2,6-Biphosphatase 3, Lipase member H, Vascular Endothelial Growth Factor A (VEGFA), P2Y purinoceptor 4, Trefoil factor 3 (TFF3) and CCL20.

When comparing CM vs. CO, 18 genes were upregulated, and the first 7 genes were: SCD, Phosphoenolpyruvate carboxykinase 1, Insulin-like growth factor 1 (IGF1), Neurolysin, Proline-Rich Protein 19, Unc-5 Homolog C (C. Elegans)-Like (UNC5CL), Peroxisome proliferator-activated receptor γ coactivator 1-α (PPARGC-1), phosphatidylethanolamine N-methyltransferase, Solute carrier family 6, member 20 and Chemokine-Like Factor Superfamily Member 7; 10 genes were downregulated, and the first 7 were: Centromere protein K, Transient receptor potential channel 1 (TRPC1), Fibrinogen-like protein 2 (FGL2), Sterile Alpha Motif Domain Containing 9, Interleukin 7 Receptor, ATP-Binding Cassette, Sub-Family C, member 2.

Discussion

Our previous companion paper reported that the preventive supplementation of Sc reduced diarrhoea in the first days and delayed mortality after infection with ETEC (Trevisi et al., 2015a); the results were similar, even when the supplementation started immediately before the ETEC challenge.

Here, to better understand how yeast administration affects the early response against pathogens, our attention was focused on the modifications occurring in the blood and in the intestinal mucosa 24 hours after ETEC infection.

The blood concentrations of CRP and Hp were not affected either by yeast or by antibiotic supplementation. In the literature, these two acute phase proteins (APP) are considered markers for inflammation stimulated by lipopolysaccharide (LPS) challenge. In the blood, the peak concentration of CRP was observed 12 hours after the injection of LPS, while Hp reached its greatest concentration after just 2 hours (Llamas Moya et al., 2006). In an ETEC infection model carried out by Badia et al. (2012), a strong increase in blood CRP concentration 48 hours after infection was observed. In this case, the delay in the inflammatory response could have been due to the two different challenge models; LPS gives a strong and immediate response while live ETEC needs to adhere to the gut wall in order to exploit its pathogenic activity. Furthermore, Badia et al. (2012) observed that live S.

cerevisiae (Sc47) reduced the CRP concentration in the blood to the same extent as colistin treatment. According to these observations, the absence of variations in APP concentration in the blood serum in our study, could be explained by the fact that 24 hours after infection with live ETEC is not enough time to completely stimulate the liver synthesis of these proteins under the effects of pro-inflammatory cytokines, such as tumor necrosis factor-alpha (TNF-a) and interleukin-6 (Vernochet et al. 2010).

Another important consequence of the ETEC adhesion to the intestinal brush border is the production of endotoxins and the consequent secretory diarrhoea (Fairbrother et al. 2005) which may be associated with alterations of the intestinal mucosa morphology. In 28-day-old healthy pigs, the administration of S. boulardii improved the ileal morphology and increased the cell mitosis rate (Bontempo et al., 2006). In the present study, notwithstanding the absence of the effect on the villus height, yeast supplementation mimes the effect of the antibiotic on crypt depth and the villus mitotic index (when given preventively). On the whole, this confirms the positive effects of Saccharomyces spp. on the small intestine morphology.

In our study the ETEC adhered to the intestinal villi of all the pigs and presumably started to activate the cell signaling involved in cell turnover and inflammation (Zhou et al., 2012). In the larger set of animals, in accordance with the worsening fecal score (Trevisi et al., 2015a), from 24 hours after infection, there were the first signs of diarrhoea. Thus, the infection may have reduced the mitotic potential in the villi while the protective effects of the antibiotic preserved the normal proliferative activity of the intestinal mucosa as observed in pigs preventively treated with yeast. Conversely, the competitive administration of yeast did not preserve a positive balance of the mitotic cells. The effect of the timing of supplementation was confirmed by the tendency observed for the number of apoptotic cells in the villi where the PR group tended to decrease the cell apoptosis as compared to the CM group.

No articles were found in the literature regarding the effect of yeast administration on the transcriptome profile in the pig intestine under ETEC stimulation. This study showed that just 24 hours after infection, before the clinical effect of colibacillosis, the transcriptome of the small intestine was deeply modified by the treatments. When compared to untreated (CO) animals, the addition of antibiotic or yeast from the beginning of the trial created the conditions for the upregulation of several clusters of gene sets after ETEC challenge. The first cluster was related to all the processes associated with cell nuclear activity, RNA processing and cell multiplication. Typically, LPS impairs the enterocyte turnover (Ruemmele et al., 2003), with several TNF- α-dependent inflammatory related effects. The jejunal transcriptome thus confirmed the observations of the higher

mitotic index in the villi and demonstrated the favorable effect of the antibiotic, as well as of the continuous supply of yeast starting immediately after weaning, in maintaining the intestinal homeostasis counteracting the ETEC infection effects. Other clusters substantially confirmed this protective effect of the two treatments (related to DNA repair and metabolism, and to the positive regulation of the transcription process).

A second interesting cluster upregulated by both the AB and the PR treatments was related to leukocyte, lymphocyte and T cell activation. We have no transcriptome data obtained before the challenge, but it could also be hypothesized that preliminary treatment for one week could have sustained the increase in immune-competent cell production as already evidenced during the supply of probiotics in infected pigs (Rieger et al., 2015). The upregulation of some genes involved in the immune response after the preventive supply of the yeast, particularly in the differentiation and proliferation of T and B lymphocytes, such as *GZMK*, *PRKCQ* and *IKZF3*, indicated a stronger stimulation of the gut mucosa and the activation of the canonical NFκB pathways which presumably determined the activation of cell apoptosis (Wullaert et al., 2011). On the other hand, the PR diet downregulated some important genes encoded for pro-inflammatory proteins, such as chemokine *CCL20* sharing the ability to reduce its expression with the colistin. Chemokine CCL20 also played an important role in maintaining the immunological barrier in the gastrointestinal tract and was upregulated in a variety of inflammatory disorders (Sibartie et al., 2009). The downregulation of the *CCL20* gene indicated that the mucosa inflammation was under control. These data were reinforced by the reduction of the expression of *IFRD1* an IFN-inducible gene affected by gut microbiota exposure and mainly by LPS (Mulder et al., 2009), and *TFF3*, a gene strongly induced after mucosal injury (Taupin et al., 2001). Moreover, the PR diet downregulated the expression of *VEGF-A*, a target gene involved in the induction of mucosal inflammation (Scaldaferri et al., 2009). These results indicated the role of *S. cerevisiae* CNCM I-4407 in maintaining mucosal homeostasis during ETEC infection. An *in vitro* study based on the IPEC-1 cell line showed the ability of this strain of *S. cerevisiae* to inhibit the ETEC-induced expression of pro-inflammatory transcripts, but, surprisingly, the yeast was not able to contain the reduction of the transepithelial electric resistance due to the ETEC infection (Zanello et al., 2011).

Both the AB and the PR treatments downregulated the specific gene sets involved in anion channel activity and cell junction, which are co-involved in modulating the active ion transport in intestinal permeability. The key genes involved in maintaining the mucosa integrity belonging to this gene set are Claudins, but the mechanisms by which Claudins can enhance or reduce paracellular permeability throughout different interactions are still not clearly understood. An example of Claudin-Claudin

interaction is observed in human chronic inflammatory bowel disease, where the upregulation of pore-forming Claudin 2, and the downregulation and redistribution of sealing Claudins 5 and 8 lead to an altered tight junction structure increasing intestinal permeability (Zeissig et al., 2007). Interestingly, inside APICAL_JUNCTION_COMPLEX gene set, the *Claudin* 2 gene was downregulated, while *Claudins* 5 and 8 were upregulated by the antibiotic. Our results indirectly confirmed the interaction between epithelial claudins in the intestine, immediately after pathogen adhesion to the intestinal villi. Mucins can be upregulated by several inflammatory cytokines and the formation of their O-glycans requires the addition of glycosyl groups to hydroxyl residues on proteins. In this study, PROTEIN_AMINO_ACID_O_LINKED_GLYCOSYLATION was downregulated by both the one-week supplementation of antibiotic and that of yeast. Within this pathway, the most affected gene was *GCNT3*, a key gene targeted to the biosynthesis of mucins and to maintaining the epithelial mucus layer; *GCNT3* was abundantly expressed by goblet cells and the intestinal mucosa. In agreement with our results Niewold et al. (2010) and Li et al. (2009) showed that this gene is upregulated by pathogen infection.

Colistin alone showed a specific node containing many gene sets involved in mitochondrial activities. Enteropathogenic and enterohemorrhagic *E. coli* impair the host cell mitochondria function (Ma et al., 2006), and suppressive effect on *E. coli* strains in the pig gut could explain the upregulation of the respiratory function of the jejeunal mucosa. Furthermore, this may also be related to the upgrade of the nodes related to the antioxidant activity observed with the antibiotic treatment.

Other genes linked to LPS and enteropathogenic infections were downregulated by the antibiotic. The expression of *DUOX2*, coding for an NADPH oxidase essential for generating reactive oxygen species, such as during gastrointestinal host defense, is stimulated by INF-γ, a potent cytokine produced in response to LPS exposure (Wu et al., 2013). Interestingly, in this study, the gene encoded for the GPR126 protein was downregulated by the colistin. The role of this G-protein is still not fully understood, but Hohenhausa et al. (2013) showed that it is downregulated by LPS infection in a cell culture system. While our *in vivo* results showed that the expression of this gene was greater in the CO than in the AB group; this could indicate that antibiotic interacts with the chemosensing complex system expressed in the intestinal mucosa.

Globally, the transcriptomic profile of the AB group supported the other results, showing that the direct effect of the colistin against ETEC increased cell energy utilization and protected the intestinal mucosa from inflammation, maintaining an optimal health status in the pigs.

Although, in the CM group, the yeast was supplied for just one day after the ETEC challenge, the transcriptome profile was enriched in some pathways versus the CO group. The upgrade of a complex group of nodes related to the nucleotide metabolic process and to the positive regulation of transcription seen with one day of yeast addition overlapped with the effect of the longer treatment. This upgrade was less evident after treatment with antibiotic and may thus be a particular effect of the yeast. Conversely, a cluster of upregulated gene sets related to antioxidant activity matched with the antibiotic treatment. The upregulation of the patterns of genes involved in cell proliferation and intestinal inflammation is also seen by observations made on single genes. One of the gene strongly upregulated in the CM group was *IGF-1*, a key gene capable of reducing the induction of cell apoptosis while maintaining cell growth under oxidative stress (Baregamian et al., 2006). The expression of *IGF-1* was supported by other genes involved in the maintenance of cell integrity, such as *THOC5* which reduces bacteria translocation and is a major regulator of homeostatic self-renewal within the intestinal crypt (Saran et al., 2013). Moreover, the CM diet was able to reduce the ETEC effect, increasing the expression of *PPAR-γ* a gene widely recognized for its anti-inflammatory effect (Ortuño Sahagún et al., 2012). This observation confirmed the data obtained *in vitro* from Zanello et al. (2011) where *Saccharomyces cerevisiae* upregulated the expression of *PPAR-γ* in porcine intestinal epithelial IPEC-1 cells infected by ETEC. Furthermore, our results showed that the CM diet strongly downregulated *TRPC1* and *FGL2*, target genes involved in the mucosal inflammation process (Marasa et al., 2008). On the other hand, the competitive administration of yeast did not completely protect the intestinal mucosa from the activation of several genes involved in mucosal inflammation, such as *UNC5CL*, a gene mainly expressed in mucosal epithelia, described for its pro-inflammatory activity and putative candidate causally involved in mucosal diseases, such as IBD (Heinz et al., 2012). In summary, the brief administration of Sc slightly restrained the detrimental effects of ETEC infection, partially maintaining some metabolic processes and reducing the impact of post-challenge inflammation. This effect agrees with the other results obtained with this kind of treatment, pairing thus increasing the ability of inhibiting the severity of diarrhoea (Trevisi et al., 2015a).

In conclusion, the results showed that a dose of 5 x 1010 CFU of Sc/kg of feed was effective in counteracting the detrimental effect of ETEC infection in susceptible pigs by means of the modulation of the transcriptomic profile of the intestinal mucosa. Moreover, this study provided evidence that the strain of *S. cerevisiae* tested was able to delay the impairment of the intestinal mucosa even when supplied at the same time as ETEC; perhaps a dose response trial could help to elucidate this preliminary evidence. However, highlighting the partial similar effects on the gene expression of the small intestine between Colistin and live *S. cerevisiae* reinforces the interest in using yeast probiotics

in a preventive or competitive way against *E. coli* around the time of weaning in order to limit the use of antibiotics for curative purposes.

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Table 1. Effect of dietary supplementation of *S. cerevisiae* CNCM I-4407 at different times or of antibiotic, on the mucosa of distal jejunum (5 pigs/group)

	Diet [2]					Statistical significance[1], *P*-values		
	CO	AB	PR	CM	SEM	AB vs CO	YEAST vs CO	PR vs CM
Villus (µm)								
Height	294	334	322	354	32.2	ns	ns	ns
Crypt (µm)								
Depth	199	236	246	232	12.0	0.05	<0.05	ns
Mucosal surface area, M index [3]	5.7	6.5	6.4	6.8	0.5	ns	ns	ns
Mitotic index (number of cells)								
Villus	16.5	28.2	28.9	19.6	3.7	<0.05	ns	0.10
Crypt	37.7	47.5	50.6	39.4	4.3	ns	ns	0.08
Apoptotic index (number of cells)								
Villus	4.2	3.9	3.6	7.2	3.7	ns	ns	0.07
Zonula-1 (number of cells)								
Villus	2.2	2.4	2.2	1.9	3.7	ns	ns	ns

[1] ns = *P*-value > 0.10. [2] CO: no live yeast + challenge; AB: antibiotic + challenge; PR: Preventive administration pattern of live yeast ($5x10^{10}$ CFU/kg of feed from day 0 to sacrifice) + challenge; CM: Competitive administration of live yeast ($5x10^{10}$ CFU/kg of feed for 1 day after ETEC challenge) + challenge. [3] The mucosal surface area was calculated as described by Kisielinski et al.(2002).

Table 2. First gene sets enriched in the jejunum tissue of ANTIBIOTIC (AB) compared to CONTROL (CO) group

Name	*P*-value	FDR q-val
In AB		
MITOCHONDRIAL_MEMBRANE[1]	0	0
REGULATION_OF_MITOSIS[1]	0	0
PROTEIN_FOLDING[1]	0	0
ORGANELLE_ENVELOPE[1]	0	1.1E-04
MITOCHONDRION[1]	0	1.9E-04
UNFOLDED_PROTEIN_BINDING[1]	0	2.7E-04
SPINDLE	0	2.8E-04
CHROMOSOME	0	5.6E-04
In CO		
CALMODULIN_BINDING	0	0.0472
BASOLATERAL_PLASMA_MEMBRANE[1]	0	0.0886
APICAL_JUNCTION_COMPLEX[1]	0.0045	0.0919
LIGAND_GATED_CHANNEL_ACTIVITY	0.0022	0.1010
PROTEIN_AMINO_ACID_O_LINKED_GLYCOSYLATION	0.0065	0.1028
MEMBRANE_FUSION	0.0040	0.1080
PROTEIN_HOMOOLIGOMERIZATION	0.0064	0.1119
CELL_JUNCTION[1]	0.0024	0.1501
CHLORIDE_CHANNEL_ACTIVITY[1]	0.0163	0.1737
GLUTAMATE_RECEPTOR_ACTIVITY	0.0162	0.1787
EXCRETION	0.0143	0.2440

[1]Other related pathway were also statistically significant.

Table 3. First gene sets enriched in in the jejunum tissue of PREVENTIVE (PR) compared to CONTROL (CO) group

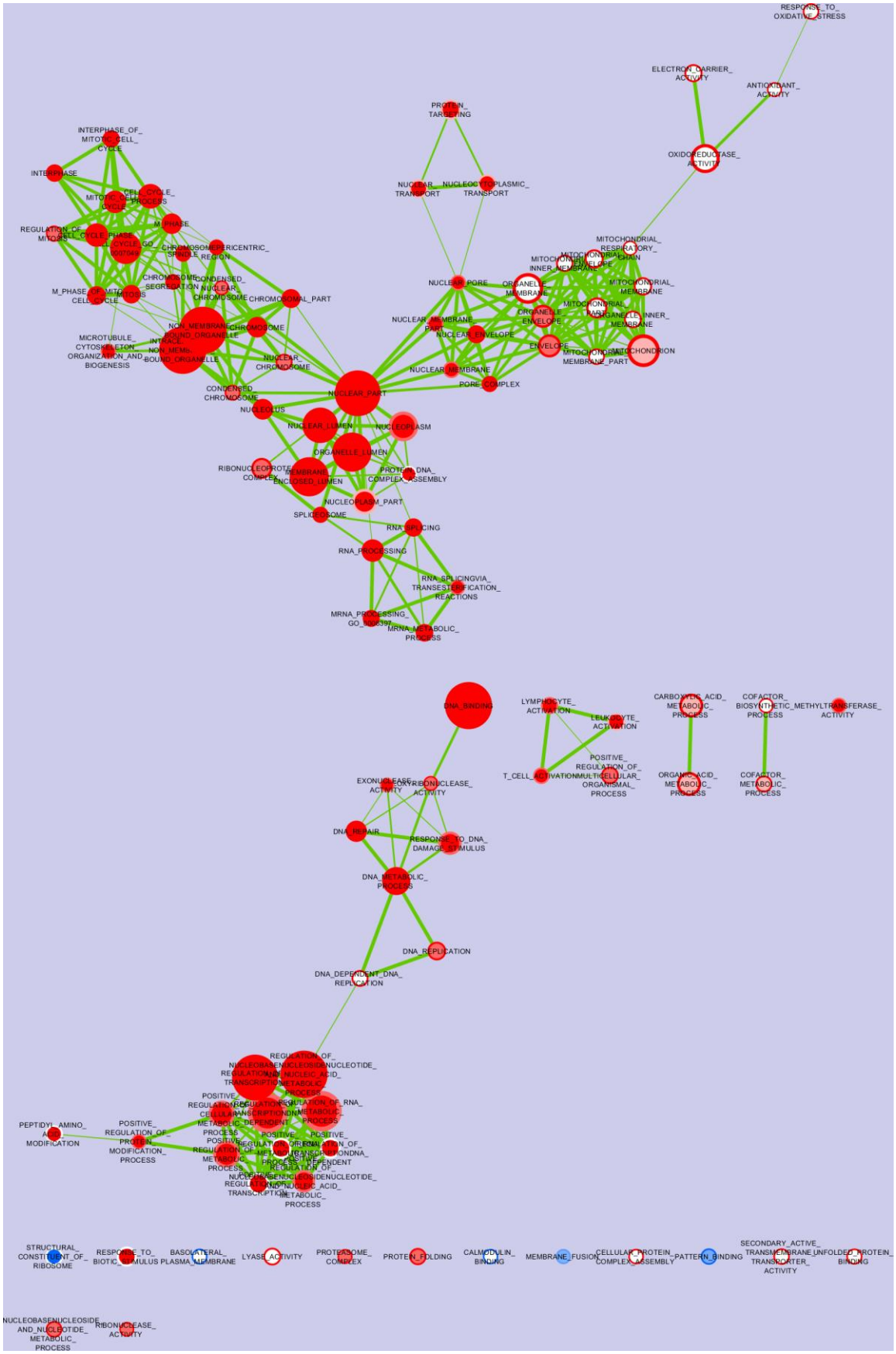
Name	*P*-value	FDR q-val
In PR		
NUCLEAR_LUMEN[1]	0	0.0042
CELL_CYCLE_PHASE[1]	0	0.0044
MRNA_PROCESSING[1]	0	0.0050
NUCLEAR_PORE[1]	0	0.0060
NUCLEOLUS	0	0.0069
CHROMOSOMEPERICENTRIC_REGION	0	0.0137
EXONUCLEASE_ACTIVITY	0.0018	0.0138
MICROTUBULE_CYTOSKELETON_ORGANIZATION_ AND_BIOGENESIS	0.0017	0.0258
NUCLEAR_TRANSPORT	0	0.0260
In CO		
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	0	0.0113
PROTEIN_AMINO_ACID_O_LINKED_GLYCOSYLATION[1]	0.0125	0.1634
ANION_TRANSMEMBRANE_TRANSPORTER_ACTIVITY[1]	0.0052	0.1692
APICAL_JUNCTION_COMPLEX[1]	0	0.1964
TRANSFERASE_ACTIVITY_TRANSFERRING_GROUPS OTHER_THAN_AMINO_ACYL_GROUPS	0.0077	0.2369
ANATOMICAL_STRUCTURE_FORMATION	0.0053	0.2466

[1]Other related pathway were also statistically significant.

Table 4. First gene sets enriched in in the jejunum tissue of COMPETITIVE (CM) compared to CONTROL (CO) group

Name	*P*-value	FDR q-val
In CM		
NUCLEOBASENUCLEOSIDE_AND_NUCLEOTIDE_METABOLIC_PROCESS[1]	0.0016	0.1408
KINASE_ACTIVITY	0	0.1425
NEGATIVE_REGULATION_OF_GROWTH	0.0016	0.1426
L_AMINO_ACID_TRANSMEMBRANE_TRANSPORTER_ACTIVITY[1]	0.0018	0.1536
PHOSPHOTRANSFERASE_ACTIVITY_ALCOHOL_GROUP_AS_ACCEPTOR[1]	0	0.1627
COFACTOR_METABOLIC_PROCESS[1]	0	0.1836
POSITIVE_REGULATION_OF_TRANSCRIPTION[1]	0.0014	0.1926
ANTI_APOPTOSIS	0	0.198
CARBOXYLIC_ACID_METABOLIC_PROCESS	0	0.2006
INTRINSIC_TO_ORGANELLE_MEMBRANE	0.0048	0.2010
OXIDOREDUCTASE_ACTIVITY_ACTING_ON_THE_ALDEHYDE_OR_OXO_GROUP_OF_DONORS	0.0184	0.2046
CARBOHYDRATE_METABOLIC_PROCESS	0	0.2062
CYTOSOLIC_PART	0.0156	0.2080
HEART_DEVELOPMENT	0.0175	0.2094
In CO		
STRUCTURAL_CONSTITUENT_OF_RIBOSOME	0	0
CENTROSOME	0	0.1027
STRUCTURAL_MOLECULE_ACTIVITY	0	0.1045
MICROTUBULE_ORGANIZING_CENTER	0	0.1760

[1]Other related pathway were also statistically significant.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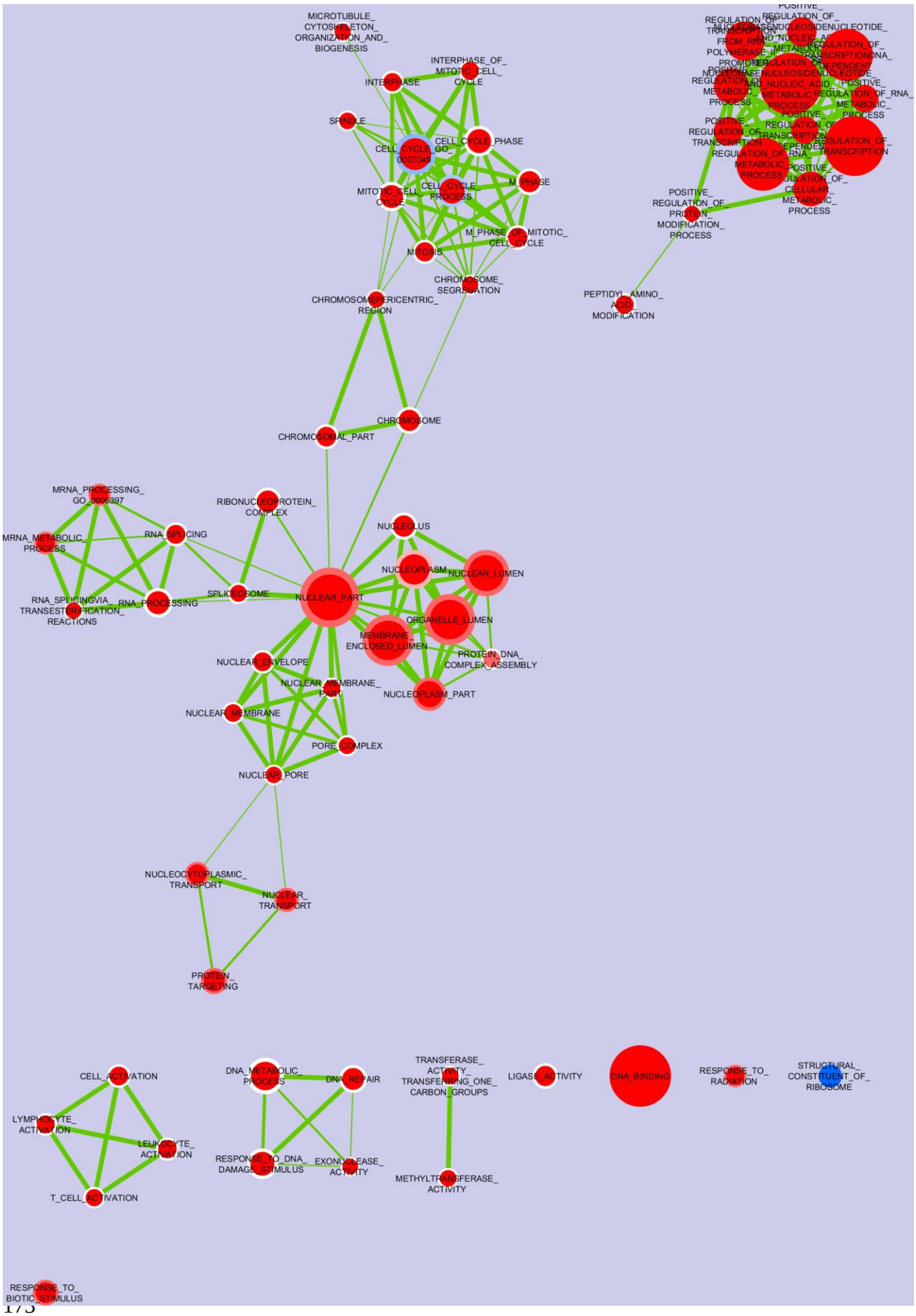
OXIDOREDUCTASE_ACTIVITY
PROTEIN_TARGETING
NUCLEAR_TRANSPORT
NUCLEOCYTOPLASMIC_TRANSPORT
INTERPHASE_OF_MITOTIC_CELL_CYCLE
INTERPHASE
CELL_CYCLE_PROCESS
MITOTIC_CELL_CYCLE
M_PHASE
REGULATION_OF_CELL_CYCLE_PHASE_TRANSITION
CELL_CYCLE_GO_0007049
CHROMOSOME_PERICENTRIC_REGION
M_PHASE_OF_MITOTIC_CELL_CYCLE
MITOSIS
CHROMOSOME_SEGREGATION
CONDENSED_NUCLEAR_CHROMOSOME
CHROMOSOMAL_PART
CHROMOSOME
NON_MEMBRANE_BOUND_ORGANELLE
INTRACELLULAR_NON_MEMBRANE_BOUND_ORGANELLE
MICROTUBULE_CYTOSKELETON_ORGANIZATION_AND_BIOGENESIS
NUCLEAR_CHROMOSOME
NUCLEAR_PART
CONDENSED_CHROMOSOME
NUCLEOLUS
NUCLEAR_LUMEN
NUCLEOPLASM
ORGANELLE_LUMEN
RIBONUCLEOPROTEIN_COMPLEX
MEMBRANE_ENCLOSED_LUMEN
PROTEIN_DNA_COMPLEX_ASSEMBLY
NUCLEOPLASM_PART
SPLICEOSOME
RNA_SPLICING
RNA_PROCESSING
RNA_SPLICINGVIA_TRANSESTERIFICATION_REACTIONS
MRNA_PROCESSING_GO_0006397
MRNA_METABOLIC_PROCESS
NUCLEAR_PORE
NUCLEAR_MEMBRANE_PART
NUCLEAR_ENVELOPE
NUCLEAR_MEMBRANE
PORE_COMPLEX
ORGANELLE_MEMBRANE
ORGANELLE_ENVELOPE
ENVELOPE
MITOCHONDRIAL_RESPIRATORY_CHAIN
MITOCHONDRIAL_ENVELOPE
MITOCHONDRIAL_INNER_MEMBRANE
MITOCHONDRIAL_MEMBRANE
MITOCHONDRIAL_ORGANELLE_INNER_MEMBRANE
MITOCHONDRIAL_MEMBRANE_PART
MITOCHONDRION
DNA_BINDING
LYMPHOCYTE_ACTIVATION
LEUKOCYTE_ACTIVATION
T_CELL_ACTIVATION
POSITIVE_REGULATION_OF_MULTICELLULAR_ORGANISMAL_PROCESS
CARBOXYLIC_ACID_METABOLIC_PROCESS
ORGANIC_ACID_METABOLIC_PROCESS
COFACTOR_BIOSYNTHETIC_PROCESS
COFACTOR_METABOLIC_PROCESS
METHYLTRANSFERASE_ACTIVITY
EXONUCLEASE_ACTIVITY
RIBONUCLEASE_ACTIVITY
DNA_REPAIR
RESPONSE_TO_DNA_DAMAGE_STIMULUS
DNA_METABOLIC_PROCESS
DNA_REPLICATION
DNA_DEPENDENT_DNA_REPLICATION
REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_REGULATION_NUCLEIC_ACID_TRANSCRIPTION
REGULATION_OF_NUCLEOBASENUCLEOSIDENUCLEOTIDE_NUCLEIC_ACID_METABOLIC_PROCESS
POSITIVE_REGULATION_OF_RNA_METABOLIC_PROCESS
POSITIVE_REGULATION_OF_CELLULAR_METABOLIC_PROCESS
POSITIVE_REGULATION_OF_TRANSCRIPTION_DNA_DEPENDENT
POSITIVE_REGULATION_OF_METABOLIC_PROCESS
PEPTIDYL_AMINO_ACID_MODIFICATION
POSITIVE_REGULATION_OF_PROTEIN_MODIFICATION_PROCESS
STRUCTURAL_CONSTITUENT_OF_RIBOSOME
RESPONSE_TO_BIOTIC_STIMULUS
BASOLATERAL_PLASMA_MEMBRANE
LYASE_ACTIVITY
PROTEASOME_COMPLEX
PROTEIN_FOLDING
CALMODULIN_BINDING
MEMBRANE_FUSION
CELLULAR_PROTEIN_COMPLEX_ASSEMBLY
PATTERN_BINDING
SECONDARY_ACTIVE_TRANSMEMBRANE_TRANSPORTER_ACTIVITY
UNFOLDED_PROTEIN_BINDING
NUCLEOBASENUCLEOSIDE_AND_NUCLEOTIDE_METABOLIC_PROCESS
RIBONUCLEASE_ACTIVITY

Figure 1. Nodes of the gene sets regulated jointly or separately in the jejunum tissue, 24 h after ETEC F4 administration, in pigs supplemented with oral supplemented with *Saccharomyces cerevisiae* CNCM I-4407 from day 0 (the centre of the nodes, PR group) or with colistin (the ring of the nodes, AB group), both compared with untreated pigs (CO group). Enrichment significance (P-value) is conveyed as node colour intensity, where red stands for upregulation, and blue for downregulation with PR or CO treatments, and white for no effect of the treatments. The size of the nodes represents how many genes are in the gene set.


MICROTUBULE_ CYTOSKELETON_ ORGANIZATION_AND_ BIOGENESIS
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INTERPHASE
SPINDLE
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CELL_CYCLE_PHASE
M_PHASE
MITOTIC_CELL_ CYCLE
CELL_CYCLE_ PROCESS
M_PHASE_OF_MITOTIC_ CELL_CYCLE
MITOSIS
CHROMOSOME_ SEGREGATION
CHROMOSOME_CENTRIC_ REGION
CHROMOSOME
CHROMOSOMAL_PART
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MRNA_METABOLIC_ PROCESS
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NUCLEOLUS
NUCLEOPLASM
NUCLEAR_LUMEN
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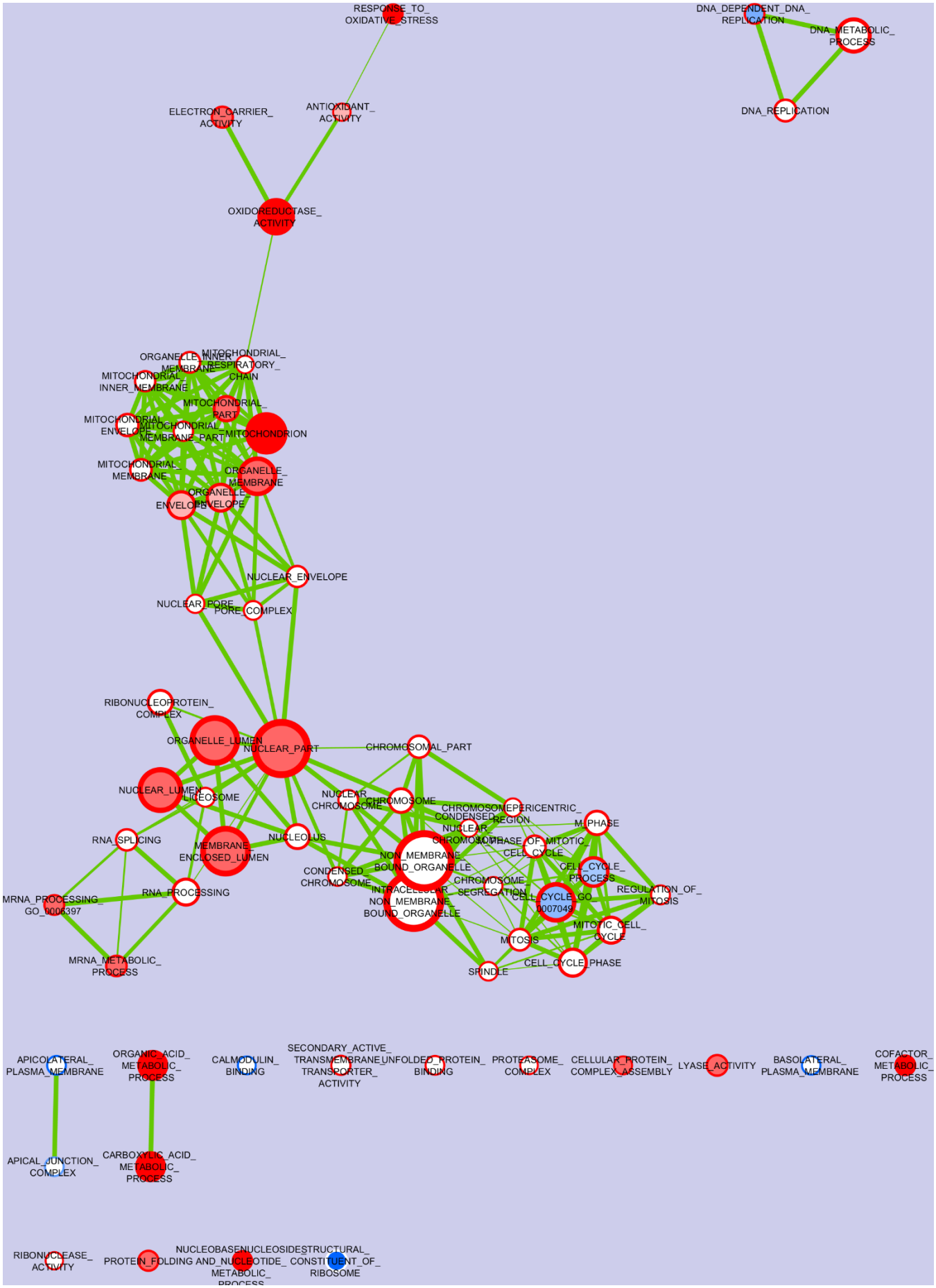
RNA_PROCESSING
SPLICEOSOME
NUCLEAR_PART
ORGANELLE_LUMEN
MEMBRANE_ ENCLOSED_LUMEN
PROTEIN_DNA_ COMPLEX_ASSEMBLY
NUCLEAR_ENVELOPE
NUCLEAR_MEMBRANE_ PART
NUCLEOPLASM_PART
NUCLEAR_MEMBRANE
PORE_COMPLEX
NUCLEAR_PORE
NUCLEOCYTOPLASMIC_ TRANSPORT
NUCLEAR_ TRANSPORT
PROTEIN_ TARGETING
PEPTIDYL_AMINO_ ACID_ MODIFICATION
POSITIVE_ REGULATION_OF_ PROTEIN_ MODIFICATION_ PROCESS
CELL_ACTIVATION
LYMPHOCYTE_ ACTIVATION
LEUKOCYTE_ ACTIVATION
T_CELL_ACTIVATION
DNA_METABOLIC_ PROCESS
DNA_REPAIR
RESPONSE_TO_DNA_ DAMAGE_STIMULUS
EXONUCLEASE_ ACTIVITY
TRANSFERASE_ ACTIVITY_ TRANSFERRING_ONE_ CARBON_GROUPS
METHYLTRANSFERASE_ ACTIVITY
LIGASE_ACTIVITY
DNA_BINDING
RESPONSE_TO_ RADIATION
STRUCTURAL_ CONSTITUENT_OF_ RIBOSOME
RESPONSE_TO_ BIOTIC_STIMULUS

Figure 2. Nodes of the gene sets regulated jointly or separately in the jejunum tissue, 24 h after oral F4 (ETEC) administration, in pigs supplemented from day 0 (the centre of the nodes, PR group) or from the day of the infection (the ring of the nodes, CM group) with *Saccharomyces cervisiae* CNCM I-4407, both compared with untreated pigs (CO group). Enrichment significance (P-value) is conveyed as node colour intensity, where red stands for upregulation, and blue for downregulation with PR or CM treatments, and white for no effect of the treatments. The size of the nodes represents how many genes are in the gene set.


RESPONSE_TO_OXIDATIVE_STRESS
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DNA_METABOLIC_PROCESS
DNA_REPLICATION
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OXIDOREDUCTASE_ACTIVITY
ORGANELLE_INNER_MEMBRANE
MITOCHONDRIAL_RESPIRATORY_CHAIN
MITOCHONDRIAL_INNER_MEMBRANE
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MITOCHONDRIAL_ENVELOPE
MITOCHONDRIAL_MEMBRANE_PART
MITOCHONDRION
MITOCHONDRIAL_MEMBRANE
ORGANELLE_MEMBRANE
ORGANELLE_ENVELOPE
NUCLEAR_ENVELOPE
NUCLEAR_PORE
PORE_COMPLEX
RIBONUCLEOPROTEIN_COMPLEX
ORGANELLE_LUMEN
NUCLEAR_PART
CHROMOSOMAL_PART
NUCLEAR_LUMEN
NUCLEOSOME
NUCLEAR_CHROMOSOME
CHROMOSOME_PERICENTRIC_REGION
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UNFOLDED_PROTEIN_BINDING
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LYASE_ACTIVITY
BASOLATERAL_PLASMA_MEMBRANE
COFACTOR_METABOLIC_PROCESS
APICAL_JUNCTION_COMPLEX
CARBOXYLIC_ACID_METABOLIC_PROCESS
RIBONUCLEASE_ACTIVITY
PROTEIN_FOLDING
NUCLEOBASENUCLEOSIDENUCLEOTIDE_METABOLIC_PROCESS
STRUCTURAL_CONSTITUENT_OF_RIBOSOME

Figure 3. Nodes of the gene sets regulated jointly or separately in the jejunum tissue, 24 h after oral F4 (ETEC) administration, in pigs supplemented with *Saccharomyces cervisiae* CNCM I-4407 (the centre of the nodes, CM group) or whit colistin (the ring of the nodes, AB group), both compared with untreated pigs (CO group). Enrichment significance (P-value) is conveyed as node colour intensity, where red stands for upregulation, and blue for downregulation with PR or CM treatments, and white for no effect of the treatments. The size of the nodes represents how many genes are in the gene set.

GENERAL DISCUSSION

Weaning is the most stressful phase for pigs, in the modern swine industry. It comprises a series of changes, like separation from the mothers, environmental changes and the litters mixing (Campbell et al. 2013). All these factors, together with the sudden shift from the liquid milk diet to the solid one cause a transient anorexia immediately after weaning and imply in a lack of nutrients and passive immune protection derived from sow's milk. This complex situation and the consequent stress condition, increase the secretion of factors which can perturb the gut homeostasis of piglets (Smith et al., 2009) that, associated whit the immaturity of the immune system (Stokes et al., 2004), expose the animals to an inflammatory status that results in a reduction of their growth rate and in an increase of risk for colibacillosis infection (Campbell et al., 2013; McLamb el al., 2013). To face this complex problem, antibiotics have been widely used since today, however, the emerging global problem of antimicrobial resistance, make pressure to identify strategies for containing colibacillosis and sustain piglet performance around weaning.

Among these strategies, approaches involving both feeding strategies and genetic breeding schemes are now under investigation (Heo et al., 2013; Roselli et al., 2017; Reiner et al., 2009). Furthermore, the recent advantages in omics techniques allowed to study more in deep the relation between host-genetics, gut microbial community and the host physiological and immunological responses. Is now recognized the critical role of genetics in modulating the gut microbial balance and its involvement in the host-microbial cross-talk as a key factor to regulate the gut health. Some specific genetic markers have been identified drive the piglet resistances to ETEC F4 and F18 infection (Jørgensen et al., 2003; Meijerink et al., 1997; Vogeli et al., 1997), two of the main pathogens responsible for the post-weaning diarrhoea in piglets. However, little information about the effect of those genetic markers in shaping the microbial community and the host-microbial cross-talk are available.

In chapter one we verify the hypothesis that putative genetic markers associated to immune response and susceptibly for colibacillosis infection may influence the gut eubiosis, the intestinal gut immune response, the host metabolomics profile and the intestinal glycomic binding profile of weaned pigs upon normal healthy condition. Our findings show that *MUC4* and *FUT1* genetic variants, that have been previously associated to ETEC F4ac and ETEC F18 susceptibility, respectively, influence the host response even in pigs reared in high hygienic condition and not challenged with specific pathogens. The effect of *MUC4* in the small intestinal was mainly ascribed to the intestinal gene expression and glycomic binding profile, furthermore, variations in the blood metabolic profile were noted. In the same way, *FUT1* affected the piglets' metabolic profile, while the effect in the small

intestine was mainly ascribed to a shift of the gut microbial population partially due to the different glycomic binding profile identified for the opposite genotype variants.

In the second chapter, the attention was mainly focused on the effect of *FUT1* gene. We verified its impact upon blood metabolomics profile, intestinal homeostasis along gastrointestinal tract and faecal bacteria population in healthy piglets during the sucking and post-weaning phase. We studied both the pre- and post-weaning phases to evaluate the dynamic of the responses of piglets in relation with its genotype for *FUT1* effect on blood metabolome was mainly observed during the suckling period respect to the post-weaning one.

Merging the results obtained with these two studies we can, therefore, assert that host genetics could play an important role in the modulation on host-microbial cross-talk and that *MUC4* and *FUT1* genetic variants influence the host physiological status of healthy piglets even when reared in good sanitary conditions.

Interesting the first study shown that the genetic variants for both genes affect the glycominc binding profile for fucose with the same trend indeed a higher level of staining UEA, which is fucose specific, was observed for the *FUT1* and *MUC4* susceptible genotypes ($FUT1^{AG}$ and $FUT1^{GG}$; and $MUC4^{GG}$ and $MUC4^{CG}$) compared to the resistant genotypes ($FUT1^{AA}$ and $MUC4^{CC}$) in jejunal brush border and for the goblet cells in villi. This different degree of fucosylation among the genetic variants could have influenced the microbial composition in jejunum, as the terminal fucose residues on the oligosaccharides that constitute the mucine could be implicated in the adhesion of specific bacteria (Hasson et al., 2005) and affect also the commensal bacteria population. Both the first and the second studies verified this hypothesis for *FUT1* using the NGS and classical colture-based dependant techniques respectively. Indeed, differences in both alpha and beta diversity indices were identified in the first study through the NGS sequencing techniques of V3-V4 regions of 16S rRNA gene. Specific OTUs discriminated the *FUT1* genotypes: Lactobacillus discriminate the $FUT1^{AA}$ genotype, while $FUT1^{GG}$ genotype was discriminated by *Fusobacterium* and *Veillonella* genus, which have been previously associated to Crohn's disease (CD) in human patients (Geves et al., 2015). In the same way, the second study, using the classical culture-based techniques, identified differences in bacterial concentration along the gastrointestinal tract and in faeces of 34 days'old healthy weaners pigs. The $FUT1^{AG}$ genotype (susceptible) showed a higher number of Enterobacteriaceae and haemolytic bacteria than $FUT1^{AA}$ both in the small and large intestine and only of haemolytic bacteria in faeces. These results confirm that the two opposite genotypes for *FUT1* can affect not only the degree of mucosal fucosylation of the small intestine but also the microbial composition of the piglet gut; particularly the susceptible $FUT1^{GG}$ and $FUT1^{AG}$ genotype may predispose piglet to the ETEC

infection as a higher level of non-beneficial bacteria have been identified in both studies. The hypothesis that *MUC4* genotype may affect the intestinal microbial composition was not disproved in these studies, however, we should consider that the effect of *MUC4* on the glycominc binding profile was lower respect the one noted for *FUT1*.

In addition, we found that *FUT1* genetic variants have affected the host metabolism and we can speculate that the observed differences could be the results of a modification of the microbial-host cross talk due to the different microbial composition observed for the opposite *FUT1* genetic variants. Though the two studies lack significant results for the effect of *FUT1* genetic variants in terms changes of SCFA concentration along the gastrointestinal tract and faeces (second study) and on the expression of genes related to immune response (both studies) and intestinal barrier integrity (second study), our hypothesis is supported by the fact that differences in the blood metabolomics profile were found in both studies using two different metabolomics approaches. In the first study, the target metabolomics approach revealed that *FUT1*^{GG} have a higher level of phosphatidylcholine, generally associated with a perturbation of fatty acid metabolism and of the host homeostasis, than *FUT1*^{AA} (Vorkas et al., 2015). In the second study using an un-target approach technique, different clusters for *FUT1*^{AA} and *FUT1*^{AG} pigs were identified mainly during the suckling period. Discriminant analysis allow to identify specific markers in piglets plasma profile and interesting those compounds were sticky related to microbial metabolism and microbial-host cross talk indeed at day 7 after birth the *FUT1*^{AG} (susceptible genotype) showed higher level of hippuric acid that in literature showed to depend on a perturbed microbial metabolism (Williams et al., 2010), and higher level of oxindole which is a secondary metabolite produced through the tryptophan pathway by bacteria metabolism (Arora and Bae 2014). In the same way, at day 28 after birth, guanosine, a nitrogenous base implicated in the brain-gut axis that improves the inflammatory response due to oxidative damage induced by LPS, discriminates the *FUT1*^{AG} piglet (Bellaver et al., 2015). The clusters due to *FUT1* variants were less visible in the plasma metabolomics profile after weaning. That result contrasted our expectation since the post-weaning period represents the phase in which the ETEC F18 infection mainly occurs, and the ETEC F18 receptors, as well as the *FUT1* gene expression in the intestine result most expressed (Coddens et al., 2007; Bao et al, 2012). However, we should consider that different factors perturb the host metabolism after weaning, and since the blood metabolomics represent the complex physiological status of the host, these different factors could have masked the genotype effect on plasma metabolome, as the differences in terms of microbiota composition were mainly observed during the post-weaning period than during the suckling phase.

In the same way, considering *MUC4*, differences in the blood metabolomics profile ascribing to the host–microbial cross-talk were noted, particularly serotonin, pointed out also for its role in the interaction between brain and gut, seems to be influenced by *MUC4* genetics variants (Clarke et al., 2012). Furthermore, the results obtained in the first study of this thesis allow reinforcing the evidence that this genetic marker could affect the mucosal transcriptional profile by the up or down-regulation of genes associated to intestinal immune response and intestinal mucosal integrity (*CCL20*, *REG3G* and *TFF3*). These results confirm what already observed previously by Trevisi et al. (2012) and suggest that *MUC4* gene could be associated not only with the pathogen adhesion but also with the regulation of piglet intestinal homeostasis.

Taking advantages from the results obtained on healthy animal experiments, the last study was designed to evaluate the efficacy of a feeding strategy, a probiotic supply, using a challenge experimental model and taking into account the host genetics effect. In detail, since the study one shown the impact of *MUC4* genotype on the jejunal expression of relevant genes for gut homeostasis, the piglets of this third study were selected to have the same *MUC4* susceptible genotype to reduce the internal variability and thus the number of required piglet for the study.

Nowadays, several studies aim to verify the utilization of probiotics as reliable strategy to improve the piglet robustness at weaning, however, the mode of action of these probiotics, their effective dose and their timing of administration remain to be elucidate and defined in order to make them become a real strategy to reduce the antibiotic use in pig industry. Among probiotics, *Saccaromyces cervisae* already proved its beneficial effects in *in vitro* and in *in vivo* studies (Collier et al., 2014), but the in deep understating of its mechanism of protection against pathogens in pig was not completely explained. Thus, the third study aims to evaluate the effect of *Saccaromyces cervisae* supplementation at the different timing: one-week prior the infection and the day of infection on acute phase proteins in blood and on the intestinal mucosal homeostasis with specific attention to the jejunal transcriptomic profile of piglets challenged with ETEC F4ac. This study started by the results obtained in our previous larger experiment which shown that preventive and competitive *Saccaromyces cervisae* supplementation was able to reduce the piglet's mortality and to maintain the piglet growth performances in the two weeks after ETEC infection, and aimed to elucidate the mechanisms by which S*accaromyces cervisae* administration was able to prevent the diametrical effect of ETEC F4ac. The results showed that preventive and competitive S*accaromyces cervisae* administration modified the intestinal mucosal integrity and permeability in terms of crypt depth, villus mitotic index and mucosal transcriptomic profile. The preventive administration showed a similar transcriptomics pattern profile than colistin supplemented group with an up-regulation of genes involved in the

activation of B and T cells through the NF-kB pathway, a down-regulation of genes related to the pro-inflammatory protein, the anion channel activity, the cell junction and the protein_amino_acid_o_linked_glycosilation pathway, resulting thus in a regulation of the intestinal integrity and permeability. Interesting both the competitive and the preventative administrations were related to the nucleotide metabolic process suggesting a specific implication of the yeast in this pathway. In addition, the competitive probiotic supply enriched some pathway compared to the control such as the antioxidant activity and intestinal inflammation (*IGF-1*, *PPAR-γ*, *TRPC1*, *FGL2*) and explain the partial efficacy of the probiotic administration in reducing the detrimental effect of ETEC F4ac infection. This study partially explains how *Saccaromices cervisae* administration interacts with the host mucosal response and helped to elucidate some microbial-host interaction. It remains to study how the probiotic administration can influence the overall microbial eubiosisis and its composition, thus this aspect could be studied in the future using experimental models based on not infected pigs selected for their genetic background for *MUC4* and *FUT1* genes.

CONCLUSION

Overall the studies included in the present thesis contribute to understanding the interaction between the host and the gut microbiota in the pre- and post-weaning phases of pigs. The host genetics, particularly referred to *MUC4* and *FUT1* genes, showed to influence the intestinal glycomics profile, the intestinal microbial population as well as the gut homeostasis and the host metabolism in healthy animals. Thus, the genotyping of *MUC4* and *FUT1* genes may be used to stratify the output of studies focusing on gut homeostasis and may contribute to a deeper understanding of host-microbial cross-talk. In addition, as the SNPs on *MUC4* and *FUT1* genes are correlated to phenotypic characters such as the expression of specific ETEC receptors; the early piglets genotyping for these SNPs may improve the experimental protocol reliability as well as reduce the number of animals needed, especially for study focused on the effect of feeding additive to contrast the ETEC infection. This approach was applied in the third study, where it was observed that the preventive use of probiotic could represent a potential strategy to reduce the detrimental effect of ETEC F4 infection.

The implementation of the animal model with the inclusion of genetic markers and feeding strategies may provide data to develop an innovative strategy to improve the piglet robustness by modulation of host-microbial cross talk and it can contribute in reducing the occurrence of antibiotic resistance issue by decreasing the use of antibiotic in pig production system.

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