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Effect of *Mucine 4* and *Fucosyltransferase 1* genetic variants on gut homoeostasis of growing healthy pigs

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Abstract

Putative genetic markers have been associated with ETEC F4 (Mucine 4 [*MUC4*]; *MUC4*$^{GG;CG}$ as susceptible; *MUC4*^{CC} as resistant) and F18 (Fucosyltransferase 1 [*FUT1*]; *FUT1*$^{GG;AG}$ as susceptible; *FUT1*^{AA} as resistant) resistances respectively. In this study, 71 post-weaning pigs were followed from d0 (35 days old) to d42 (77 days of age) to investigate the effect of *MUC4* or *FUT1* genotypes on the mid-jejunal microbiota composition, pigs expression of genes related to inflammation (*IL8*, *GPX2*, *REG3G*, *TFF3*, *CCL20* and *LBPI*) and glycomic binding pattern profile (*Ulex europaeus* agglutinin I [UEA] fucose-binding lectin and peanut agglutinin [PNA] galactose-specific), and on blood plasma targeted metabolomics profile, faecal score and performance parameters of growing healthy pigs. The *MUC4* and *FUT1* resistant genotypes improved the pigs' growth performance and had firmed faecal score susceptible genotypes in d0–d21 period. Pigs with *MUC4*^{GG} genotype had a higher jejunal expression of genes relate to immune function (*CCL20* and *REG3G*) than *MUC4*^{CG} and *MUC4*^{CC} pigs ($p < 0.05$). *MUC4*^{CG} pigs had higher expression of *TFF3* (implicated in mucosal integrity) than *MUC4*^{GG} and *MUC4*^{CC} ($p < 0.05$). *FUT1* influenced the alpha- and beta-jejunal microbial indices. The *FUT1*^{AA} group had a higher number of OTUs belonging to *Lactobacillus* genus, while *FUT1*^{GG} group had a higher number of OTUs belonging to *Veillonella* genus. *MUC4*^{CC} pigs had lower scores for UEA on brush borders and goblet cells in villi than *MUC4*^{GG} ($p < 0.05$). *FUT1*^{AA} pigs had lower UEA positivity and higher PNA positivity on brush borders and goblet cells than *FUT1*^{AG} and *FUT1*^{GG} ($p < 0.05$). Both *FUT1* and *MUC4* influenced the metabolic profile of healthy pigs. Results highlight the role of *MUC4* and *FUT1* on pig intestinal homoeostasis and improved the knowledge regarding the potential interaction between host genetics, gut microbiota composition and host metabolism in a healthy status.

KEYWORDS
16S rRNA, fucose, *FUT1*, intestinal mucosa, *MUC4*

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1 | INTRODUCTION

In pigs, the post-weaning period is frequently characterized by sub-optimal growth and a high prevalence of intestinal disorders, mainly due to colibacillosis infection (Schokker et al., 2015), that impact on the pig morbidity and mortality and increase the use of antibiotics (Gresse et al., 2017). Preserving the gut homoeostasis by maintaining a favourable intestinal bacteria balance and preserving the functionality of the mucosa to ensure the digestion and absorption of nutrients and the immunological functions are crucial to sustain the gut health (Wells et al., 2017).

Several genetic markers have been associated with pigs post-weaning diarrhoea (PWD) due to enterotoxigenic *Escherichia coli* (ETEC) F4 and F18. The single nucleotide polymorphism (SNP) 8227C>G located on Mucine 4 (*MUC4*) gene has been associated with the expression of the F4ac receptor in the jejunum brush border and with the susceptibility to ETEC F4. Piglets with $MUC4^{CG/GG}$ genotypes result as susceptible and express F4 receptor, while piglets with $MUC4^{CC}$ genotype are most associated with the resistant phenotype (Jørgensen et al., 2003). The SNP located on Fucosyltransferase 1 (*FUT1*) gene has been associated with piglet susceptibility to ETEC F18ab/ac. Piglets with $FUT1^{AG/GG}$ genotypes result as susceptible; piglets with $FUT1^{AA}$ genotype results as resistants (Meijerink et al., 2000). *FUT1* encodes for the FUT1 enzyme which catalyses the addition of terminal alpha (1,2) fucose residues on the carbohydrates expressed on the surface of epithelial cells and in mucosal secretions (Henry et al., 1996) that has been highlighted as a putative receptor for ETEC F18 adhesion in the small intestine. For this reason, piglets with different *FUT1* genotypes have been associated with a different phenotype for ETEC F18 receptors in the jejunum brush border and with their relative susceptibility to ETEC F18 (Coddens et al., 2007).

Both host genetic background and intestinal bacteria can influence the intestinal gut homoeostasis, and the overall host metabolism and health status (McKnite et al., 2012; Sommer & Backhed, 2013). Studies suggested that *MUC4* and *FUT1* genotypes are not only associated with piglets susceptibility to PWD but can also influence the intestinal homoeostasis. For instance, healthy piglets with different *MUC4* and *FUT1* genotypes are known to differ for their intestinal microbial profile (*MUC4*, Messori, Trevisi, Simongiovanni, Priori, & Bosi, 2013; *FUT1*, Poulsen et al., 2018) and for their intestinal protein glycosylation (Hesselager, Everest-Dass, Thaysen-Andersen, Bendixen, & Packer, 2016). Furthermore, the intestinal mucosa of piglets with the susceptible genotype ($MUC4^{CG/GG}$) had an up-regulation of genes related to antimicrobial peptide and immune function such as lipopolysaccharide binding protein (*LBPI*) and regenerating islet-derived 3 gamma (*REG3G)* compared to pigs with a resistant genotype (Trevisi et al., 2012).

Those differences in the intestinal characteristics can, therefore, concur to determine differences in piglets' metabolism (Poulsen et al., 2018) and in piglet nutritional requirement (Trevisi et al., 2012) and result in different growth performance as already reported by Fontanesi et al.. (2012) for *MUC4* and by Bao et al. (2011) for *FUT1* in adult pigs.

However, the effects of *MUC4* and *FUT1* genetics variances in influencing the intestinal homoeostasis and microbial profile in healthy piglets are still poorly investigated.

We hypotheses that *MUC4* and *FUT1* genetic variances can influence the glycomic pattern and the microbial profile of the small intestine giving a different stimulation of the mucosal immune response, resulting in the modulation of piglets' metabolism and growth performance. The aim of the present study was to evaluate the effect of *MUC4* and *FUT1* genetic variances on the growth performance, physiological status and the intestinal homoeostasis of growing healthy pigs.

2 | MATERIAL AND METHODS

The procedures complied with the Italian law 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 by the approval number 801/2015-PR.

2.1 | Animal and sampling

One week post-weaning (35 days of age – d0), seventy-one piglets were randomly selected from 10 litters based on their individual body weight (7,063 ± 936 g average body weight), moved to the experimental facility of the University of Bologna and located in individual pens with a mesh floor for 6 weeks (d42). The study was carried out in three consecutive batches composed by 24, 24 and 23 pigs respectively. Pigs were kept at a controlled temperature (28°C at d0 to 25°C at d42) and heated additionally by infrared lamps for the first 7 days. Animals were fed two consecutive diets: a pre-starter diet from d0 to d21 and a starter diet from d22 to d42 (Table 1). Feed intake was daily recorded, and piglets were individually weighted every week until the end of the trail. To evaluate the pigs' health status, the individual faecal score was daily recorded by visual appraisal using a five-point scoring system (1–5): 1: hard, 5: watery faeces, where a faecal score >3 was considered as diarrhoea. Weekly faecal score was then calculated individually. From each pig, bristles were collected for the genotyping analysis. On d42, all animals were anaesthetized and sacrificed via an intracardiac injection of Tanax (0.5 ml/kg body weight). The jejunum was isolated at 50% of the small intestine length. Jejunum content was collected and mixed with the mucous layer obtained from a gentle scraping of the jejunum mucosa. The sample was snap-frozen in liquid nitrogen and stored at −20°C for microbiota analysis. From the same emptied jejunal site, the mucosa was gently scraped, snap-frozen in liquid nitrogen and preserved at −80°C for gene expression analyses.

From a sub-group of 24 pigs, selected to be balanced for *FUT1* and *MUC4* genotypes, an additional sample of mid-jejunum tissue was collected, washed with 0.01 M phosphate-buffered saline (PBS), pinned tightly to balsa wood, fixed in 10% buffered formalin for

TABLE 1 Ingredients and composition of piglet pre-starter diet (from d0 to d21) and starter diet (from d22 to 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	–
Soya bean meal	4.80	20.00
Soya bean 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 and 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

24 hr at room temperature (RT) and dehydrated in a graded series of ethanol and embedded in paraffin for immunohistochemistry analysis. A blood sample from the same sub-group was obtained by venipuncture of vena cava on a collection tube with K3 EDTA (Vacutest Kima Srl, Padova, Italy) before the animal sacrifice. Blood samples were centrifuged at 3,000 *g* for 10 min at 4°C to obtain the plasma. Plasma samples were stored at −80°C until further targeted metabolomics profile analysis.

2.2 | Animal genotyping

Genomic DNA of each pig was extracted from bristles. The bristle bulbs were incubated in Proteinase K solution (10 mg/ml of proteinase K in buffer [20 mM Tris–HCl (pH 8.4), 50 mM KCl]) for 2 hr at 50°C; then, the proteinase was inactivated at 95°C. Samples were stored at −20°C. Genotyping of the *MUC4* g.8227C>G, *FUT1* g.307 G>A, was carried out by Restriction Fragment Length Polymorphism PCR (PCR-RFLP) using specific primers, annealing temperature, condition enzymes reported by Jørgensen et al. (2003) and Meijerink et al. (2000).

2.3 | Gene expression

The total RNA was isolated from scraped mid-jejunum mucosa 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. cDNA was synthesized using 1,000 ng of RNA as template (ImProm-II Reverse Transcription System Promega Corporation, Milan, Italy). The semi-quantitative PCR (qPCR) of interleukin-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). The genes related to immune function (*IL8*, *CCL20* and *REG3G*), antimicrobial activity (*LBPI*) and inflammatory response (*GPX2*, *TFF3*) were selected because highlighted in several studies as effective markers able to disclose alterations of the gut mucosa homoeostasis, especially in ETEC-infected pigs (Bosi et al., 2004; Sargeant et al., 2010; Trevisi, ~~Latorre~~ et al., 2017; Trevisi et al., 2018).

Primers' sequence, amplified fragments' length and qPCR conditions are reported in Luise et al. (2017). 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 relative quantification of gene expressions was calculated using standard curve methods and normalized to a housekeeping gene hydroxymethylbilane synthase (*HMBS*).

2.4 | Microbiota Analysis

Total bacterial DNA was extracted from all samples using QIAamp Stool Mini Kit (Qiagen, Hilden, Germany). DNA concentration and its purity (absorbance ratio 260/280 and 260/230) were measured by ND 1000 Spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and visualized on 1.5% of agarose gel. All samples had absorbance ratios 260/280 higher than 1.8 and 260/230 ratios lower than 2. In addition, a PCR for lactic acid bacteria (LAB) was performed following the protocol reported by Walter et al. (2001), to further confirm that bacterial DNA was extracted. The library formation and sequencing of the 16S rRNA gene were performed with MiSeq® Reagent Kit V3-V4 on MiSeq-Illumina® platform. Generated sequences (approximately ~460 bp) were analysed using subsampled open-reference OTU strategy with default settings in QIIME (v1.9.1) (Caporaso et al., 2010). The reads of the 16S rRNA gene were paired-end and demultiplexed. Subsampled open-reference OTU-picking was performed

using UCLUST with 97% sequence similarity. 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.

2.5 | Immunohistochemistry analysis

Transvers[illegible] µm thick) sections were obtained from jejunum and analysed for *Ulex europaeus* agglutinin I (UEA; Vector Laboratories, Peterborough, UK; catalogue n. B-1065- fucose-specific) and peanut agglutinin (PNA; Vector Laboratories, Peterborough, UK; catalogue n. B-1075- galactose-specific) following the protocol described by Priori et al. (2016). 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) and for labelled cells frequency in the villi and crypts. The intensity of 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; and 3 = very marked positivity of immunoreactivity and great thickness. 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; and 4 = many IR cells.

2.6 | Plasma targeted metabolomics 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). 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). 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).

2.7 | Statistical analysis

Except for microbiota and metabolomics data, all data were analysed on SAS software (version 3.4 SAS Institute), using the GLM procedure including *MUC4* and *FUT1* genotypes, litter within the batch and batch as fixed factors. Sex was included in the model when significant. A restricted model including only significant factors was then carried out.

Biostatistics on OTUs table was performed using the vegan and phyloseq packages in R software (v.3.3.0). The richness and alpha-diversity indices (Chao1 and Shannon indices) were calculated on raw data while beta-diversity ordination and differential abundance analysis were carried out after rarefaction correction. 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, and differences were tested by pairwise Wilcoxon signed-rank post hoc test. In order to identify the discriminant OTUs belonging to each variant of *FUT1* (the sole significant gene), the sparse partial least squares discriminant analysis (sPLS-DA) was carried out using the MixOmics (mixOmics_6.1.3) package. Microbial data were previously normalized using the total sum scaling normalization coupled with the centred log-ratio (CLR) transformation as recommended by MixOmics. The optimal number of components and the optimal number of selected variables for each component included in the sPLS-DA model were 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. To validate the results, the stability frequency scores of the selected OTUs were calculated ("perf" function) with 10-fold cross-validation and 100 repetitions. The plotVar function was then used to identify the structure of the correlation between OTUs matrix and *FUT1* genotypes. OTUs showing a correlation with *FUT1* >0.5 and a stability ≥65% were considered discriminative.

For targeted metabolomics analysis, data were imported into the MetaboAnalyst 2.0 software (http://www.metaboanalyst). Metabolites with more than 20% of missing value were removed, and the remaining 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. An ANOVA model for each genotype was then carried out for all the metabolites, and *P* values were corrected for false discovery rate. Furthermore, the sPLS-DA method was applied to describe the *FUT1* and *MUC4* genotype variant influence the targeted metabolome profile and to obtain the most discriminate metabolites among the different genotypes. *p* value <0.05 was considered as significant.

3 | RESULTS

3.1 | Animal genotypes

For *MUC4*, 38 pigs had $MUC4^{CC}$ genotype, 26 pigs had the $MUC4^{CG}$ genotype and seven had the $MUC4^{GG}$ genotype where the $MUC4^{GG}$ is the susceptible genotype for ETEC F4. For *FUT1*, 33 pigs $FUT1^{GG}$, 31 pigs $FUT1^{AG}$ and seven pigs $FUT1^{AA}$ were observed, where the $FUT1^{AA}$ is the resistant genotype for ETEC F18. A total of 28 pigs had the genotype indicating the susceptibility to both ETECs (three had $MUC4^{GG}$, $FUT1^{GG}$; three had $MUC4^{GG}$, $FUT1^{AG}$; 11 had $MUC4^{CG}$, $FUT1^{GG}$; *and* 11 had $MUC4^{CG}$, $FUT1^{AG}$). Four pigs had the susceptible genotype for ETEC F4 and the resistant one for the F18 (two had $MUC4^{CG}$, $FUT1^{AA}$ *and* two had $MUC4^{CG}$, $FUT1^{AA}$). A total of 32 pigs had the resistant genotype for the ETEC F4 and the susceptible

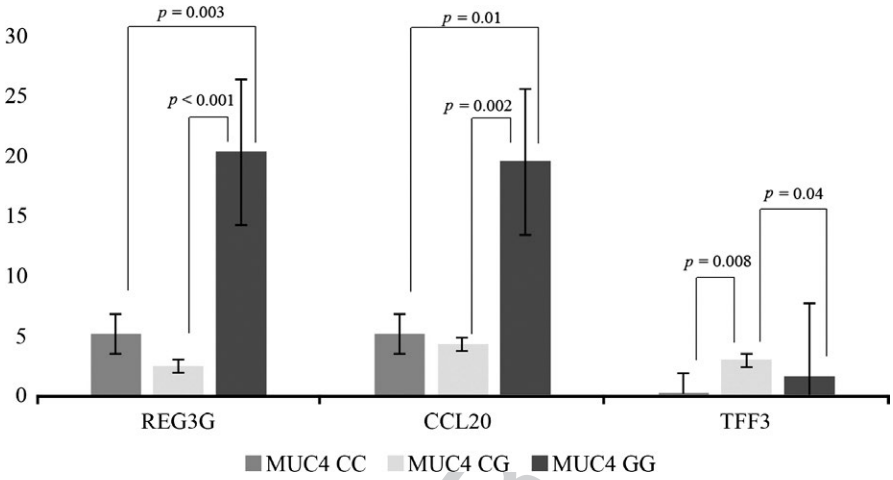

FIGURE 1 Effect of *MUC4* genotypes on the expression of *REG3G*, *CCL20* and *TFF3* in the mid-jejunal mucosa of healthy young pigs. Gene expression of *REG3G*, *CCL20* and *TFF3* and *P*-values of GLM model for the comparisons between $MUC4^{CC}$, $MUC4^{CG}$ and $MUC4^{GG}$ pigs. Data are expressed as the ratio of mRNA copy number of target gene and mRNA copy number of HMBS, multiplied by 10

one for the ETEC F18 (13 pigs had $MUC4^{CC}$, $FUT1^{AG}$ and 19 pigs had $MUC4^{CC}$, $FUT1^{GG}$). One pig had the genotype indicating the resistance against both ETECs, showing the variants $MUC4^{CC}$ and $FUT1^{AA}$.

3.2 | Monitoring of pigs' health status and growth performance

The pigs remained healthy throughout the experiment. The average weekly faecal score was lower than 3 (cut-off value for diarrhoea). Results showed that $MUC4^{CC}$ genotype had a lower (harder) faecal score than $MUC4^{CG}$ and $MUC4^{GG}$ genotypes during the second week of the study ($p < 0.05$). The $MUC4^{CG}$ tended to have higher (softer) faecal score than $MUC4^{GG}$ during the fourth and fifth weeks ($p < 0.1$). The $FUT1^{AA}$ genotype had a faecal score lower than $FUT1^{GG}$ genotype during the first week and tended to have a faecal score lower during the third week. During the third week, the $FUT1^{AA}$ genotype had a faecal score lower than $FUT1^{AG}$ genotype ($p < 0.05$; Supporting Information Table S1).

$MUC4^{CC}$ genotype had a BW higher than $MUC4^{GG}$ genotype at d21 and d42 ($p < 0.05$). $MUC4^{CC}$ genotype had an ADG higher than $MUC4^{GG}$ and $MUC4^{GG}$ genotypes in the period from d0 to d21 ($p < 0.05$) and higher than $MUC4^{GG}$ genotype in the period from d0 to d42 ($p < 0.05$). Feed intake was higher in $MUC4^{CC}$ genotype than in $MUC4^{GG}$ genotype from d0 to d21 ($p < 0.05$). F:G ratio was lower in $MUC4^{CC}$ genotype than $MUC4^{GG}$ and $MUC4^{GG}$ genotypes in the period from d0 to d21 ($p < 0.05$). $FUT1^{AA}$ genotype had a BW at d0 lower than $FUT1^{GG}$ and an ADG higher than $FUT1^{AG}$ and $FUT1^{GG}$ in the period from d0 to d21 ($p < 0.05$; Supporting Information Table S2).

3.3 | Jejunum gene expression

Results showed that $MUC4^{GG}$ genotype had a higher expression of *CCL20* and *REG3G genes* than $MUC4^{CG}$ and $MUC4^{CC}$ genotypes ($p < 0.05$). For *TFF3* expression, the $MUC4^{CG}$ genotype had a higher expression than $MUC4^{GG}$ and $MUC4^{CC}$ ($p < 0.05$; Figure 1). *FUT1* genotype did not influence the jejunum expression of the selected genes.

3.4 | Microbiota composition of mid-jejunum

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 nine bacterial 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. *Lactobacillus* genus (39.2%) was the most abundant, followed by *Streptococcus* (19.8%). *MUC4* polymorphism did not influence the alpha- (Chao1 and Shannon indices) and beta-diversity (Bray–Curtis distance) indices.

FUT1 polymorphism did not influence the Chao1 index, while the Shannon index differed for *FUT1*. The $FUT1^{AG}$ group (3.02 ± 0.72) had a Shannon value higher than $FUT1^{GG}$ (2.76 ± 0.76) and $FUT1^{AA}$ (2.26 ± 1.01) groups ($p = 0.01$). A significant effect was observed for *FUT1* polymorphism ($p = 0.004$) on Bray–Curtis distance matrix. The pairwise adonis procedure showed a difference between the $FUT1^{AA}$ genotype and the other two genotypes ($FUT1^{AA}$ vs. $FUT1^{AG}$, $p = 0.01$; $FUT1^{AA}$ vs. $FUT1^{GG}$, $p = 0.02$). Results of the sPLS-DA for *FUT1* were reported in Table 2. The $FUT1^{AA}$ group was discriminated by a higher number of OTUs belonging to *Lactobacillus* genus, mainly belonging to *Lactobacillus reuteri* species, while the $FUT1^{GG}$ group was discriminated by a higher number of OTUs belonging to *Veillonella* genus.

3.5 | Plasma targeted metabolome profile

The MS/MS targeted analysis performed on plasma samples of the sub-selected group of pigs provided results for 134 metabolites (Supporting Information Table S2). ANOVA indicated that carnitine (C0), *O*-acyl-*O*-acyl phosphatidylcholine (PC_aa) C36:1 and asymmetric dimethylarginine (ADMA) differently responded to the *MUC4* genotypes and spermidine to the *FUT1* genotypes. Higher level of C0 was observed for $MUC4^{CC}$ genotype (5.92 μM) than $MUC4^{CG}$ (4.53 μM) and $MUC4^{GG}$ (4.50 μM); a lower amount of PC aa C36:1 was observed in $MUC4^{CC}$ (32.90 μM) than $MUC4^{CG}$ (36.80 μM) and

FUT1[a]	Family	Genus	Species	Value.var[b]	Freq[c]
AA	Lactobacillaceae	*Lactobacillus*	NA	0.19	0.67
AA	Lactobacillaceae	*Lactobacillus*	NA	0.23	0.77
AA	Lactobacillaceae	*Lactobacillus*	NA	0.20	0.71
AA	Lactobacillaceae	*Lactobacillus*	NA	0.18	0.70
AA	Lactobacillaceae	*Lactobacillus*	NA	0.10	0.65
AA	Lactobacillaceae	*Lactobacillus*	NA	0.28	0.81
AA	Lactobacillaceae	*Lactobacillus*	NA	0.22	0.75
AA	Lactobacillaceae	*Lactobacillus*	*mucosae*	0.23	0.72
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.30	0.79
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.26	0.75
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.39	0.80
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.24	0.74
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.27	0.78
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.22	0.75
AA	Lactobacillaceae	*Lactobacillus*	*reuteri*	0.25	0.77
GG	Veillonellaceae	NA	NA	0.51	0.65
GG	Veillonellaceae	*Veillonella*	*dispar*	0.48	0.68

[a]*FUT1*, 1-fucosyltransferase gene. Genotypes had the following distribution: $FUT1^{AA}$ = 5; $FUT1^{AG}$ = 27; $FUT1^{GG}$ = 33. [b]Value.var expresses the variance explained by the single OTU. [c]Freq expresses the frequencies by which the OTUs were chosen among the 100 repetitions of the cross-validation.

TABLE 2 Significant discriminant OTUs for *FUT1* polymorphism obtained using the sparse partial least squares discriminant analysis methods coupled with 10-fold cross-validation and 100 repetitions

$MUC4^{GG}$ variants (36.36 μM); and a higher level of ADMA was observed for $MUC4^{GG}$ (2.42 μM) than $MUC4^{CC}$ and $MUC4^{CG}$ (1.68 μM; $p < 0.05$). A higher level of spermidine was observed in the $FUT1^{AA}$ genotype (0.53 μM) than the $FUT1^{AG}$ (0.24 μM) and $FUT1^{GG}$ variants (0.29 μM; $p < 0.05$). The multivariate analysis showed that metabolomics profile was partially affected by *MUC4* and *FUT1* genotypes variants as shown by the sPLS-DA score and loading plot (Figure 2a,b). The 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 C0, 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 characterized by a higher value of PC_aa_C42:1, PC_aa_C40:3, PC_aa_34:1, PC_aa_C38:5, and 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. For *FUT1*, the sPLS-DA score plot 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* showed that levels of spermidine, spermine, SM_OH_C24:1, glycine, putrescine and taurine were higher in the $FUT1^{AA}$ group than $FUT1^{AG}$ and $FUT1^{GG}$ groups, while the $FUT1^{GG}$ group was characterized by higher level of PC_ae_C36:5, PC_ae_C42:4, PC_ae_C44:5, and PC_ae_C40:4 than $FUT1^{AA}$ and $FUT1^{AG}$ groups.

3.6 | Immunohistochemistry

~~*Ulex europaeus*~~ agglutinin I and PNA immunoreactivities were observed on the surface of the jejunum villi, while IR goblet cells were observed both in villi and in crypts and scored as shown in Figure 3. Immunoreactivity in the villi showed up as a layer that followed the entire profile of the villus. The IR goblet cells presented a typical columnar/pear shape morphology containing mucin granules intensely marked and were distributed along the axis of the villus and/or aggregated in the crypts. Opposite intensities for UEA and PNA were observed in the same brush borders. The degree of intensity seen in the goblet cells with PNA was averagely lower than with UEA. The effect of *MUC4* and *FUT1* genotypes on the average scores for the UEA or PNA immunoreactivity in the jejunal brush borders and IR goblet cells is shown in Table 3. The $MUC4^{CC}$ pigs had a lower score for UEA in brush borders and goblet cells in villi than $MUC4^{GG}$ ($p < 0.05$) and $MUC4^{CG}$ pigs (marginally, $p = 0.081$ for brush borders; $p < 0.05$ for goblet cells). No effect of *MUC4* on the UEA-IR goblet cells in crypt and of PNA for brush borders and goblet cells in villi and crypts was found. The $FUT1^{AA}$ genotype had lower UEA immunoreactivity score in brush border than $FUT1^{AG}$ and $FUT1^{GG}$ genotypes ($p < 0.001$). UEA-IR goblet cells in the villi and crypts were less in the $FUT1^{AA}$ genotype than in $FUT1^{AG}$ genotype and in $FUT1^{GG}$ genotype ($p < 0.05$). $FUT1^{AG}$ had less UEA-IR goblet cells in crypts ($p < 0.05$), and a trend of significant lower UEA immunoreactivity score on the brush border ($p < 0.1$) than $FUT1^{GG}$. No difference was observed for UEA-IR goblet cells in crypts. For PNA, the $FUT1^{AA}$ genotype had

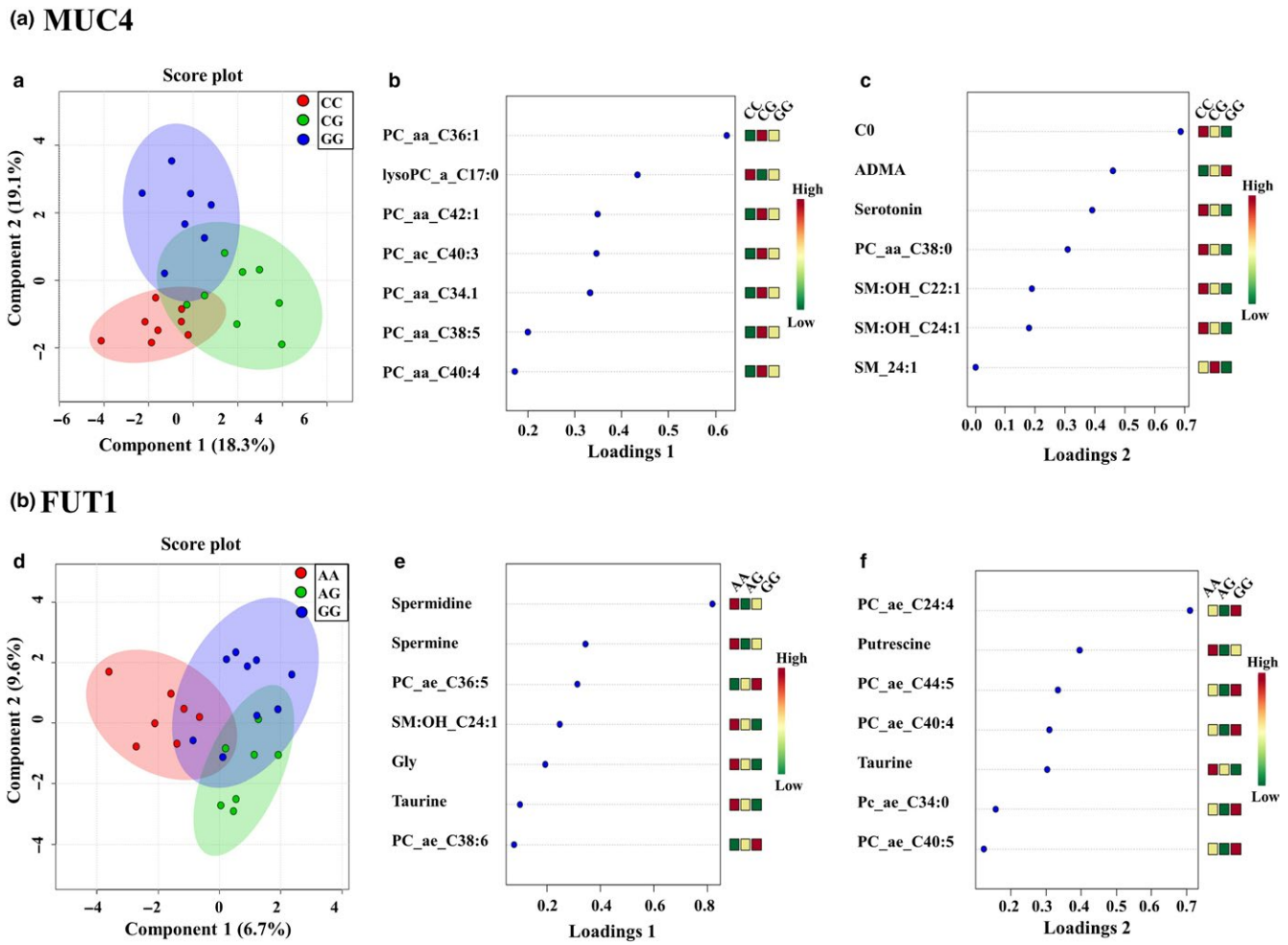

FIGURE 2 Score plots and loading plots of sparse partial least squares discriminant analysis (sPLS-DA) on serum target metabolomics profile of young healthy pigs according to *MUC4* (A) and *FUT1* (B) genotypes. The (a and d) show the individual score plot for *MUC4* and *FUT1* variances respectively. The (b and c) represent the loading plots showing the metabolites that contribute toward the separation of sPLS-DA scores between *MUC4* variances. The ~~(d and e)~~ represent the loading plots showing the metabolites that contribute toward the separation of 15 sPLS-DA scores between *FUT1* variances

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 more abundant in the $FUT1^{AA}$ genotype than in $FUT1^{AG}$ genotype and in $FUT1^{AG}$ genotype ($p < 0.05$). No difference between $FUT1^{AG}$ and $FUT1^{GG}$ for PNA immunoreactivity in bush border and goblet cells was observed.

4 | DISCUSSION

The present study investigated the effect of *MUC4* or *FUT1* genotypes on gut homoeostasis of pigs by evaluating their effect on the host response in the absence of specific infections during the post-weaning phase.

Our study highlighted that the *MUC4* SNP, which is associated with piglet susceptibility for ETEC F4ac (Jørgensen et al., 2003), can influence piglet growth performance and the faecal score during the first weeks post-weaning. The pigs had no diarrhoea and remained apparently healthy during the test; nevertheless, our results indicate that the pigs with the resistant genotype ($MUC4^{CC}$) had firmer faeces than the pigs with the susceptible genotypes. In addition, our study indicates that improved growth performance and improved feed conversion can be observed in the post-weaning pigs with the resistant genotype, in accord with the study of Trevisi et al. ~~(2009)~~ in 18 which post-weaning piglets susceptible to ETEC F4 and fed a standard nutritional diet had a lower ADG and higher feed intake. This could be explained by the different intestinal homoeostasis of pigs differ for the genotypes. This hypothesis is supported by the up-regulation of genes related to inflammation and immune response such as *REG3G*, *CCL20* and *TFF3* observed in piglets with the susceptible genotypes in our study. These results are consistent with the findings of Trevisi, ~~Latorre~~ et al. (2017) for ETEC-infected pigs. Particularly, REG3G protein is mainly expressed in the intestinal crypts; it is associated with the host C-type lectins and with antimicrobial activity, and indeed, *REG3G* expression increases in infected pigs (Soler, Miller, Nöbauer, Carpentier, & Niewold, 2015; Trevisi et

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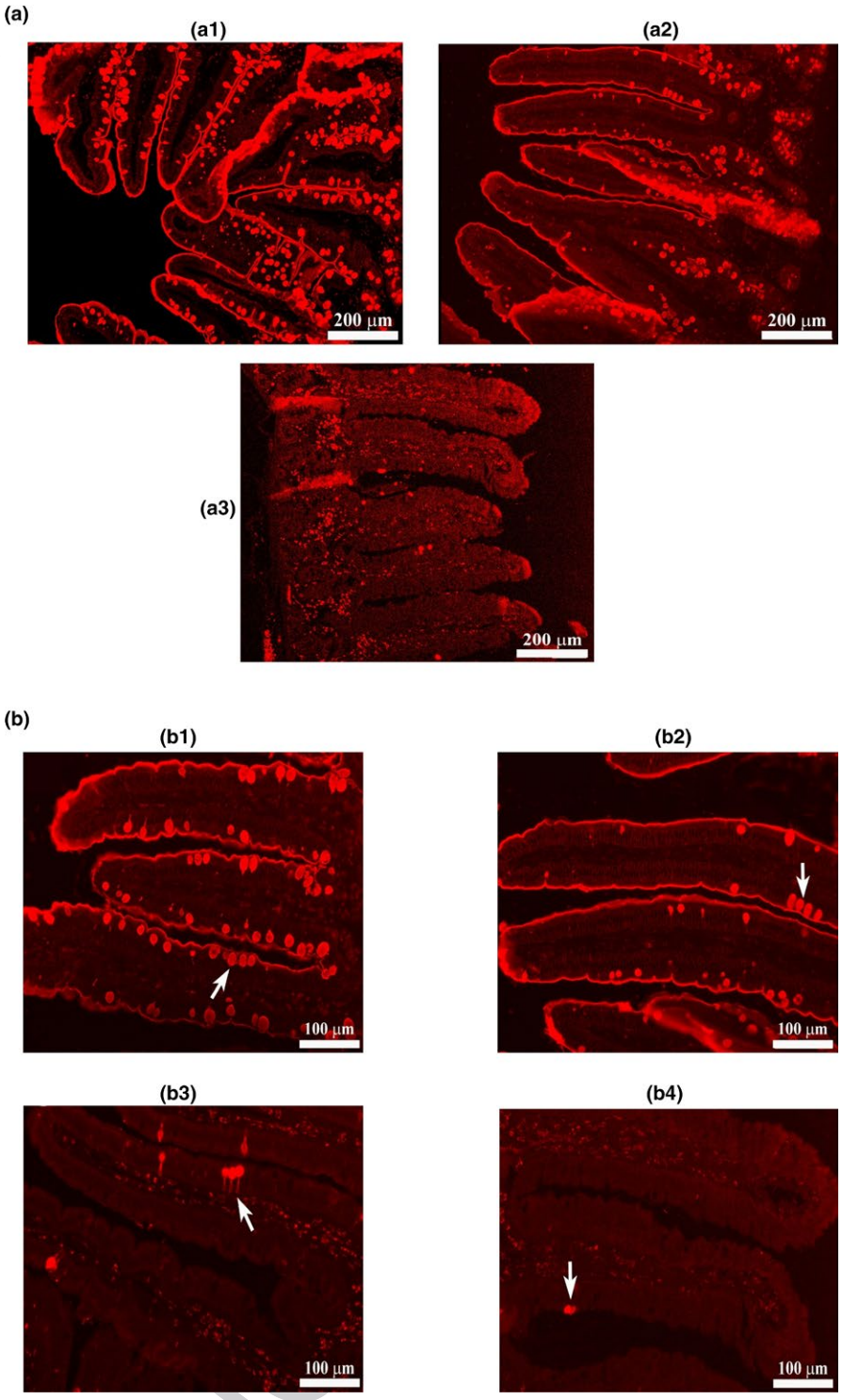

FIGURE 3 Images referred to the immunofluorescence method obtained using ~~*Ulex europaeus*~~ agglutinin I (UEA, fucose-binding) and peanut agglutinin (PNA; galactose-binding) in the pig jejunum sections for brush border (in 3.~~A~~) and for the distribution of immunoreactive (IR) goblet cells in the villi (in 3.~~B~~). The image ~~A.1~~ shows the UEA high immunoreactivity of brush border in correspondence of the mucosal surface with score 2. ~~In the villages of image A.2~~ 16 represents the mucosal surface with score 1 (less intense and thick than previous image ~~A.1~~). The image ~~A.3~~ represents the mucosal surface with score 0 devoid of any immunofluorescence at the brush border. The image ~~B.1~~ (UEA marker) shows the relevant number of IR goblet cells that scored 4: Many IR goblet cells with round and/or pear shape were observed along the axis of the villus. The image ~~B2~~ image shows the mucosal surface of samples scoring 3 (PNA marker): Some isolated or grouped IR goblet cells were observed (arrow). The image ~~B.3~~ shows the mucosal surface of samples scoring 2: The number of immunofluorescent cells ~~(slag)~~ is reduced (UEA marker). Rare IR cells (score 1) are shown in the image ~~B.4~~ (PNA marker) 17

al., 2018). Furthermore, an increase in *REG3G* expression with the phenotype for ETEC intestinal adhesion was seen in healthy pigs fed a low dose of oral tryptophan (17% to lysine), but not with a higher dose (22% to lysine) (Trevisi et al., 2012). In the same way, the increasing of chemokine *CCL20* expression has been linked to bacteria stimulation of pro-inflammatory signals (Skovdahl et al., 2015) while the expression of *CCL20* was reduced by the oral supplementation with antibiotic (colistin) or probiotic (*Saccharomyces cerevisiae* CNCM I-4407) (Trevisi, ~~Latorre~~ et al., 2017). Overall, this suggests that apparently healthy $MUC4^{GG}$ pigs can have a more stimulated immune system than the $MUC4^{CC}$ pigs.

Furthermore, in our study, the *TFF3* expression was higher in the $MUC4^{CG}$ genotype than $MUC4^{CC}$ and $MUC4^{GG}$ genotypes indicating that, although $MUC4^{CG}$ and $MUC4^{GG}$ are generally considered equally susceptible to the ETEC F4ac adhesion and infection (Bijlsma & Bouw, 1987), the response in terms of gut homoeostasis of healthy pigs could be partially different between the two susceptible genotypes. Differences between the two susceptible genotypes are also

TABLE 3 Effect of *MUC4* or *FUT1* polymorphisms 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 pigs on d42

	MUC4[a]							*FUT1*[b]					
	Mean[c]				*p*			Mean[c]				*p*	
Item	CC	CG	GG	*SEM*	CC versus CG	CC versus GG	CG versus GG	AA	AG	GG	*SEM*	AA versus AG	AA versus GG	AG versus 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	<0.001	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	<0.001	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	<0.001	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	<0.001	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	<0.001	0.118

[a]*MUC4*, Mucin 4 gene; genotypes had the following distribution: $MUC4^{CC}$ = 8;$MUC4^{CG}$ = 8; $MUC4^{GG}$ = 8. [b]*FUT1*, 1-fucosyltransferase gene; genotypes had the following distribution: $FUT1^{AA}$ = 7;$FUT1^{AG}$ = 8; $FUT1^{GG}$ = 9. [c]Mean is expressed as mean of assigned score values.

supported by the results reported by Roubos-van den Hil, Litjens, Oudshoorn, Resink, and Smits (2017), which highlighted a different response in terms of ETEC F4ac faecal shedding between $MUC4^{GG}$ and $MUC4^{CG}$ in ETEC F4ac-infected piglets.

Furthermore, the modifications of the blood plasma metabolomics profile in pigs differing for *MUC4* SNP, observed in our study, are relevant to disentangle the relationship between the genetic background of pigs and its physiological functions. Our results indicated that $MUC4^{CG}$ 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 (Vorkas et al., 2015). The $MUC4^{CC}$ genotype displayed a higher plasma serotonin level than the $MUC4^{GG}$ genotype. Serotonin is considered a neurotransmitter and paracrine signalling molecule involved in the brain–gut interactions. In our study, no signal of diarrhoea was reported; thus in this study, plasma serotonin could not be directly ascribed to the intestinal disorder. Furthermore, it is worth to mention that tryptophan is the precursor of serotonin and that a higher dietary amount of tryptophan was required for pigs with an ETEC-susceptible phenotype than not-susceptible pigs to optimize the growth response and to regulate the gene expression (Trevisi et al., 2012). Here, dietary tryptophan was provided at a sufficient level (22% to lysine), but we cannot exclude that tryptophan was limiting for plasma serotonin level in $MUC4^{GG}$ genotype. In the present trial, pig behaviour was not objected of specific observations; however, based on this metabolic observation and on the inverse correlation of hypothalamic serotonin with salivary cortisol (Shen, Voilqué, Kim, Odle, & Kim, 2012) and aggressive behaviour (D'Eath, Ormandy, Lawrence, Sumner, & Meddle, 2005), it will be interesting in the future to follow-up the present study considering the behaviour in growing pigs differing for *MUC4* genotype.

The immunohistochemistry results for *MUC4* are particularly interesting. Based on the literature, we expected an effect of *MUC4* genotypes on PNA staining, since galactose has been implicated in ETEC F4ac adherence (Grange, Erickson, Anderson, & Francis, 1998; Trevisi, Miller et al., 2017), and no effect on fucosylation of the intestinal mucosa. The absence of differences in the scores for PNA in the brush borders and goblet cells of pigs for *MUC4* genotype, found in our study, makes questionable the association of ETEC F4 diarrhoea with a galactose-related receptor for F4ac fimbriae. Indeed, a number of additional putative receptors for ETEC F4ac such as lactosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, globotriaosylceramide, lactotetraosylceramide and lactotetraosylceramide have been described and characterized (Coddens et al., 2011; Grange, Mouricout, Levery, Francis, & Erickson, 2002). However, our data do not exclude the sialoglycoprotein (Grange et al., 1998) or *n*-acetyl-hexosamine nature of the receptor, where galactose may play as an enhancer of the adhesion of ETEC F4 (Grange et al., 2002). Furthermore, we cannot directly exclude the galactose implication in ETEC F4 adhesion since we analysed the intestinal PNA score of pigs under normal healthy conditions, while the galactose amount in the brush border may be affected by the ETEC presence or by

an inflammatory bowel condition (Amador et al., 2008; Peuhkuri, Vapaatalo, & Korpela, 2010).

On the other hand, the reduced degree of fucosylation that we observed for the goblet cells in the villi in ETEC F4ac genetically susceptible pigs could be a possible indirect effect of other transfer enzymes involved in fucosylation, or maybe a consequence of a commensal bacteria stimulation able to modify the degree of mucosal fucosylation (Pickard et al., 2014).

This hypothesis is not completely supported by our results on gut microbiota composition since we did not identify any effect of *MUC4* genotype for that. Our result partially contrast with the previous study by 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, Rodriguez-R, Luo, Tsementzi, & Konstantinidis, 2014); thus, we cannot conclude that gut microbiota is not influenced by *MUC4* genotype and further investigations are required.

On the other hand, we found significant differences in the jejunal microbiota associated with *FUT1* genotypes, both in alpha and beta indices. The animals in $FUT1^{AG}$ group had higher internal microbial variability than the other two genotypes. Furthermore, the sPLS-DA coupled with 10-fold cross-validation revealed differences between *FUT1* variants. Our results indicated that $FUT1^{AA}$ genotype, associated with ETEC F18 resistance, was discriminated by OTUs belonging to the *Lactobacillus* genus. *Lactobacillus* presence in the gut is mostly associated with gut eubiosis, while its reduction has been observed in case of pathogen presences and under stress condition such as weaning (Heo et al., 2013). On the other hand, the $FUT1^{GG}$ genotype (ETEC F18 susceptible) was discriminated by OTUs belonging to the *Veillonella* genus. Bacteria belonging to *Veillonella* genus have been recognized as non-beneficial and associated with Crohn's disease human patients (Gevers et al., 2015). Little information is reported on the *FUT1* effect on intestinal microbiota. However, our results reinforce the observations reported by Poulsen et al. (2018) in which $FUT1^{AG}$ ETEC F18 susceptible piglets showed a higher number of non-beneficial bacteria, including haemolytic bacteria, both in faeces and in digesta, and of Enterobacteriaceae in digesta than $FUT1^{AA}$ piglets, even without the presences of diarrhoea. Thus, our results suggest that *FUT1* genotype is not only associated with ETEC F18 resistance but can also play a role in the modulation of the commensal gut bacteria in healthy pigs. A reason of the potential relevant role of *FUT1* on the microbiota could be ascribed to its functionality in the gut; *FUT1* catalyses the addition of fucose to a terminal galactose in a α1,2-linkage. The mutation on *FUT1* leads to a higher expression of *FUT1* and *FUT2* and an increase in the activity of the enzyme 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 UEA than $FUT1^{AG}$ and $FUT1^{GG}$ and an opposite response for the *PNA* immunoreactivity both in brush border and goblet cell. The *FUT1* effect on brush borders is mainly important because it represents the site where the ETEC F18 adhesion occurs. Our results contribute to explain the reason why the $FUT1^{GG}$ genotype is more susceptible to the ETEC F18 infection. Our findings support that *FUT1* susceptible pigs have a higher active fucose metabolism and display a higher level of fucose in the intestinal mucosa which can be used by the *E. coli* to increase its colonization. In addition, we observed a reverse pattern for UEA and PNA immunoreactivity between the F18-susceptible ~~or not pigs~~. This result can be explained by the fact that in the F18-resistant genotype, the genetically determined impaired activity of the α(1,2)-fucosyltransferase could have 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, resulting in an increase in galactose.

The role of *FUT1* in the glycosylation in the gut and on the modulation of gut microbiota could also explain the variation in the plasma metabolic profile that we observed in our study. Previous observation showed the influence of *FUT1* genotypes in modulating the plasma metabolome and some specific plasma metabolites related with gut microbial metabolism (hippuric acid, oxindole, betaine) and inflammation (guanosine) (Poulsen et al., 2018). Since different techniques were applied in our and Poulsen et al. (2018) studies, and no univocal metabolites were detected, results cannot directly be compared. Here, we detected a higher amount of several phosphatidylcholines in the $FUT1^{GG}$ genotype than in the $FUT1^{AA}$ genotype. Phosphatidylcholines are indicative compounds related to inflammation and disturbed homoeostasis for their function in increasing processes of cells proliferative growth and programmed cell death (Vorkas et al., 2015). Thus, we hypothesize that gut microbiota, particularly the not beneficial genera, identified as discriminating in the $FUT1^{GG}$ animals, could have altered the gut eubiosis and caused stimulation of the immune system, which resulted in an alteration of the plasma metabolic profile. Furthermore, our results on gut homoeostasis in late growing phase may contribute to explain the reduced growth performance and the softer faecal score observed in the susceptible piglets during the first 3 weeks post-weaning.

In conclusion, our results improved the knowledge regarding the interplay between host genetics, gut microbiota and host physiological status. Our study indicates that *MUC4* and *FUT1* genotypes are not only related respectively to ETEC F4 and F18 susceptibility but can also affect the gut homoeostasis in healthy piglets in terms of fucose and galactose glycomic profiles, expression of genes related to inflammation in jejunal mucosa (*MUC4*) and jejunal microbiota (*FUT1*), and in plasma metabolic profile and growth performance. These observations could be mainly important for studies aimed to study the interplay between host and microbiota in which the genotyping of *MUC4* and *FUT1* genes may be used to stratify the output of studies and may contribute to a deeper understanding of host-microbial crosstalk. In addition, the early piglets' genotyping for *MUC4* and *FUT1* could improve the experimental protocol reliability of studies with ETEC challenge model by decreasing the number of animals needed and improving the ethicality of in vivo studies.

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CONFLICT OF INTEREST

The authors declare that they have no competing interests.

ETHICAL APPROVAL

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.

SOFTWARE AND DATA REPOSITORY RESOURCES

The data sets on microbiota data supporting the conclusions of this article are available in the Sequence Read Archive (SRA) repository with number SRP128419.

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SUPPORTING INFORMATION

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