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Host genotype and amoxicillin administration affect the incidence of diarrhoea and faecal microbiota of weaned piglets during a natural multiresistant ETEC infection

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Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the aetiological agent of postweaning diarrhoea (PWD) in piglets. The SNPs located on the *Mucine 4* (*MUC4*) and *Fucosyltransferase 1* (*FUT1*) genes have been associated with the susceptibility to ETEC F4 and ETEC F18, respectively. The interplay between the *MUC4* and *FUT1* genotypes to ETEC infection and the use of amoxicillin in modifying the intestinal microbiota during a natural infection by multiresistant ETEC strains have never been investigated. The aim of this study was to evaluate the effects of the *MUC4* and *FUT1* genotypes and the administration of amoxicillin through different routes on the presence of diarrhoea and the faecal microbiota composition in piglets naturally infected with ETEC. Seventy-one piglets were divided into three groups: two groups differing by amoxicillin administration routes—parenteral (P) or oral (O) and a control group without antibiotics (C). Faecal scores, body weight, presence of ETEC F4 and F18 were investigated 4 days after the arrival in the facility (T0), at the end of the amoxicillin administration (T1) and after the withdrawal period (T2). The faecal bacteria composition was assessed by sequencing the 16S rRNA gene. We described that *MUC4* and *FUT1* genotypes were associated with the presence of ETEC F4 and ETEC F18. The faecal microbiota was influenced by the *MUC4* genotypes at T0. We found the oral administration to be associated with the presence of diarrhoea at T1 and T2. Furthermore, the exposure to amoxicillin resulted in significant alterations of the faecal microbiota. Overall, *MUC4* and *FUT1* were confirmed as genetic markers for the susceptibility to ETEC infections in pigs. Moreover, our data highlight that group amoxicillin treatment may produce adverse outcomes on pig health in course of multiresistant ETEC infection. Therefore, alternative control measures able to maintain a healthy faecal microbiota in weaners are recommended.

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KEYWORDS

Antibiotic resistance, *Escherichia coli*, *FUT1*, gut microbiota, *MUC4*, swine

1 | INTRODUCTION

Weaning is considered the main critical period for pigs raised in intensive farms (Lallés, Bosi, Smidt, & Stokes, 2007). This phase may be associated with the onset of gastrointestinal disorders with postweaning diarrhoea (PWD), caused by Enterotoxigenic *Escherichia coli* (ETEC) that play a major role (Baker, Billey, & Francis, 1997; Luppi, 2017). PWD leads to pig morbidity and mortality causing considerable economic losses to farmers worldwide (Fairbrother & Gyles, 2012). The ETEC strains possess fimbrial adhesins, identified as F4 or F18, that mediate microbial attachment to the intestinal epithelium (Luppi, 2017). These fimbriae allow ETEC to adhere to specific receptors on the brush border membrane of the small intestine enterocytes (Fairbrother & Gyles, 2012). Besides adhesion, ETEC strains secrete enterotoxins able to impair enterocyte functions by increasing cell cation exchanges and reducing water absorption (Sun & Woo, 2017), finally resulting in a severe diarrhoea.

Piglets are not equally susceptible to ETEC infection. Susceptibility to ETEC F4 has been associated with a single nucleotide polymorphism (SNP) located in intron 7 (g.13:8227C>G) of the *Mucin 4* gene (*MUC4*) (Jørgensen et al., 2004; Luise et al., 2019; Rampoldi et al., 2011). Piglets with *MUC4*$^{G-}$ genotypes express the F4 receptor and are considered susceptible to ETEC F4 infection, while piglets with *MUC4*^{CC} genotype are associated with the resistant phenotype (Jorgensen et al., 2003). On the other hand, susceptibility to the ETEC F18 infection appears to be dependent on the activity of the alpha-fucosyltransferase-1 (*FUT1*) gene, which is the candidate gene for the adhesion to F18 receptor. The g.6:54079560T>C SNP located on *FUT1* gene has been associated with the susceptibility to ETEC F18 infection; piglets with *FUT1*$^{C-}$ genotypes appear susceptible to ETEC F18, while piglets with *FUT1*^{TT} genotype are resistant to the infection (Meijerink et al., 1997; Muñoz et al., 2018; Vogeli et al., 1997; Wang et al., 2012).

At weaning, the gut microbiota of piglets is characterized by severe compositional changes (Mach et al., 2015), which might impair the barrier effect exerted by symbiotic bacteria towards enteric pathogens (Konstantinov et al., 2006). Notably, the abrupt decrease in *Lactobacillus* spp. at weaning could increase the risk of enteritis, since bacteria belonging to this genus play a major role in disease prevention (Konstantinov et al., 2006). Moreover, the gut microbiota composition of piglets at weaning is also influenced by the host genetic background and by ETEC F4 and ETEC F18 infections (Bin et al., 2018; Messori, Trevisi, Simongiovanni, Priori, & Bosi, 2013; Poulsen et al., 2018). Finally, the administration of antibiotics, which is often recorded in this production phase, impacts the microorganism abundance and may cause a severe disruption of the gut microbiota ecosystem (Blaser, 2016; Mulder et al., 2009; Schokker et al., 2014; Soler et al., 2018; Zhang et al., 2016).

In European farms, amoxicillin is the main antimicrobial molecule used at weaning, mainly to control ETEC and *Streptococcus suis* infections (Burch & Sperling, 2018). This antibiotic is currently used for therapeutic or metaphylactic purposes, and it can be administered either by the parenteral or oral route, for animal group treatment. However, concerns have been expressed for the use of oral formulations, since they exert a selective pressure on the gut microbiota (Kim, Covington, & Pamer, 2018; Stanisavljevi et al., 2019; Zhang, Huang, Zhou, Buckley, & Wang, 2013). Consequently, antibiotic-resistant bacteria or resistance determinants may increase in the gut microbiota, making it a potential *reservoir* of antibiotic resistance. Strikingly, the oral administration of amoxicillin has been associated with an increase in extended-spectrum beta-lactamase (ESBL) *E. coli* in pigs (Cameron-Veas, Solà-Ginés, Moreno, Fraile, & Migura-Garcia, 2015). Of greater concern is the spread of multidrug-resistant ETEC strains in European pig herds (Magistrali et al., 2018; Rosager et al., 2017; Smith et al., 2010). In this scenario, a full understanding of the impact of group antimicrobial treatments on gut health in field conditions is long overdue.

The interplay between the resistance/susceptibility genotypes to ETEC infection and the use of amoxicillin in modifying the intestinal microbiota during a natural outbreak of PWD has never been investigated.

The hypothesis of this study was that the host genotypes for *MUC4* and *FUT1* and the route of administration of amoxicillin could affect the development of PWD and the faecal microbiota composition in weaning piglets naturally infected by ETEC.

2 | MATERIALS AND METHODS

2.1 | Animal experimental design

Animals were allocated at the animal experimental facility of the Istituto Zooprofilattico Sperimentale dell' Umbria e delle Marche "Togo Rosati" (Perugia, Italy) and were left to acclimatize 4 days before the onset of the experiment. The experiment was authorized by the Italian Ministry of Health

(Authorization no 68/2018-PR of 31-01-2018), according to the Italian and European regulations (Directive 2010/63/EU, D.L. 26/2014), and was carried out under the supervision of certified veterinarians.

Seventy-two animals were purchased from an Italian herd, positive for ETEC infection, and neither piglets nor sows were vaccinated against ETEC and piglets never received antibiotic before entering in the experimental facilities. One piglet was removed from the study, because the animal died within the first week of the experiment. A diagnosis of colibacillosis was made based on lesions and the isolation of ETEC F4 from the gut, according to Luppi (2017).

Seventy-one piglets (35 females and 36 males) were divided into three groups (P, O and C) balanced for litter of origin, sex, age at weaning and weight (Figure S1).

Group P (23 piglets) received parenteral administration of amoxicillin (Longocilline L.A.; CEVA), group O (24 piglets) was administrated with oral amoxicillin (Amoxione; Vetoquinol), and group C (24 piglets) received a placebo made with water and was considered the control group. Each pig of group P received the antibiotic *via* intramuscular injection with the recommended dosage of 15 mg/kg bodyweight two administrations at 48-hr interval. The group O received 12–20 mg/kg bodyweight of the suspension orally twice a day, approximately 7:00 a.m. and 7:00 p.m. for 5 days. Animals were fed with a starter diet from the day of the arrival (d0) until the end of the experiment (d16). The composition of the diet is shown in Table S1.

Animals arrived in the facility the day of weaning (d31, $N = 36$ and d38, $N = 35$). Animals were evaluated 4 days after their arrival (T0), following a 4-day period for acclimatization, at the end of the amoxicillin administration (T1) and again 7 days corresponding to the withdrawal period of the antibiotic (T2).

Individual faecal samples were collected, and faecal consistency scores were individually evaluated at each time point. Faecal scores were categorized after visual observation of the certified veterinarian supervising the experiment as follows: 0 = normal stools; 1 = loose stools; 2 = watery diarrhoea. The individual body weight was also recorded at each time point.

2.2 | Microbiological culture, antimicrobial susceptibility testing

To evaluate the susceptibility profiles to antibiotics of the ETEC strains, standard bacteriological tests at each time point were performed.

Briefly, the primary isolation from individual faecal samples was carried out on blood agar plates (Blood Agar Base, Biolife Italiana Srl), supplemented with 5% sheep red blood cells. Plates were incubated at 37°C overnight. Haemolytic *E. coli* isolates were identified using standard biochemical procedures (RapidAPI32E, bioMérieux Italia Spa), followed by species-specific PCR as described in the following section "ETEC PCR for adhesin detection". The isolates resulting positive for the fimbriae factors F4 and F18 were tested for antimicrobial susceptibility using the agar diffusion method on Muller Hinton Agar (Oxoid Ltd), according to the EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing, 2017a, 2017b). *E. coli* ATCC 25922 was used as control strain. The following antimicrobial discs (Oxoid Ltd) were tested: ampicillin (10 μg), amoxicillin/clavulanic acid (30 μg), cefotaxime (30 μg), cephazolin (30 μg), chloramphenicol (30 μg), ciprofloxacin (5 μg), gentamicin (10 μg), kanamycin (30 μg), nalidixic acid (30 μg), streptomycin (10 μg), sulphonamides (300 μg), tetracycline (30 μg) and sulphamethoxazole/ trimethoprim (25 μg). The interpretation of inhibition diameters was carried out following the EUCAST breakpoint tables (The European Committee on Antimicrobial Susceptibility Testing, 2017a, 2017b) with the exception of cefazolin, enrofloxacin, kanamycin, nalidixic acid, sulphonamides, tetracycline and sulphamethoxazole/ trimethoprim for which CLSI M100 breakpoints were used (CLSI, 2018). Intermediate results were classified as resistant.

2.3 | Blood sample collection and DNA analysis from blood samples

Blood samples were collected by venepuncture of *v. jugularis* on all piglets at T0. Genomic DNA was extracted from blood samples following the procedure of the kit NucleoSpin Blood (Macherey Nagel-740951.250). The Nanodrop instrument was used to assess the quality and quantity of the extracted DNA.

Genotyping of the g.13:8227C>G SNP located on the *MUC4* gene and the g.6:54079560T>C SNP located on *FUT1* gene was carried by using the PACE™ Genotyping approach (https://3crbio.com/wp-content/uploads/2019/01/PACE-IR-User-Guide-v1.5.pdf).

To assess the genotype of the *MUC4* gene, the following primers were used: 5′-GAAGGTGACCAAGTTCATGCTATTTGTACCTCAGTTTCTGTATCTG-3′ for the allele C (allele 1), 5′-GAAGGTCGGAGTCAACGGATTCTATTTGTACCTCAGTTTCTGTATCTC-3′ for the allele G (allele 2) and the common primer 5′-ACAACAACCCCATGAAGGAGATCTATTTT-3′. Regarding the *FUT1* gene, the following primers were used: 5′-GAAGGTGACCAAGTTCATGCTGCGGCCGTTGAGCTGCGC-3′ for the allele C (allele 1), 5′-GAAGGTCGGAGTCAACGGATTGCGGCCGTTGAGCTGCGT-3′ for the allele T (allele 2) and the common primer 5′-GATGGCCGGTTTGGGAACCAGAT-3′ were used in the genotyping assay. After thermal cycling was complete, the

fluorescent signal was detected by reading the plate in the QuantStudio 12k Flex instrument (Applied BioSystems).

2.4 | Faecal sample collection and DNA analysis from faecal samples

Faecal samples were collected from the piglet rectum at three different time points: at T0, at T1 and at T2. All faecal samples were directly frozen in liquid nitrogen and further stored at −80°C until use. Genomic DNA of each faecal sample was extracted the Qiagen QIAamp DNA stool kit, following the modified protocol of Dore et al. (2015).

The DNA extracted from faecal samples was analysed by PCR endpoint in order to assess the presence/absence of the genes encoding adhesins F4 and F18 (Casey & Bosworth, 2009).

Microbial profiling was performed using high-throughput sequencing of the V3–V4 hypervariable region of the 16S rRNA gene (2 × 250 bp paired-end reads) on an Illumina MiSeq platform following the standard Illumina sequencing protocol and by using primers PCR1F_343 (5′-CTTTCCCTACACGACGCTCT TCCGATCTACGGRAGGCAGCAG-3′) and PCR1R_784 (5′-GGAGTTCAGACGTGTGCTCTTCC GATCTTACCAGGGTATCTAATCCT-3′). The generated FastQ files were first quality checked through the FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and then analysed using the Quantitative Insights Into Microbial Ecology (QIIME) v1.9.1 package (Caporaso et al., 2010) by following the open-reference subsampled OTU calling strategy (Rideout et al., 2014). Singleton Operational Taxonomical Units (OTUs) and OTUs with a number of sequences less than 0.005% of the total number of sequences were removed from the dataset (Bokulich et al., 2012). Chimeric sequences were removed using QIIME and by using the BLAST algorithm. All samples with less than 10,000 postquality control reads were removed from the analysis, which resulted in eliminating only one sample (pig number 622 sampled at T2).

2.5 | Biostatistical analysis

Basic statistics for the analysis of pig weight were estimated in R v.3.6.0 (TeamCore, 2018) by performing ANOVA analyses with the "aov" function. The Fisher test was used to correlate the *MUC4* and *FUT1* genotypes with the excretion of ETEC F4 and ETEC F18 and the faecal scores. Moreover, the Fisher test was carried out to evaluate the links between the presence of ETEC F4 and ETEC F18 with the faecal scores. In our analyses, the faecal categories 0 and 1 were considered as "negative" and the score 2 as "positive" for the presence of diarrhoea. Regarding the *MUC4* and *FUT1* genes, we have considered as "resistant" the animals $MUC4^{CC}$ and $FUT1^{TT}$ and "susceptible" the animals harbouring $MUC4^{CG}$, $MUC4^{GG}$, $FUT1^{CT}$ and $FUT1^{CC}$ genotypes. Differences among the pig weight and the sex, age, litter of origin, administration routes, *MUC4* and *FUT1* genotypes, susceptibility to ETEC F4 and ETEC F18, and presence/absence of diarrhoea were assessed using ANOVA test, and if showing a significant *p*-value, we performed a post hoc test using the Tukey's honest significant differences (HSD) test.

For the analysis of microbiota composition, the biom OTU table was imported into R with Phyloseq package (v.1.28.0) (McMurdie & Holmes, 2013). Vegan v2.5-5 package (Oksanen et al., 2019) was used for the rarefaction on the OTU level of each experimental group. Richness and diversity analyses were performed at the OTU level. Alpha diversity was calculated with Shannon index, beta diversity through the Whittaker's index, and richness was evaluated as the total number of OTUs present in each sample. To assess the diversities, the ANOVA was performed on α and β diversity and on log10 richness using the "aov" procedure in R. The Tukey's HSD was also calculated. Vegan's Non-Metric Multidimensional Scaling (NMDS), using the Bray–Curtis distance and with the "metaMDS" function that standardizes the scaling in the result, was used to represent the global diversity of faecal microbiota composition between samples. The function "envfit" in Vegan was used to fit environmental factors onto the NMDS ordination to compare the groups and evaluate the statistical significance. The permutational multivariate analysis of variance (PERMANOVA) using the Bray–Curtis distance was performed using the "adonis" function in order to assess the community differences between groups. The significance threshold was set at $p < .05$.

The differential abundance analysis was performed using the function "fitZig" in the metagenomeSeq (v.1.26.0) package at the OTU level (Paulson, Stine, Bravo, & Pop, 2013). The *MUC4* genotype and the age at T0, the antimicrobial treatment at T1, the faecal score (categories: 0, 1, 2), and the antimicrobial treatment at T2 were taken into account in the model as co-factors. In order to make a pairwise comparison of differentially abundant OTUs between the experimental groups (C vs. P, C vs. O, P vs. O), we performed the differential abundance analysis at T1 and at T2, using "fitZig" function. The resulting differentially abundant (DA) OTUs have been plotted in Venn diagrams using Venny 2.1 (Oliveros, 2013).

3 | RESULTS

3.1 | Microbiological culture and antimicrobial susceptibility testing

Results showed that the piglet groups were naturally infected by ETEC F4 ($N = 50$) and F18 ($N = 20$) at T0, while only F18 ($N = 61$) was still detected at T1. Few animals were positive for ETEC F4 ($N = 3$) and F18 ($N = 8$) at T2 (Figure S2; Table S2). In particular, at T0 43 animals tested positive for ETEC F4 and negative for ETEC F18, while

seven piglets were positive for both; eight animals were negative for both ETEC F4 and F18 and 13 animals were negative for ETEC F4 and positive for ETEC 18. Regarding the susceptibility testing, both the ETEC F4 and ETEC F18 isolates were classified as multiresistant, showing resistance to beta-lactams (ampicillin and amoxicillin/clavulanic acid), phenicols (chloramphenicol), quinolones (ciprofloxacin and nalidixic acid), sulphonamides (sulphonamides and sulphamethoxazole/trimethoprim) and tetracycline. The ETEC F4 isolates, differently from the ETEC F18 ones, were also resistant to streptomycin. Both ETEC F4 and ETEC F18 showed susceptibility to cephalosporins (cefazolin and cefotaxime), gentamicin and kanamycin.

3.2 | Animal genotypes for MUC4 and FUT1

For *MUC4*, 19 pigs had *MUC4*^{CC} resistant genotype for ETEC F4 and 52 had the susceptible genotype for ETEC F4 (36 *MUC4*^{CG} and 16 *MUC4*^{GG}). As regards to *FUT1*, 13 *FUT1*^{TT} for ETEC F18-resistant pigs and 58 for ETEC F18-susceptible pigs (25 *FUT1*^{CC} and 33 *FUT1*^{CT}) were observed (Figure S2). Overall, 52 and 58 pigs had a genotype susceptible to ETEC F4 and F18, respectively.

Forty-one pigs were susceptible to both ETECs (six were *MUC4*^{GG}, *FUT1*^{CC}; six were *MUC4*^{GG}, *FUT1*^{CT}; nine were *MUC4*^{CG}, *FUT1*^{CC} and 20 were *MUC4*^{CG}, *FUT1*^{CT}). Nine pigs were susceptible for ETEC F4 while being resistant for F18 (two had *MUC4*^{GG}, *FUT1*^{TT} and seven had *MUC4*^{CG}, *FUT1*^{TT}). In addition, 17 pigs were resistant for ETEC F4 and susceptible for ETEC F18 (seven pigs were *MUC4*^{CC}, *FUT1*^{CT} and 10 pigs were *MUC4*^{CC}, *FUT1*^{CC}). Two pigs were resistant to both ETECs, showing the variants *MUC4*^{CC} and *FUT1*^{TT}. The composition of the experimental groups according to the pigs' genotypes is reported in Table S3.

3.3 | Animal phenotypes and correlation with genotypes

All phenotypic traits are summarized in Table S2.

ANOVA tests on the individual body weights did not show significant differences among the groups at any of the three time points ($p > .05$). Moreover, the sex of the animals and the presence/absence of diarrhoea did not affect the weight of the animals ($p > .05$). Using the ANOVA analysis, the weight was different between the two ages of the piglets at the three time points (T0, $p = .003$; T1, $p = .0005$; T2, $p = .0004$) and consequently by litter of origin (T0, $p = .002$; T1, $p = .0001$; T2, $p = .0003$). The younger piglets (d31) weighted less than the older piglets (d38) at weaning; however, animals were balanced in all the three groups. At T0, *MUC4* and *FUT1* genotypes, presence of ETEC F4 and ETEC F18 did not affect the weight of animals.

At T1, ANOVA showed differences in the piglets body weight according to the *FUT1* gene (ANOVA, $p = .01$). The post hoc test showed differences between *FUT1*^{CC} and *FUT1*^{CT} genotypes (Tukey's HSD, $p = .01$), but did not show differences between the comparison of *FUT1*^{CC} versus *FUT1*^{TT} and between *FUT1*^{CT} versus *FUT1*^{TT} (Tukey's HSD, $p > .05$). *MUC4* genotypes and the presence of ETEC F18 did not affect the weight of animals ($p > .05$).

Moreover, at T2 we described that the weight was influenced by the *FUT1* gene (ANOVA, $p = .02$), which were referred to *FUT1*^{CC} and *FUT1*^{CT} (Tukey's HSD, $p = .04$) and not to *FUT1*^{CC} versus *FUT1*^{TT} or *FUT1*^{CT} versus *FUT1*^{TT} (Tukey's HSD, $p > .05$). *MUC4* genotypes and the presence of ETEC F4 and ETEC F18 did not affect the weight of animals ($p > .05$).

The faecal scores were recorded, and the results at each time points are reported in Figure S2. At T0, we described 43, 11 and 17 animals with 0, 1 and 2 categories of faecal score, respectively; at T1, we observed a higher number of animals with diarrhoea (faecal score 2; $N = 25$) than without diarrhoea (faecal score 0, $N = 17$; faecal score 1, $N = 29$). At T2, the faecal consistencies of piglets fell in categories 0 ($N = 34$) and 1 ($N = 27$), with only 10 animals presenting diarrhoea.

At T0, Fisher tests showed that susceptible *MUC4* genotypes were significantly associated with the presence of ETEC F4 ($p = .003$) and the occurrence of diarrhoea (categories 0, 1 = negative for diarrhoea; category 2 = positive for diarrhoea) ($p = .01$). However, the *MUC4* resistant genotype was associated with an ETEC F4-negative status but also with a higher diarrhoea score. In this case, 9/19 animals with a *MUC4* resistant genotype and 8/52 animals with a *MUC4* susceptible genotype showed diarrhoea (Figure S2, Table 1). At T1, no ETEC F4 was detected. We found that *FUT1* genotypes were significantly associated with the presence of ETEC F18 ($p = .01$) but not with the faecal scores ($p > .05$) at T1; however, the cases of diarrhoea were more frequent in susceptible *FUT1* animals than in the resistant *FUT1* piglets. At T2, we did not describe any effect taking into account the *MUC4* and *FUT1* genotypes associated with either the ETEC F4 and ETEC F18 infections or the faecal scores (Figure S2, Table 1). No association was found between the faecal score and the presence of ETEC F4 or F18 ($p > .05$) at each time point.

3.4 | Correlation between the antibiotic administration routes and the ETEC status

Antibiotic administration did not influence the ETEC F4 status of the animals at the three time points ($p > .05$). Conversely, antibiotic administration showed a significant association with the status of ETEC F18 at T1 ($p = .017$), with the group P having less ETEC F18-positive pigs ($N = 17$) than the other two groups (Group O, $N = 24$ and Group C, $N = 20$). At T2, a difference in the number of ETEC F18-positive pigs was observed

TABLE 1 Distribution of animal status for the presence of diarrhoea according to the *MUC4* and *FUT1* genotypes at T0, T1 and T2. Statistical differences calculated using the Fisher exact test in the different comparisons and the *p*-values are reported

Time point	Gene	Susceptibility (S) Resistance (R)	Individual diarrhoea status		Fisher test (p-value)
			Negative	Positive	
T0	*MUC4*	S	44	8	**.01**
		R	10	9	
	FUT1	S	45	13	*.49*
		R	9	4	
T1	*MUC4*	S	34	18	*1*
		R	12	7	
	FUT1	S	38	20	*.94*
		R	8	5	
T2	*MUC4*	S	45	7	*1*
		R	16	3	
	FUT1	S	49	9	*.76*
		R	12	1	

Significative value is in bold and the non-significative values in italics.

TABLE 2 Distribution of animals status for the presence of diarrhoea according to the experimental groups (C = control, P = parenteral administrated, O = oral administrated) at T0, T1 and T2. Statistical differences calculated using the Fisher exact test in the different comparisons, and the p-values are reported

Time point	Group	Presence of diarrhoea		Fisher test (*p*-value)
		Negative	Positive	
T0	P	17	6	.61
	O	17	7	
	C	20	4	
T1	P	11	10	**.009**
	O	14	10	
	C	21	3	
T2	P	19	4	**.02**
	O	18	6	
	C	24	0	

The significative values are in bold.

in the three groups ($p = .004$): seven animals were ETEC F18 positive in the group treated orally, while only one ETEC F18-positive piglet was found in the group C and none in the group P. Moreover, the antibiotic treatments were associated with the faecal score at T1 ($p = .009$) and at T2 ($p = .02$), with more animals showing diarrhoea in the group O compared to the other two groups (Figure S2, Table 2).

3.5 | Faecal microbiota sequencing, identification and annotation of OTUs

After quality control, a mean of 36706 reads was available for each sample. OTU counts per sample and OTU taxonomical assignments are available in Table S4. Sequences across the whole sample sets were successfully clustered into 1080 OTUs, and only (10/1,080) 0.92% of the OTUs could not be assigned to any phylum. Globally, 553 out of 1080 OTUs were annotated at the genus level (51%). The *Firmicutes* (584/1080) and *Bacteroidetes* (391/1080) phyla represented 54% and 36% of the annotated OTUs, respectively. The 97% (567/584) OTUs belonging to the *Firmicutes* phylum were assigned to the *Clostridiales* order, 48% (254/567) to the Ruminococcaceae family and 27% (152/567) to the *Lachnospiraceae* family. The 54% (209/391) OTUs annotated to the *Bacteroidetes* phylum were assigned to the *Prevotella* genus. Other phyla were also present but with lower percentages of OTUs (e.g., 5% Proteobacteria, 2% Spirochaetes, 0.5% Actinobacteria, 0.3% Fusobacteria, 0.3 Fibrobacteres, 0.3% Actinobacteria, 0.2% Deferribacteres, 0.04% Tenericutes; Figure S3). The effect of the time resulted to be significant between time points, showing clusters in the NMDS plot (envfit test, $p = .004$; Figure S4).

3.6 | Differences in the faecal microbiota at T0 in piglets

The overall composition of the microbiota at T0 (NMDS, Figure 1) was mainly driven by *MUC4* gene (Adonis test, $p = .004$), the age of the piglets (Adonis test, $p = .001$) and the faecal score (Adonis test, $p = .001$), whereas *FUT1* genotype and the presence of ETEC F4 and ETEC F18 had no influence (Adonis test, $p > .05$). The beta diversity was different only between the class of ages of the piglets (ANOVA test, $p = .001$; Figure S5B) showing that the group weaned at 38 days of age had a lower beta diversity, comparing to the animals of 31 days of age, but animals were equally

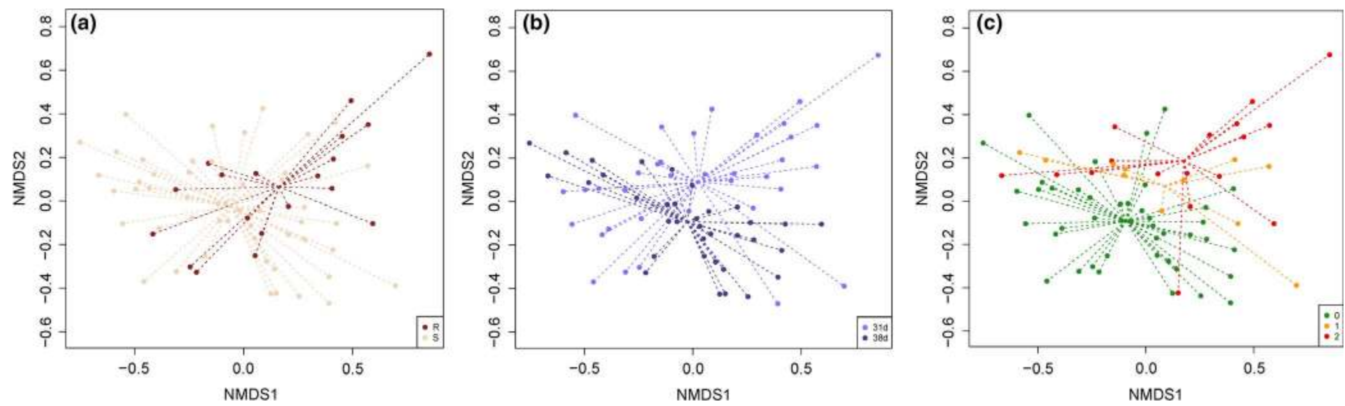

FIGURE 1 Plots include only the samples obtained from T0. Dissimilarities in gut microbiota composition represented by the non-metric multidimensional scaling (NMDS) ordination plot, with Bray–Curtis dissimilarity index calculated on unscaled OTU abundances. The centroids of each group are features as the group name on the graph ('envfit'; Vegan R package). Samples are coloured by *MUC4* gene (a): resistant (R, red) and susceptible (S, pink) genotypes; by age (b): 31 days old (31d, light blue) and 38 days old (38d, blue) and by faecal score (c): category 0 (green), 1 (orange) and 2 (red)

distributed in groups P, C and O (Figure S1B). In the NMDS plot, the *MUC4* genotypes (envfit test, $p = .018$; Figure 1a), the age of piglets (envfit test, $p = .039$; Figure 1b) and the faecal score (envfit test, $p = .0004$; Figure 1c) showed significant values for the envfit analysis. The alpha diversity at OTU level was not different between the groups taking into account the *MUC4* gene and the faecal score (ANOVA test, $p > .05$; Figure S5A,C), but the co-factor age of the piglets revealed differences (ANOVA test, $p = .002$; Figure S5B), showing the 38-day-old piglets had a higher alpha diversity. Moreover, the same finding was described in the observed microbial richness between the groups when analysing the *MUC4* gene and the faecal score effect (ANOVA test, $p > .05$; Figure S5A,C) and the age of piglets (ANOVA test, $p = .001$; Figure S5B).

Since the presence of diarrhoea was correlated with the *MUC4* gene, the *MUC4* genotype and the age at T0 were used in the model of the differential analysis at the OTUs level, describing 68 DA OTUs (Table S5; Figure S6A). Globally, OTU belonging to *Oscillospira* genera and the *Actinobacillus porcinus* were more abundant in the resistant *MUC4* genotype. Moreover, the same differential analysis was carried out taking into account in the model only the diarrhoea phenotype (faecal scores 0 and 1 = negative; faecal score 2 = positive)

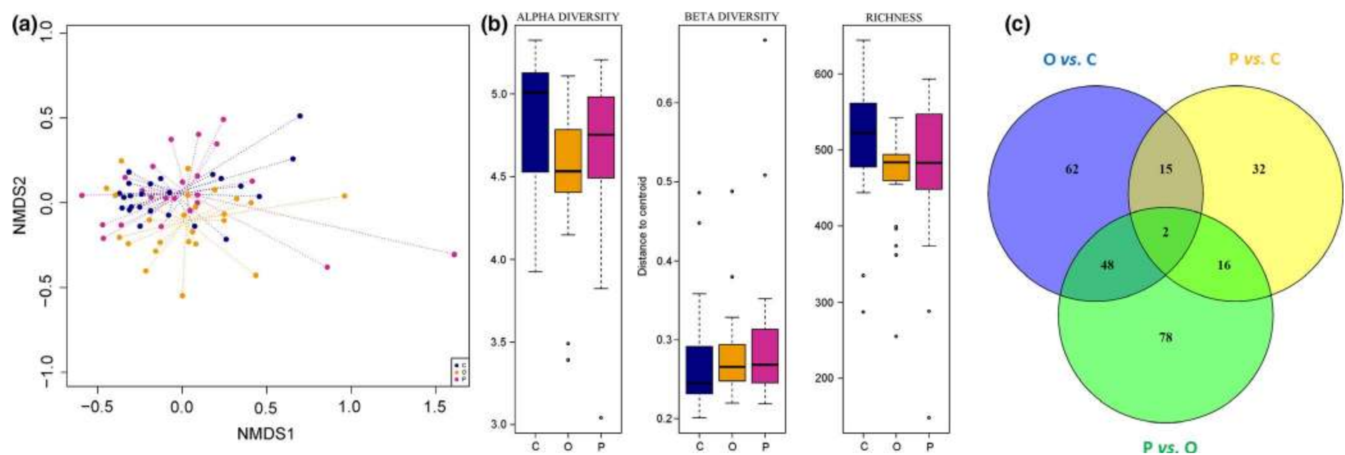

FIGURE 2 Plots include only the samples obtained from T1. (a) Dissimilarities in gut microbiota composition represented by the non-metric multidimensional scaling (NMDS) ordination plot, with Bray–Curtis dissimilarity index calculated on unscaled OTU abundances. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral administered (O, orange) and amoxicillin parenteral administered (P, purple). (b) Box plot graph representation of the alpha diversity (Shannon index), beta diversity (Whittaker's index) and richness (total number of OTUs present in each sample) using the rarefied OTU table for each group and time point. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral administered (O, orange) and amoxicillin parenteral administered (P, purple). (c) Venn diagram representing the overlaps of differentially abundant OTUs more abundant belonging to the comparison of two experimental groups (C vs. P, C vs. O, P vs. O) ('fitZig'; MetagenomeSeq R package). Group is coloured by comparisons: control versus amoxicillin parenteral administered (C vs. P, yellow), control versus amoxicillin oral administered (C vs. O, blue) and amoxicillin oral administered versus amoxicillin parenteral administered (O vs. P, green)

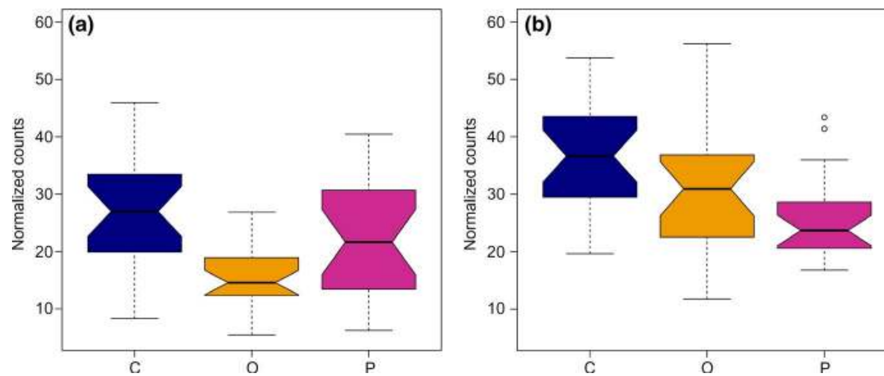

FIGURE 3 Abundances of *Lactobacillus* spp. at T1 (a) and T2 (b) among the experimental groups. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral administered (O, orange) and amoxicillin parenteral administered (P, purple). Abundances were calculated as the addition of normalized for OTUs annotated as *Lactobacillus* spp. in the whole dataset (MetagenomeSeq R package). The notched boxplots display the confidence interval around the median. If two box notches do not overlap, there is "strong evidence" (95% confidence) their medians differ, and consequently, the difference is described as "statistically significant at the 0.05 level"

and we identified 153 DA OTUs (Table S6; Figure S6B). Among them, 71 DA OTUs were more abundant in animals without diarrhoea and 82 OTUs were overabundant in piglets with diarrhoea. OTUs more abundant in pigs without diarrhoea belonged mainly to Ruminococcaceae and Christensenellaceae families. *Bacteroides*, *Parabacteroides, Fusobacterium* genera and Pasteurellaceae family were predominant among the OTUs more abundant in the diarrhoeal animals.

3.7 | Differences in the faecal microbiota at T1 in piglets

The overall composition of the microbiota at T1 (NMDS, Figure 2a) was mainly driven by the antibiotic treatment (Adonis test, $p = .0009$), whereas *MUC4* and *FUT1* genotypes, ages, faecal score and the status of ETEC F4 and ETEC F18 had no influence (Adonis test, $p > .05$). The beta diversity was not different between the antimicrobial treatment groups (ANOVA test, $p > .05$; Figure 2b). In the NMDS plot, the centroids of the group O appeared separated from the other two groups, resulting in a significant value (envfit test, $p = .02$; Figure 2a). The alpha diversity at OTU level was different between the antimicrobial groups (ANOVA test, $p = .03$; Figure 2b), showing a lower alpha diversity in the group O. Nevertheless, the observed microbial richness did not show differences between the antimicrobial treatment groups (ANOVA test, $p > .05$; Figure 2b).

The antibiotic administration groups had 187 DA OTUs (Table S7; Figure S6C) in metagenomeSeq analyses. There were several OTUs annotated as *Lactobacillus* spp. in the whole dataset. Since at least one OTU was found DA in most comparisons between experimental groups, we decided to further explore the global abundance of *Lactobacillus* spp. by adding the abundances of the OTUs in the whole dataset at T1 (OTUs 292057, 24271, 725198, 536754, 588197, 549756, 553352, 302975, 703741, 807795). Normalized global abundance of *Lactobacillus* in each group clearly showed an increase of abundance in group C and in the group P comparing to the group O (Figure 3a). Accordingly, ANOVA analyses showed significant differences ($p = 8.56 \times 10^{-5}$) among the three groups at the OTUs level. In addition, the post hoc test showed differences between the O versus C group (Tukey's HSD, $p = .0001$) and P versus O group (Tukey's HSD, $p = .01$) and did not show a significant p-value among C versus P group (Tukey's HSD, $p > .05$). When comparing two groups, we have described 144 DA OTUs in the comparison P versus O, 127 O versus C and 65 by comparing P versus C (Tables S8, S9 and S10, respectively). In the Venn diagram, the overlapping DA OTUs between the two by two groups comparison are shown (Figure 2c).

3.8 | Differences in the faecal microbiota at T2 in piglets

The overall composition of the microbiota at T2 (NMDS, Figure 4) was mainly linked to the antibiotic treatment (Adonis test, $p = .0001$) and the faecal score (Adonis test, $p = .0002$), whereas *MUC4*, *FUT1* genotypes, the age and the presence of ETEC F4 and ETEC F18 had no influence (Adonis test, $p > .05$). The beta diversity was not significantly different across the antimicrobial treatment groups (ANOVA test, $p > .05$). In the NMDS plot, the centroids of the group O appeared separated from the P group and the C group, resulting in a significant value (envfit test, $p = .03$; Figure 4a). The alpha diversity at OTU level and the observed microbial richness did not show differences among the groups (ANOVA test, $p > .05$; Figure 4b). Moreover, the antibiotic administration differential analysis at the OTUs level identified 124 DA OTUs (Table S11; Figure S6D). Since at least one OTU was found DA in most comparisons between experimental groups, we decided

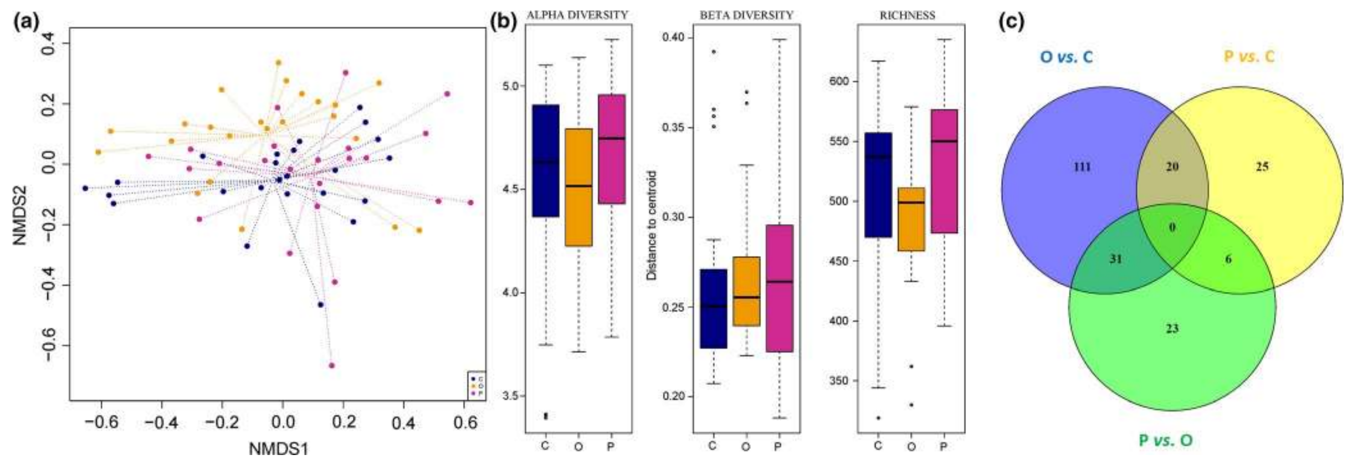

FIGURE 4 Plots include only the samples obtained from T2. (a) Dissimilarities in gut microbiota composition represented by the non-metric multidimensional scaling (NMDS) ordination plot, with Bray–Curtis dissimilarity index calculated on unscaled OTU abundances. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral administered (O, orange) and amoxicillin parenteral administered (P, purple). (b) Box plot graph representation of the alpha diversity (Shannon index), beta diversity (Whittaker's index) and richness (total number of OTUs present in each sample) using the rarefied OTU table for each group and time point. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral administered (O, orange) and amoxicillin parenteral administered (P, purple). (c) Venn diagram representing the overlaps of differentially abundant OTUs more abundant belonging to the comparison of two experimental groups (C vs. P, C vs. O, P vs. O) ("fitZig"; MetagenomeSeq R package). Group is coloured by comparisons: control versus amoxicillin parenteral administered (C vs. P, yellow), control versus amoxicillin oral administered (C vs. O, blue) and amoxicillin oral Lachnospiraceae (O vs. P, green)

to further explore the global abundance of *Lactobacillus* spp. by adding the abundances of the OTUs in the whole dataset at T2 (OTUs 292057, 24271, 725198, 536754, 588197, 581474, 549756, 553352, 302975, 703741, 807795). We described that *Lactobacillus* spp. was more abundant in group C (Figure 3b). ANOVA analyses showed significant differences ($p = .001$) between the experimental groups. In addition, the post hoc test showed significant differences between P versus C group (Tukey's HSD, $p = .0009$) and a significant trend between the O versus C group (Tukey's HSD, $p = .055$). No differences were described between O and P group (Tukey's HSD, $p > .05$). When comparing two groups, we have described 162 DA OTUs in the comparison O versus C, 61 P versus O and 51 when comparing P versus C (Tables S12, S13 and S14, respectively). In the Venn diagram, the overlapping DA OTUs among the different comparisons are showed (Figure 4c). In the DA OTUs belonging to the O versus C comparison, we have described *Prevotella copri*, *Ruminococcus* and *Lactobacillus* to be more abundant in the C than in the O group.

4 | DISCUSSION

The present study investigates a common situation occurring in commercial pig herds during the weaning period, when animals are naturally infected by ETEC strains and simultaneously treated with antibiotics. The postweaning period is associated with multiple stressors, causing a faecal microbiota dysbiosis, which is among the leading causes of postweaning diarrhoea in piglets. The study was focused on the interactions among the host genetics, the phenotype traits and the faecal microbiota composition in field conditions.

In our study, the weight gain was not affected by the genotypes of animals: this finding is in accordance with other reports (Casini et al., 2016; Poulsen et al., 2018). We found an association between a susceptible genotype for *MUC4* gene and the shedding of ETEC F4, confirming the role of this gene in the host susceptibility to the infection. Similarly, we showed an association between the susceptible *FUT1* genotype and the presence of ETEC F18. The association of the *MUC4* and *FUT1* genes with diarrhoea has been largely described in literature (Casini et al., 2016; Jørgensen et al., 2004; Luise et al., 2019; Meijerink et al., 1997; Poulsen et al., 2018; Vogeli et al., 1997; Zhang et al., 2017). However, the *MUC4* resistant genotype was characterized by a higher diarrhoea score, which is in contrast with a previous study (Luise et al., 2019). It should be noted that a small percentage of animals with the resistant genotype could show susceptible phenotypes (Joller et al., 2009) and this may explain our findings. Likewise, the susceptible *FUT1* genotype was not associated with the presence of diarrhoea. In this experiment, we decided to use naturally infected piglets; therefore, the infectious load was not homogeneous in the animals and this has to be considered as a possible source of bias in our study. In addition, dysbiosis, which is associated with diarrhoea, is commonly reported in this phase and may have confounded our results (Gresse et al., 2017; Lallés et al., 2007). Taking together, our results confirm the role of host genotype on the susceptibility to ETEC infection, but our data suggest that other factors may

play a role in determining the presence of diarrhoea in field conditions.

The investigation on the faecal microbiota composition showed that in animals without antimicrobial treatments during weaning, the intestinal microbiota is mainly influenced by the *MUC4* genotypes, as reported in previous studies (Luise et al., 2019; Messori et al., 2013). We associated *Actinobacillus porcinus* to the *MUC4* resistant group. Interestingly, *Actinobacillus porcinus* has been described in weaned piglets with a high weight gain (Nowland, Plush, Barton, & Kirkwood, 2019), thus confirming its beneficial role in porcine gut health. Contrary to what reported by Messori et al. (2013), we did not describe *Clostridium barlettii* in the resistant *MUC4* piglets, in accordance with the recent study of Luise et al. (2019). Furthermore, the *Oscillospira* genus was also more abundant in the resistant *MUC4* animals: this is not surprising since this genus belong to the Ruminococcaceae family, which usually increases after weaning and it is associated with a non-dysbiotic gut (Frese, Parker, Calvert, & Mills, 2015; Huang et al., 2019; Mach et al., 2015).

Moreover, we described a different composition of the faecal microbiota in diarrhoeic animals compared to non-diarrhoeic animals, confirming the role of dysbiosis in the development of diarrhoea. DA OTUs showed that in the piglets with diarrhoea the *Bacteroides*, *Parabacteroides, Fusobacterium* genera and the bacteria belonging to the Pasteurellaceae family dominated. Our results about *Fusobacterium* are in accordance with what already reported in the literature, where a higher abundance of this genus in dysbiotic animals than in healthy piglets is described (Huang et al., 2019).

Finally, we confirmed the role of age at weaning as a major influencer of the intestinal microbiota in piglets, as reported in previous papers (Bian et al., 2016; Massacci et al., 2019; Soler et al., 2018). In our study, we described a more homogeneous and richer microbiota composition in the oldest piglets compared to the younger ones, which is in accordance with other finding produced by the same group (Massacci et al., 2019).

Besides the genotype, the antibiotic treatment seems to have an effect on the presence of diarrhoea at T1 and T2. Pigs administered with amoxicillin were at higher risk for diarrhoea when compared to non-treated piglets. Likewise, the risk of shedding ETEC F18 was higher in piglets treated with amoxicillin by the oral route than in not-treated animals or piglets with parenteral administration route. Amoxicillin could not exert an anti-bacterial effect on the ETEC strains, since both the ETEC F4 and ETEC F18 were resistant to this antibiotic.

On the contrary, the amoxicillin treatment affected the faecal microbiota of piglets, at T1 and T2. The amoxicillin exposure resulted in significant alterations of the faecal microbiota population evaluated immediately after the end of the treatment, showing a lower alpha diversity in the orally administered group and thus confirming a more direct effect on the microbiota composition. The shifts were different according to the two administration routes. In the group that received amoxicillin orally, we described a decreased abundance of the commensal *Lactobacillus.* This finding is in accordance with what was reported in a previous study (Connelly, Subramanian, Hasan, Colwell, & Kaleko, 2018), where a lower abundance of *Lactobacillus* was associated with the administration of amoxicillin through the oral route. This is consistent with the clinical activity of amoxicillin (Burch & Sperling, 2018), which may affect the abundance of Gram-positive commensals, such as *Lactobacillus* species. Moreover, it has been described that the abrupt decrease in *Lactobacillus* spp. at weaning could increase the risk of enteritis, since bacteria belonging to this genus play a major role in disease prevention (Konstantinov et al., 2006). Our data suggest that the oral administration of amoxicillin can deeply modify the faecal microbiota, therefore reducing its barrier effect towards ETEC infection and finally resulting in an increased colonization by the pathogen. The same effect was not recorded after a parenteral administration, since the faecal microbiota of piglets in the group treated by the parenteral route were close to the one of the control group. After the withdrawal period of amoxicillin, the control group showed a higher abundance of OTUs belonging to the *Lactobacillus* genus compared to both groups administered with amoxicillin, demonstrating that even the parenteral administration had a long-term effect on the abundance of *Lactobacillus* in piglets gut.

However, the differential analysis after the withdrawal period confirmed the parenteral administration of amoxicillin had a lower impact on the faecal microbiota composition compared to the oral administration. In fact, taking the control group as a reference, the number of differentially abundant OTUs was higher in the group receiving amoxicillin by the oral route than in the one receiving amoxicillin by the parenteral route. In our investigation, we have described that the control and the parenteral administered group had a higher abundance of *Prevotella copri*, *Ruminococcus* and *Lactobacillus* species compared to the oral administered group, in accordance with previous studies (Connelly et al., 2018; Konstantinov et al., 2006). These results highlight that the microbiota composition of the intestine of piglets is highly affected by the antimicrobial administrations by the oral route.

It has to be noted that in commercial pig herds, amoxicillin is mainly administered through feed or water as a metaphylactic treatment to control Streptococcosis and PWD (Burch & Sperling, 2018; Haas & Grenier, 2016; Waack & Nicholson, 2018). Amoxicillin is currently considered an

extremely valuable antimicrobial in both human and animal medicine and remains in the critically important category of antibiotics by the World Health Organization (WHO, 2017). In our study, the ETEC F4 and ETEC F18 were multidrug resistant, which is a common feature of ETEC strains in Europe (Magistrali et al., 2018). When amoxicillin is used in group treatment, there is the risk of creating a selective pressure favourable to amoxicillin-resistant ETEC strains, thus making colonization easier. Since pathogenic bacteria are becoming increasingly resistant to antimicrobials, new practices, aimed to limit the administration of antimicrobials, should be encouraged.

In our study, we confirm that the *MUC4* and *FUT1* genotypes are associated with the susceptibility to ETEC F4 and F18 infection, respectively. The association between diarrhoea and the piglets' *FUT1* genotype was not shown, probably due to the presence of multiple variables at the same time. Overall, the *MUC4* and *FUT1* were confirmed as genetic markers for the susceptibility to ETEC infections in pigs. Moreover, our data highlight that group amoxicillin treatment may produce adverse outcomes on pig health in course of multiresistant ETEC infection and this effect is stronger when the antibiotic is orally administered than parenterally. Alternative control measures, such as the selection of resistant genotypes or vaccination, should be included in farm management practices to preserve a balanced and stable gut microbiota in weaners.

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CONFLICT OF INTEREST

The authors declared that they had no conflict of interests with respect to their authorship on the publication of this article.

DATA AVAILABILITY STATEMENT

The raw sequencing data have been submitted to NCBI's Sequence Read Archive (SRA) repository (BioProject: PRJNA543556; Biosample: SUB5638166, accessions 11771978 to 11772198).

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REFERENCES

Baker, D. R., Billey, L. O., & Francis, D. H. (1997). Distribution of K88 *Escherichia coli*-adhesive and nonadhesive phenotypes among pigs of four breeds. *Veterinary Microbiology*, *54*(2), 123–132. https://doi.org/10.1016/S0378-1135(96)01277-1

Bian, G., Ma, S., Zhu, Z., Su, Y., Zoetendal, E. G., Mackie, R., … Zhu, W. (2016). Age, introduction of solid feed and weaning are more important determinants of gut bacterial succession in piglets than breed and nursing mother as revealed by a reciprocal cross-fostering model. *Environmental Microbiology*, *18*(5), 1566–1577. https://doi.org/10.1111/1462-2920.13272

Bin, P., Tang, Z., Liu, S., Chen, S., Xia, Y., Liu, J.,& Zhu, G. (2018). Intestinal microbiota mediates Enterotoxigenic Escherichia coli induced diarrhea in piglets. *BMC veterinary research.*, *14*(1), 385. https://doi.org/10.1186/s12917-018-1704-9

Blaser, M. J. (2016). Antibiotic use and its consequences for the normal microbiome. *Science*, *352*(6285), 544–545. https://doi.org/10.1126/science.aad9358

Bokulich, N. A., Subramanian, S., Faith, J. J., Gevers, D., Gordon, J. I., Knight, R., … Caporaso, J. G. (2012). Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nature Methods*, *10*(1), 57–59. https://doi.org/10.1038/nmeth.2276

Burch, D. G. S., & Sperling, D. (2018). Amoxicillin — current use in swine medicine. *Veterinary Pharmacology and Therapeutics*, *41*, 356–368. https://doi.org/10.1111/jvp.12482

Cameron-Veas, K., Solà-Ginés, M., Moreno, M. A., Fraile, L., & Migura-Garcia, L. (2015). Impact of the use of beta-Lactam antimicrobials on the emergence of *Escherichia coli* isolates resistant to Cephalosporins under standard pig-rearing conditions. *Applied and Environment Microbiology*, *81*(5), 1782–1787. https://doi.org/10.1128/AEM.03916-14

Caporaso, J. G., Kuczynski, J., Stombaugh, J., Bittinger, K., Bushman, F. D., Costello, E. K., … Knight, R. (2010). QIIME allows analysis of high-throughput community sequencing data. *Nature Methods*, *7*(5), 335–336. https://doi.org/10.1038/nmeth.f.303

Casey, T. A., & Bosworth, B. T. (2009). Design and evaluation of a multiplex polymerase chain reaction assay for the simultaneous identification of genes for nine different virulence factors associated with *Escherichia coli* that cause diarrhea and edema disease in swine. *Veterinary Diagnostic Investigation*, *21*, 25–30.

Casini, L., Bosi, P., Gremokolini, C., Trevisi, P., Mazzoni, M., & Bonilauri, P. (2016). Oral challenge with *E. coli* K88 as a tool to assess growth and health performance in feeding trials of-weaned pigs to assess growth and health performance. *Italian Journal of Animal Science*, *2*, 358–360. https://doi.org/10.4081/ijas.2003.11676010

CLSI (2018). In M. P. Weinstein (Ed,). *Performance standards for antimicrobial susceptibility testing* (29th ed.). Wayne: Clinical and Laboratory Standards Institute. 2019

Connelly, S., Subramanian, P., Hasan, N. A., Colwell, R. R., & Kaleko, M. (2018). Distinct consequences of amoxicillin and ertapenem exposure in the porcine gut microbiome. *Anaerobe*, *53*, 82–93. https://doi.org/10.1016/j.anaerobe.2018.04.012

Dore, J., Ehrlich, S. D., Levenez, F., Pelletier, E., Alberti, A., Bertrand, L., … IHMS Consortium (2015). *IHMS_SOP 06 V1: Standard operating procedure for fecal samples DNA extraction, Protocol Q. International Human Microbiome Standards.* Retrieved from http://www.microbiome-standards.org

Fairbrother, J., & Gyles, C. (2012). Colibacillosis. In J. Zimmerman, L. Karriker, A. Ramirez, K. Schwartz & G. Stevenson (Eds.), *Disease of swine*. (10th ed, pp. 723–747). Hoboken, NJ: Wiley-Blackwell.

Frese, S. A., Parker, K., Calvert, C. C., & Mills, D. A. (2015). Diet shapes the gut microbiome of pigs during nursing and weaning. *Microbiome*, *3*(28), https://doi.org/10.1186/s40168-015-0091-8

Gresse, R., Chaucheyras-durand, F., Fleury, M. A., VandeWiele, T., Forano, E., & Blanquet-Diot, S. (2017). Gut microbiota dysbiosis in postweaning piglets: Understanding the keys to health. *Trends in Microbiology*, *25*(10), 851–873. https://doi.org/10.1016/j.tim.2017.05.004

Haas, B., & Grenier, D. (2016). Impact of Sub-inhibitory concentrations of amoxicillin on *Streptococcus suis* capsule gene expression and inflammatory potential. *Pathogens*, *5*(2), https://doi.org/10.3390/pathogens5020037. pii: E37.

Huang, A., Cai, R., Wang, Q., Shi, L., Li, C., & Yan, H. (2019). Dynamic change of gut microbiota during porcine epidemic diarrhea virus infection in suckling piglets. *Frontiers in Microbiology*, *10*, 322. https://doi.org/10.3389/fmicb.2019.00322

Joller, D., Jørgensen, C. B., Bertschinger, H. U., Python, P., Edfors, I., Cirera, S., … Vögeli, P. (2009). Refined localization of the *Escherichia coli* F4ab/F4ac receptor locus on pig chromosome 13. *Animal Genetics*, *40*(5), 749–752. https://doi.org/10.1111/j.1365-2052.2009.01881.x

Jorgensen, C. B., Cirera, S., Anderson, S. I., Archibald, A. L., Raudsepp, T., Chowdhary, B., … Fredholm, M. (2003). Linkage and comparative mapping of the locus controlling susceptibility towards *E. coli* F4ab/ac diarrhoea in pigs. *Cytogenetic and Genome Research*, *102*(1–4), 157–162 https://doi.org/75742.

Jørgensen, C. B., Cirera, S., Archibald, A. L., Andersson, L., Fredholm, M., & Edfors-Lilja, I. (2004). *Porcine polymorphisms and methods for detecting them*. Patent Number: WO2004048606.

Kim, S., Covington, A., & Pamer, E. G. (2018). The intestinal microbiota: Antibiotics, colonization resistance, and enteric pathogens. *Immunological Reviews*, *279*(1), 90–105. https://doi.org/10.1111/imr.12563.

Konstantinov, S. R., Awati, A. A., Williams, B. A., Miller, B. G., Jones, P., Stokes, C. R., … De Vos, W. M. (2006). Post-natal development of the porcine microbiota composition and activities. *Environmental Microbiology*, *8*(7), 1191–1199. https://doi.org/10.1111/j.1462-2920.2006.01009.x

Lallés, J.-P., Bosi, P., Smidt, H., & Stokes, C. R. (2007). Nutritional management of gut health in pigs around weaning. *Proceedings of the Nutrition Society*, *2007*(66), 260–268. https://doi.org/10.1017/S0029665107005484

Luise, D., Trevisi, P., Bertocchi, M., Salvarani, C., Pagotto, U., Bosi, P., … Fanelli, F. (2019). Effect of Mucine 4 and Fucosyltransferase 1 genetic variants on gut homoeostasis of growing healthy pigs. *Animal Physiology and Animal Nutrition*, *103*, 801–812. https://doi.org/10.1111/jpn.13063

Luppi, A. (2017). Swine enteric colibacillosis: Diagnosis, therapy and antimicrobial resistance. *Porcine Health Management*, *3*(1), 16. https://doi.org/10.1186/s40813-017-0063-4

Mach, N., Berri, M., Estelle, J., Levenez, F., Lemonnier, G., Denis, C., … Lepage, P. (2015). Early-life establishment of the swine gut microbiome and impact on host phenotypes. *Environmental Microbiology Reports*, *7*(3), 554–569. https://doi.org/10.1111/1758-2229.12285

Magistrali, C., Curcio, L., Luppi, A., Pezzotti, G., Orsini, S., Tofani, S., … Villa, L. (2018). Mobile colistin resistance genes in *Escherichia coli* from pigs affected by colibacillosis. *International Journal of Antimicrobial Agents*, *52*, 744–746. https://doi.org/10.1016/j.ijantimicag.2018.08.008

Massacci, F. R., Berri, M., Lemonnier, G., Guettier, E., Blanc, F., Jardet, D., Estellé, J. (2019). *Late weaning is associated with increased microbial diversity and higher* F. prausnitzii *abundance in piglet's gut microbiota*. Submitted.

McMurdie, P. J., & Holmes, S. (2013). Phyloseq: An R package for reproducible interactive analysis and graphics of microbiome census Data. *PLoS ONE*, *8*(4), e61217 https://doi.org/10.1371/journal.pone.0061217

Meijerink, E., Fries, R., Vogeli, P., Masabanda, J., Wigger, G., Stricker, C., … Stranzinger, G. (1997). Two alpha(1,2) fucosyltransferase genes on porcine chromosome 6q11 are closely linked to the blood group inhibitor (S) and *Escherichia coli* F18 receptor (ECF18R) loci. *International Mammalian Genome Society*, *8*(10), 736–741.

Messori, S., Trevisi, P., Simongiovanni, A., Priori, D., & Bosi, P. (2013). Effect of susceptibility to enterotoxigenic *Escherichia coli* F4 and of dietary tryptophan on gut microbiota diversity observed in healthy young pigs. *Veterinary Microbiology*, *162*(1), 173–179. https://doi.org/10.1016/j.vetmic.2012.09.001

Mulder, I. E., Schmidt, B., Stokes, C. R., Lewis, M., Bailey, M., Aminov, R. I., … Kelly, D. (2009). Environmentally-acquired bacteria influence microbial diversity and natural innate immune responses at gut surfaces. *BMC Biology*, *7*(79), https://doi.org/10.1186/1741-7007-7-79

Muñoz, M., Bozzi, R., García, F., Núñez, Y., Geraci, C., Crovetti, A., … Óvilo, C. (2018). Diversity across major and candidate genes in European local pig breeds. *PLoS ONE*, *13*(11). https://doi.org/10.1371/journal.pone.0207475

Nowland, T. L., Plush, K. J., Barton, M., & Kirkwood, R. N. (2019). Development and function of the intestinal microbiome and potential implications for pig production. *Animals (Basel)*, *9*(3), https://doi.org/10.3390/ani9030076. pii: E76.

Oksanen, J., Blanchet, F. G., Friendly, M., Kindt, R., Legendre, P., McGlinn, D., … Szoecs, E. (2019). *vegan: Community ecology package.* R Package Version 2.5-5. Retrieved from https://cran.r-project.org/package=vegan

Oliveros, J. C. (2013). *Venny. An interactive tool for comparing lists with Venn's diagrams*. Retrieved from http://bioinfogp.cnb.csic.es/tools/venny/index.html

Paulson, J. N., Stine, O. C., Bravo, H. C., & Pop, M. (2013). Differential abundance analysis for microbial marker-gene surveys. *Nature Methods 10*, 1200–1202.

Poulsen, A. R., Luise, D., Curtasu, M. V., Sugiharto, S., Canibe, N., Trevisi, P., & Lauridsen, C. (2018). Effects of alpha-(1,2)-fucosyltransferase genotype variants on plasma metabolome, immune responses and gastrointestinal bacterial enumeration of pigs pre- and post- weaning. *PLoS ONE*, *13*(8), e0202970.

Rampoldi, A., Jacobsen, M. J., Bertschinger, H. U., Joller, D., Bürgi, E., Vögeli, P., … Neuenschwander, S. (2011). The receptor locus for *Escherichia coli* F4ab/F4ac in the pig maps distal to the MUC4-LMLN region. *Mammalian Genome*, *22*(1–2), 122–129. https://doi.org/10.1007/s00335-010-9305-3

Rideout, J. R., He, Y., Navas-Molina, J. A., Walters, W. A., Ursell, L. K., Gibbons, S. M., … Caporaso, J. G. (2014). Subsampled open-reference clustering creates consistent, comprehensive OTU definitions and scales to billions of sequences. *PeerJ*, *2*, e545. https://doi.org/10.7717/peerj.545

Rosager, W. N., Peter, N. J., Lind, J. S. E., Svend, H., Matthew, D., & Steen, P. K. (2017). Comparison of antimicrobial resistance in *E. coli* isolated from rectal and floor samples in pens with diarrhoeic nursery pigs in Denmark. *Preventive Veterinary Medicine*, *147*, 42–49. https://doi.org/10.1016/j.prevetmed.2017.08.007

Schokker, D., Zhang, J., Zhang, L. L., Vastenhouw, S. A., Heilig, H. G. H. J., Smidt, H., … Smits, M. A. (2014). Early-life environmental variation affects intestinal microbiota and immune development in new-born piglets. *PLoS ONE*, *9*(6), https://doi.org/10.1371/journal.pone.0100040

Smith, M. G., Jordan, D., Chapman, T. A., Chin, J. J., Barton, M. D., Do, T. N., … Trott, D. J. (2010). Antimicrobial resistance and virulence gene profiles in multi-drug resistant enterotoxigenic *Escherichia coli* isolated from pigs with post-weaning diarrhoea. *Veterinary Microbiology*, *145*, 299–307. https://doi.org/10.1016/j.vetmic.2010.04.004

Soler, C., Goossens, T., Bermejo, A., Migura-garcı, L., Cusco, A., Francino, O., & Fraile, L. (2018). Digestive microbiota is different in pigs receiving antimicrobials or a feed additive during the nursery period. *PLoS ONE*, *13*, e0197353.

Stanisavljevi, S., Aleksa, Č., Boji, S., Veljovi, K., Mihajlovi, S., Neda, Đ., … Goli, N. (2019). Oral neonatal antibiotic treatment perturbs gut microbiota and aggravates central nervous system autoimmunity in Dark Agouti rats. *Scientific Reports*, *9*, 918. https://doi.org/10.1038/s41598-018-37505-7

Sun, Y., & Woo, S. (2017). Intestinal challenge with enterotoxigenic *Escherichia coli* in pigs, and nutritional intervention to prevent postweaning diarrhea. *Animal Nutrition*, *3*(4), 322–330. https://doi.org/10.1016/j.aninu.2017.10.001

TeamCore (2018). *R: A language and environment for statistical computing*. Vienna, Austria: R Foundation for Statistical Computing. Retrieved from https://www.r-project.org/

The European Committee on Antimicrobial Susceptibility Testing (2017a). *Breakpoint tables for interpretation of MICs and zone diameters*. Retrieved from http://www.eucast.org

The European Committee on Antimicrobial Susceptibility Testing (2017b). *EUCAST disk diffusion method for antimicrobial susceptibility testing*. Retrieved from http://www.eucast.org

Vogeli, P., Meijerink, E., Fries, R., Neuenschwander, S., Vorlander, N., Stranzinger, G., & Bertschinger, H. U. (1997). A molecular test for the detection of *E. coli* F18 receptors: A breakthrough in the struggle against edema disease and post-weaning diarrhea in swine. *Schweizer Archiv Fur Tierheilkunde*, *139*(11), 479–484.

Waack, U., & Nicholson, T. L. (2018). Subinhibitory concentrations of amoxicillin, lincomycin, and oxytetracycline commonly used to treat swine increase *Streptococcus suis* biofilm formation. *Frontiers in Microbiology*, *9*(2707), https://doi.org/10.3389/fmicb.2018.02707.

Wang, S. J., Liu, W. J., Yang, L. G., Sargent, C. A., Liu, H. B., Wang, C., … Zhang, S. J. (2012). Effects of FUT1 gene mutation on resistance to infectious disease. *Molecular Biology Reports*, *39*, 2805–2810. https://doi.org/10.1007/s11033-011-1039-0

WHO (2017). *The selection and use of essential medicines: report of the WHO Expert Committee*.

Zhang, D., Ji, H., Liu, H., Wang, S., Wang, J., & Wang, Y. (2016). Changes in the diversity and composition of gut microbiota of weaned piglets after oral administration of Lactobacillus or an antibiotic. *Applied Microbiology and Biotechnology*, *9*, 10081–10093. https://doi.org/10.1007/s00253-016-7845-5

Zhang, L., Huang, Y., Zhou, Y., Buckley, T., & Wang, H. (2013). Antibiotic administration routes significantly influence the levels of antibiotic resistance in gut microbiota. *Antimicrobial Agents and Chemotherapy*, *57*(8), 3659–3666. https://doi.org/10.1128/AAC.00670-13

Zhang, W., Zhu, Y.-H., Zhou, D., Wu, Q., Song, D., Dicksved, J., & Wang, J.-F. (2017). Oral administration of a select mixture of bacillus probiotics affects the gut microbiota and goblet cell function following *Escherichia coli* challenge in newly weaned pigs of genotype muc4 that are supposed to be enterotoxigenic *E. coli* F4ab/ac Receptor Negative. *Applied and Environmental Microbiology*, *83*(3). https://doi.org/10.1128/AEM.02747-16. pii: e02747-16.

SUPPORTING INFORMATION

Additional supporting information may be found online in the Supporting Information section at the end of the article.

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