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ENTERIC DISORDERS AT WEANING: AGE, AMOXICILLIN ADMINISTRATION AND ENTEROTOXIGENIC *ESCHERICHIA COLI* INFECTION AFFECTING THE GUT MICROBIOTA OF PIGLETS.

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ABSTRACT

In pig production systems, weaning is a crucial period characterized by nutritional, environmental and social stress. During this process, piglets are susceptible to diarrhoea and the gut ecosystem needs to adapt to dietary changes, from a milk-based diet to a solid and more complex cereal-based feed, and to environmental pathogen pressure. One of the most important etiological agent of the post-weaning diarrhoea (PWD) is the Enterotoxigenic *Escherichia coli* (ETEC) able to cause severe outcomes and considerable economic losses to farmers worldwide. A role of host genetics in infection appearance is well-established, the SNPs located on the *Mucine 4* (*MUC4*) and *Fucosyltransferase 1* (*FUT1*) genes being associated with the susceptibility to ETEC F4 and ETEC F18, respectively. To investigate aspects related to weaning diarrhoea, two studies have been performed. The aim of the first study was to evaluate the impact of weaning age on gut microbiota diversification in piglets comparing animals at different weaning ages. Forty-eight Large White piglets were divided into four groups of 12 animals weaned at 14 days old (early weaning), 21 or 28 days old (main weaning ages in pig intensive farming) and 42 days old (late weaning). In each group, faecal bacteria composition was assessed by sequencing the 16S rRNA gene of faecal DNA on the weaning day, 7 days post-weaning and at 60 days of age. Our results showed that late weaning increases the gut microbiota diversity including a higher abundance of *Faecalibacterium prausnitzii*, reported as beneficial in humans. Our results suggest than the pre-weaning gut microbiota composition conferred by a late weaning at 42 days of age could enhance gut health in piglets. This would provide a competitive advantage to piglets accumulating a higher diversity of potentially beneficial microbes prior to the stressful and risky weaning transition.

The aim of the second study was to evaluate the effects of the host-genotype and different routes of amoxicillin administration on the presence of diarrhoea and the microbiota composition, during a natural infection by multi-resistant ETEC strains in weaned piglets. For this purpose, 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). Our results confirmed the *MUC4* and *FUT1* as host genetic markers for the susceptibility to ETEC infections. Moreover, our data highlighted that amoxicillin treatment may produce adverse outcomes on pig health in course of multi-resistant ETEC

infection and this effect is stronger when the antibiotic is orally administered than parenterally.
Both studies highlighted the importance of alternative control measures related to farm management in controlling weaning related diarrhoea. With a need to limit the use of antibiotics, selection of resistant genotypes, next-generation probiotics supplementation in feed, and correct procedures of weaning age, should be considered in farm management practices in order to preserve a balanced and stable gut microbiota and consequently reduce occurrence of diarrhoea at weaning.

RIASSUNTO

Lo svezzamento rappresenta un momento cruciale nell'allevamento suinicolo ed è caratterizzato da stress nutrizionale, ambientale e sociale. In questa fase, i suinetti risultano a maggior rischio di insorgenza di diarrea in quanto la microflora intestinale deve adattarsi ai cambiamenti alimentari legati al passaggio da una dieta a base lattea ad un alimento solido a base di cereali e più complesso e all'elevata pressione infettiva ambientale. Uno dei più importanti agenti eziologici responsabili della diarrea post-svezzamento (PWD) è *Escherichia coli* Enterotossigeno (ETEC) in grado di provocare gravi quadri clinici nonché ingenti perdite economiche per gli allevatori. Che ci sia una componente genetica nell'evoluzione di queste infezioni è stato ben definito attraverso l'individuazione degli SNP situati sui geni *Mucine 4* (*MUC4*) e *Fucosyltransferase 1* (*FUT1*) associati rispettivamente alla suscettibilità nei confronti di ETEC F4 e ETEC F18.

Nella presente tesi sono illustrati due studi che hanno avuto l'obiettivo di approfondire alcuni aspetti legati alla comparsa di diarrea durante lo svezzamento. Lo scopo del primo studio è stato quello di valutare l'impatto dell'età di svezzamento sulla diversità del microbiota intestinale, confrontandone la composizione in suinetti svezzati a diverse età. Quarantotto suinetti di razza Large-White sono stati suddivisi in quattro gruppi da 12 soggetti, svezzati rispettivamente a 14 giorni di età (svezzamento precoce), a 21 o 28 giorni (età di svezzamento principale nell'allevamento intensivo) e a 42 giorni (svezzamento tardivo). In ogni gruppo è stata valutata la composizione batterica fecale il giorno dello svezzamento, 7 giorni post-svezzamento e a 60 giorni di età, sequenziando il gene 16S rRNA dal DNA batterico fecale. I risultati ottenuti hanno evidenziato come lo svezzamento tardivo aumenti il grado di diversificazione del microbiota intestinale, aumentando l'abbondanza di *Faecalibacterium prausnitzii*, già considerato benefico per l'uomo. Emerge, inoltre, come la composizione del microbiota intestinale nel pre-svezzamento associata allo svezzamento tardivo incrementi il livello di salute intestinale nei suinetti. Tale condizione, comporterebbe un notevole vantaggio per gli animali che acquisiscono una maggiore differenziazione del microbiota intestinale, incrementando l'abbondanza di batteri beneficiali prima di affrontare lo stress dello svezzamento.

Lo scopo del secondo studio è stato quello di valutare gli effetti del genotipo dell'ospite e le vie di somministrazione dell'amoxicillina sulla comparsa della diarrea e sulla composizione del microbiota intestinale, durante un'infezione naturale causata da ETEC multi-resistente, in suinetti

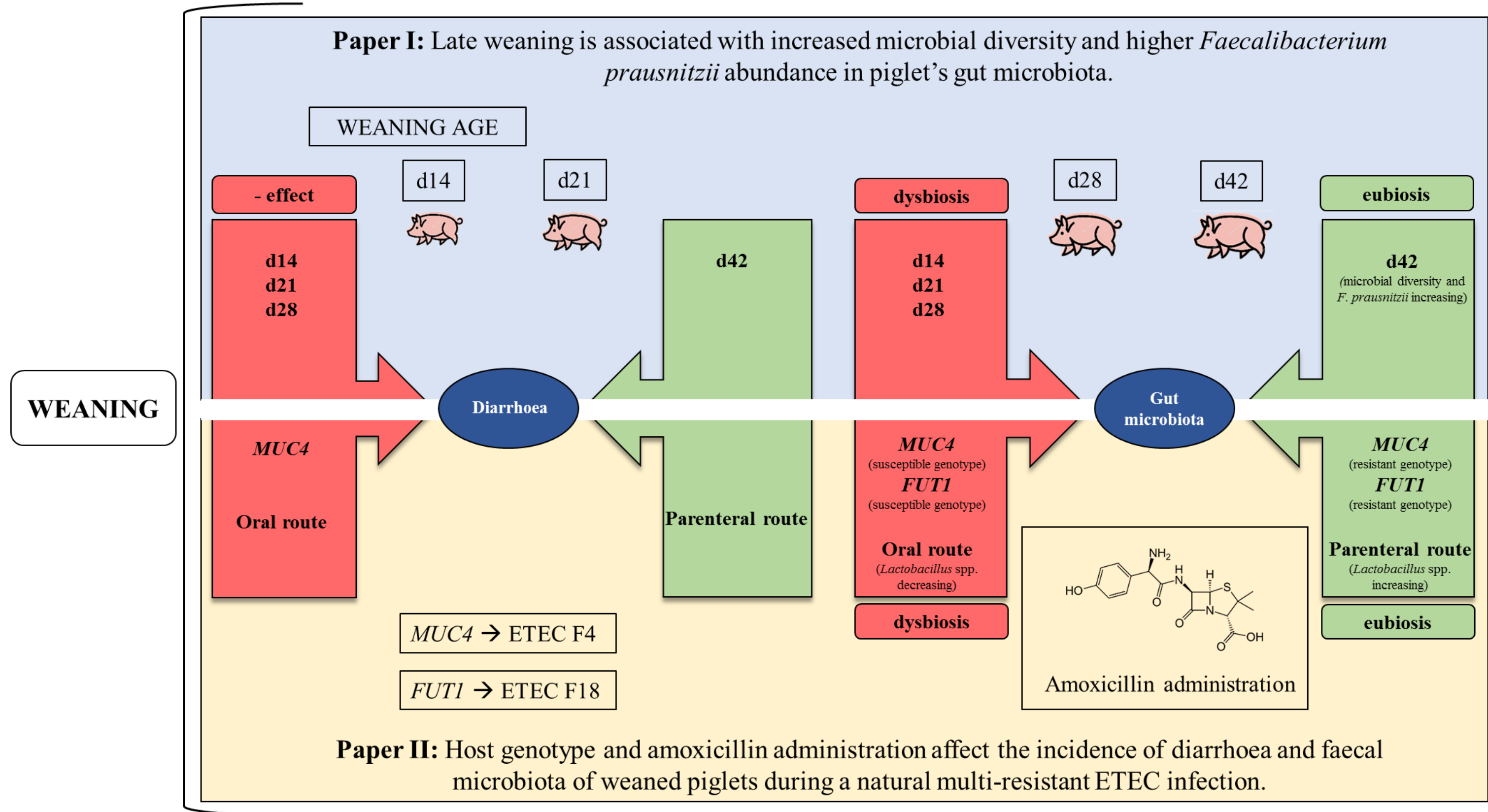
svezzati. A tale scopo, settantuno suinetti sono stati divisi in tre gruppi: due gruppi diversificati dalla via di somministrazione dell'amoxicillina - parenterale (P) o orale (O), e un terzo gruppo di controllo in cui non sono stati somministrati antibiotici (C). I risultati ottenuti hanno confermato il ruolo di *MUC4* e *FUT1* quali marcatori genetici di suscettibilità alle infezioni da ETEC. Inoltre, i nostri dati hanno evidenziato come la somministrazione di amoxicillina possa influenzare negativamente lo stato di salute dei suini in corso di infezione da ETEC, effetti ancora più evidenti quando la somministrazione antibiotica avviene per via orale.
Entrambi gli studi hanno sottolineato l'importanza di adottare misure alternative legate al management aziendale per il controllo della diarrea post-svezzamento. Nell'ottica di limitare l'utilizzo di antibiotici, azioni quali la selezione di genotipi resistenti, l'integrazione di probiotici di nuova generazione nei mangimi ed una corretta gestione dell'età di svezzamento, dovrebbero essere prese in considerazione nelle pratiche gestionali aziendali al fine di preservare un microbiota intestinale equilibrato e stabile e di conseguenza ridurre l'insorgenza di diarrea allo svezzamento.

RÉSUMÉ

En élevage porcin, le sevrage est une période critique caractérisée par un stress nutritionnel, environnemental et social, avec une forte sensibilité des animaux à la diarrhée. Le microbiote intestinal doit s'adapter à un changement alimentaire, avec le passage d'une alimentation lactée à un aliment plus complexe à base de céréales, et les animaux sont soumis à la pression exercée par les agents infectieux environnementaux. Les bactéries entérotoxiques *Escherichia coli* (ETEC) sont les principaux agents pathogènes responsables de la diarrhée post-sevrage et peuvent entrainer des pertes économiques considérables. Le rôle de la génétique de l'hôte dans la sensibilité à l'infection est bien établi, le polymorphisme des gènes *Mucine 4* (*MUC4*) et *Fucosyltransférase 1* (*FUT1*) étant associé à la sensibilité à ETEC F4 et F18, respectivement. Nous avons réalisé deux études afin d'analyser l'effet de facteurs pouvant influer sur la sensibilité des porcelets à la diarrhée au sevrage. Dans une première étude, nous avons évalué l'impact de l'âge au sevrage sur la diversification du microbiote intestinal, par comparaison du microbiote d'animaux sevrés à différents âges. Quarante-huit porcelets de race Large White ont été répartis en quatre groupes de 12 animaux sevrés à 14 jours (sevrage précoce), 21 ou 28 jours (âge au sevrage courant en élevage intensif) et 42 jours (sevrage tardif). La composition bactérienne du microbiote a été établie par séquençage du gène de l'ARNr 16S d'ADN fécal extrait de selles prélevées le jour du sevrage, sept jours après et à l'âge de 60 jours. Nous avons montré que le sevrage tardif augmente la diversité du microbiote, avec une plus grande abondance de *Faecalibacterium prausnitzii* identifiée comme bénéfique chez l'homme. Ces résultats suggèrent que la composition du microbiote intestinal pré-sevrage conférée par un sevrage à 42 jours pourrait améliorer la santé intestinale des porcelets, en leur permettant d'acquérir un microbiote plus diversifié avec des bactéries potentiellement bénéfiques lors du sevrage. La seconde étude a eu comme objectif d'évaluer, chez des porcelets sevrés, les effets du génotype des gènes *MUC4* et *FUT1* et des voies d'administration de l'amoxicilline sur la présence de diarrhée et la composition du microbiote fécal, lors d'une infection naturelle par des souches d'ETEC multirésistantes. Soixante et onze porcelets ont été répartis en trois groupes: deux groupes se différenciant par la voie d'administration de

l'amoxicilline, parentérale (P) ou orale (O), et un groupe témoin sans antibiotiques (C). Nous avons confirmé que *MUC4* et *FUT1* sont des marqueurs génétiques de l'hôte pour la sensibilité aux infections à ETEC et montré que le traitement à l'amoxicilline pouvait avoir des effets néfastes sur la santé du porc au cours d'une infection à ETEC multirésistante, accentués lors d'une administration par voie orale. Les deux études ont mis en évidence l'importance de considérer des méthodes alternatives de conduite d'élevage. Avec la nécessité de limiter l'utilisation d'antibiotiques, la sélection de génotypes résistants, la supplémentation en next-generation probiotics dans l'alimentation et une meilleure optimisation de l'âge au sevrage devraient être prises en compte dans les pratiques, afin de favoriser un microbiote intestinal diversifié, capable de réduire les diarrhées au sevrage.

GRAPHICAL ABSTRACT



LIST OF PUBLICATIONS

The present thesis is based on the work contained in the list of scientific papers below:

Paper I

Massacci F.R., M. Berri, G. Lemonnier, E. Guettier, F. Blanc, D. Jardet, M.N. Rossignol, M.J. Mercat, J. Doré, P. Lepage, C. Rogel-Gaillard, J. Estellé. Late weaning is associated with increased microbial diversity and *Faecalibacterium prausnitzii* abundance in the fecal microbiota of piglets. Animal Microbiome (2020) 2:2.
https://doi.org/10.1186/s42523-020-0020-4

Paper II

Massacci F.R., Tofani S., Forte C., Bertocchi M., Lovito C., Orsini S., Tentellini M., Marchi L., Lemonnier G., Luise D., Blanc F., Castinel A., Bevilacqua C., Rogel-Gaillard C., Pezzotti G., Estellé J., Trevisi P., Magistrali C.F. Host genotype and amoxicillin administration affect the incidence of diarrhoea and faecal microbiota of weaned piglets during a natural multi-resistant ETEC infection. Journal of Animal Breeding and Genetics. 2020; 137:60–72.
https://doi.org/10.1111/jbg.12432

LIST OF ABBREVIATIONS

ACDC	American Centre for Disease Prevention and Control
AMR	Antimicrobial resistance
CD	Crohn’s disease
CIA	Critically important antimicrobials
CRC	Colorectal cancer
ECDC	European Centre for Disease Prevention and Control
EFSA	European Food Safety Authority
EMA	European Medicines Agency
ESVAC	European Surveillance of Veterinary Antimicrobial Consumption
EPEC	Enteropathogenic *Escherichia coli*
ETEC	Enterotoxigenic *Escherichia coli*
EU	European union
FAO	Food and Agriculture Organization
GIT	Gastrointestinal tract
IBD	Inflammatory bowel disease
IBS	Irritable bowel syndrome
MDR	Multi-drug resistant
NGP	Next generation probiotic
NGS	Next generation sequencing
OTU	Operational taxonomic unit
PCR	Polymerase chain reaction
PWD	Post-weaning diarrhoea
SCFA	Short chain fatty acids
SNP	Single-nucleotide polymorphism
WHO	World Health Organization
ZnO	Zinc oxide

GENERAL INTRODUCTION

Section I

In the present section, an overview of the topics covered in this thesis will be provided.
The first part will present essential knowledge needed to understand the economic importance of pig production, from data on pork consumption to the different pig production systems. Moreover, the use and the amount of antimicrobials in pig herds will be described.
The second part will provide the biological background to understand one of the main objectives of this study, the gut microbiota and its interactions with the host for gut health. The present state of the art of gut microbiota in pigs, both in healthy and in disease status, will be reviewed.
The third part will provide a global overview of the main diseases in pigs, focusing mainly on the enteric diseases caused by bacterial pathogen. Moreover, a description of the ETEC infection occurring in weaners will carried out.

1. Pig production

With the increase in worldwide demand for meat, fast-growing species with efficient feed conversion rates, as pigs, are likely to account for a major share in the growth of the livestock subsector. The increase in animal numbers is not spread evenly around the World: Asia leads the trend, whereas pig numbers in North America and Europe are increasing more slowly or holding steady (FAO, 2017).

a. Pig data in the World

Recent reports state that the expansion in global pork production will decelerate over the next decade, but China's production growth is expected to provide nearly half of the additional global output (OECD-FAO, 2018). The total global volume will remain in line with the demand recovery, which is significantly lower relative to the past decade. Strong production growth rates over the outlook period (2018-2027) are also expected in Brazil, Mexico, Philippines, the Russian Federation, the United States and Vietnam (OECD-FAO, 2018). In March 2018, China was home to the largest number of pigs of any country with 440.6 million pigs (STATISTA, 2018). European Union and United States were second and third in the list, with over 150 and 73.2 million heads, respectively (STATISTA, 2018). In recent months, a severe outbreak of African swine fever decimated the 50% of China's pig population and it continues to spread with new cases mainly reported in South China, having a strong impact on the pig production in this country (Mallapaty, 2019).

Australia, Central and Eastern Europe have been reported to register cases of African swine fever (https://www.promedmail.org/ and https://www.gov.uk/government/publications/african-swine-fever-in-pigs-in-poland-lithuania-and-latvia). Globally, the virulent strain of African swine fever could potentially kill up to 25% of the world's pig population (https://www.theguardian.com/world/2019/oct/13/african-swine-fever-the-deadly-virus-at-australias-doorstep).

b. Data on pig production in Europe, Italy and France

With respectively 59.4 million and 47.7 million pigs slaughtered in 2016, Germany (23% of the EU total) and Spain (19%) were by far the two largest pork meat producers in the EU. They are followed by France (23.8 million, 9%), Poland (21.8 million, 8%), Denmark (18.2 million, 7%), the Netherlands (15.4 million, 6%), Italy (11.8 million, 5%), Belgium (11.2 million, 4%) and the United Kingdom (11.0 million, 4%) (EUROSTAT, 2017). It was reported that 12,301,293 and 8,570,807 pigs were produced in 2017 in France and in Italy, respectively (FAO, 2017).

c. Pig production systems

Animal welfare is of increasing interest worldwide and it is becoming a mandatory issue to face consumer's demand. Public opinion often has a favourable perception of some alternative production systems, like outdoor or organic rearing, considering it more respectful of animal welfare, sustainable and environmentally friendly. Changes in animal agriculture

over the last half of the 20th century have drastically altered farming practices and management (HSI, 2014).

Very few traditional forms of pig husbandry survive in the developed world. Along with new niche markets such as organic pig farming, they demonstrate the feasibility of alternative production systems – usually mixed farming linked to local markets instead of landless production aiming at global trade. In developing countries, half of the current pig population is still bred in traditional small-scale subsistence-driven production systems in which pigs provide a potential economic benefit.

Less intensive pig production systems are dissimilar worldwide and often connected to tradition. Therefore, they often differ by pig breeds, environmental conditions, and other natural resources. Outdoor pig farming is defined as a system that allows the pigs to have outside access including contact with soil and growing plants, with which animals can express their natural behaviour (Park *et al.*, 2017). From one side, this system is considered to be beneficial for welfare as animals are kept at low stocking density and are able to express better their natural behaviour. From the other side, outdoor systems could also present negative aspects, such increased exposure to pathogens and in some cases even the access to water and feed, and the protection against climate episode are limited. Moreover, possible attacks from wildlife and infections carried out by parasites are more frequent than in intensive herds. Moreover, it should be considered that outdoor farms have also biosecurity measures such as fences, reducing the potential contact with external animals (except for birds). The use of outdoor systems is often associated with pig husbandry of local breeds. One of the most representative local pig breed is the Iberian raised in the southwest of the Iberian Peninsula, but there are many other

breeds reared in European countries (Muñoz *et al.*, 2018). These breeds are in general characterized by a good adaptation to specific environments, high potential for fat deposition and characteristic meat quality, mostly related to high intramuscular or intramuscular fat content, which are associated with high quality pork productions. As regards for the Iberian breed, outdoor pigs are fed exclusively with acorns present in the field, and this is a request for the production of specific products becoming to local breeds, such as the *Jamón ibérico de bellota*.

Recently, the swine industry has focused on a sustainable intensification of the pig farming systems, which maximizes value over production costs and represents a shift away from antimicrobial usage. However, free range pigs seldom grow as fast as intensively farmed pigs because they expend more energy to walk around while feeding, may lose more weight due to inclement weather, and do not eat a concentrated ration.

In this scenario, there is an urgent need not only for a correct combination of sustainability and efficiency to meet consumer expectations, but also for the development of new phenotypes related to host robustness (Merks *et al.*, 2012).

d. Antibiotic practises in pig herds and the antibiotic resistance issue

The indiscriminate use of antibiotics in livestock has raised concerns that the selective pressure on the bacteria population promotes antibiotic resistance. In fact, the use of antibiotics is common not only for treatment, but also for controlling the spread of infection (metaphylaxis), preventing

infection (prophylaxis) particularly in periods of stress and vulnerability to infections (Aarestrup, 2005).

However, many classes of antibiotic used for humans are also prescribed in food producing animals. For this reason, the WHO produces a list of all antimicrobials grouped into 3 categories based on their importance in treating human infections (WHO, 2017). The classes of drugs included in the list of CIA for human medicine contain the last-resort antibiotics to treat severe infections caused by MDR. The CIA list of Highest Priority Critically Important Antimicrobial includes quinolones, 3rd and higher generation cephalosporins, macrolides and polymixins, an antibiotic class which includes colistin (WHO, 2017).

Despite the difficulties in demonstrating the transmission of resistant bacteria from animals to humans, many studies involving zoonotic pathogens, showed evidence of human infection from resistant bacteria in animals (Angeles *et al.*, 2017; Guevarra *et al.*, 2019; Marshall and Levy, 2011; Nhung *et al.*, 2016; Van Den Bogaard and Stobberingh, 2000). One of the most recent concern about the AMR is the discovery of plasmid-mediated colistin resistant genes (*mcr-1, mcr-2, mcr-3, mcr-4, mcr-5*) in commensal *Escherichia coli* from pigs (Borowiak *et al.*, 2017; Carattoli *et al.*, 2017; Liu *et al.*, 2016; Xavier *et al.*, 2016; Yin *et al.*, 2017). Before EMA recommandations on limiting the use of colistin in animals, colistin was used for over to treat infections caused by *Enterobacteriaceae* in farm animals, such as colibacillosis in piglets (EMA, 2016a). Nowadays, colistin is considered a last resort antibiotic as it is one of the only antibiotics active in severe infections caused by hospital acquired pathogens such as *Pseudomonas aeruginosa*, *Acinetobacter baumannii* and *Enterobacteriaceae* (Lekagul *et al.*, 2019).

Because of the selective pressure exerted by antibiotics and the spread of difficult-to-treat MDR pathogens observed during the last two decades, their use in human and veterinary medicine started to be considered an important issue. Though the transfer of AMR from livestock to humans may occur via several pathways, a considerable evidence suggests that the food route is the most relevant one (Murphy *et al.*, 2017). In France, the Ecoantibio plan has been developed from 2012 in order to limit the use of antibiotics in livestock (https://agriculture.gouv.fr/plan-ecoantibio-2012-2017-lutte-contre-lantibioresistance).
In EU, recent data have been estimated that resistance to antibiotics led to 25,000 deaths per year and 700,000 worldwide, while the ACDC estimated that among the USA population, 2 million people become infected with bacteria that are resistant to antibiotics (ECDC and EFSA, 2017). The projection brutally indicates that AMR has the potential to become the first economic and societal challenges worldwide, as well as one of the important worldwide disease. Since 2001, the European Commission has developed a road-map to fight antimicrobial resistance and this has included taking action at EU level under the One Health initiative (Council conclusions: press release 349/16-17/06/2016).
As regard to the AMR issue, European public-private members are working together on a sustainable and competitive European livestock production sector by fostering knowledge development and innovation in the complete animal production chain. In the recent vision paper of the Animal Task Force (ATF), priorities for research and innovation are suggested within Horizon Europe (ATF, 2019). In fact, one of the main presented proposal is to reduce vulnerability to health threats and risk of

antibiotic resistance, recognised as fundamental items for public health and livestock efficiency.

i. Antibiotics consumption data in Europe

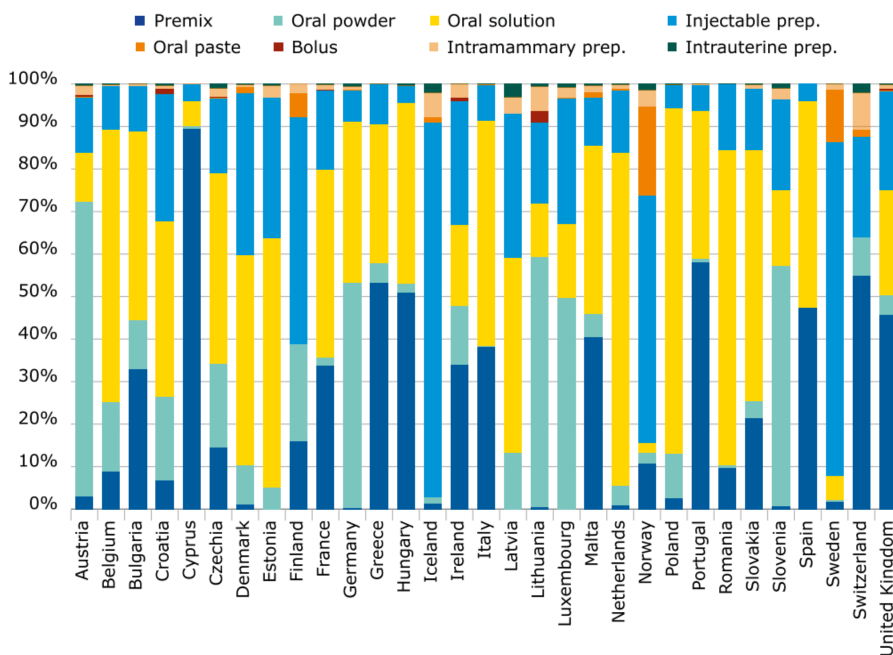
The monitoring of antimicrobial consumption serves many objectives. It monitors time trends of antimicrobial use, compares use by different antimicrobial classes, identifies high users and promotes a more prudent use, and studies the association between level of usage and bacterial resistance (Lekagul *et al.*, 2018).

The last report published by the EMA shows that the overall sales of veterinary antimicrobials across Europe decreased by more than 32.5 % between 2010 and 2017 (EMA, 2019). This continues the downward trend seen over the last few years and confirms that EU guidance and national campaigns promoting prudent use of antibiotics in animals to fight antimicrobial resistance are exerting a positive effect (EMA, 2019).

The overall national sales data of antimicrobials between 2010 and 2017 in 31 European countries for use in food-producing animals are shown in Table 1. Italy, with 1067.7 tonnes, resulted to be the second country for the consumption of antibiotics just after Spain (1770.4 tonnes) and followed by Germany (775.2 tonnes), Poland (751.6 tonnes) and France (498.1 tonnes) at the 5th position (EMA, 2019).The sales of veterinary antimicrobial agents for food-producing animals, stratified into pharmaceutical forms, by country, are shown in Figure 1.

Either in Italy or in France, the oral solution is the most used pharmaceutical form of used antibiotics (EMA, 2019).

Figure 1: sales of veterinary antimicrobial agents for food-producing animals, stratified into pharmaceutical forms, by country (EMA, 2019).



Generally, a breakdown per class of antimicrobials shows there was a drop of almost 66.4% in sales of polymyxins for veterinary use. Sales of 3rd and 4th generation cephalosporins decreased by 20.9%, while sales of quinolones declined by 10.3% (EMA, 2019). The ESVAC report also shows that the situation across Europe is not homogenous. Given the substantial decline observed, there is also a potential for a decrease of antimicrobial use in other countries, especially in those with a high consumption (EMA, 2019).

Table 1: Distribution of overall sales, in tonnes of active ingredient, split into tablets (used in companion animals) and all other pharmaceutical forms (used in food-producing animals)(EMA, 2019).

Country	Tablets		All other pharmaceutical forms		Total tonnes
	Tonnes	% of overall sales	Tonnes	% of overall sales	
Austria	0.6	1.4	44.6	98.6	45.2
Belgium	1.9	0.8	221.0	99.2	222.8
Bulgaria	0.2	0.3	49.6	99.7	49.7
Croatia	0.1	0.5	21.1	99.5	21.2
Cyprus	0.05	0.1	45.4	99.9	45.5
Czech Republic	1.0	2.3	44.1	97.7	45.1
Denmark	0.8	0.9	94.4	99.1	95.2
Estonia	0.1	2.1	6.3	97.9	6.4
Finland	1.2	11.0	9.8	89.0	11.0
France	15.2	3.0	482.9	97.0	498.1
Germany	8.6	1.1	766.6	98.9	775.2
Greece	0.1	0.1	116.7	99.9	116.8
Hungary	0.3	0.2	147.2	99.8	147.5
Iceland	0.04	7.1	0.6	92.9	0.6
Ireland	1.2	1.2	98.5	98.8	99.7
Italy	9.9	0.9	1057.8	99.1	1067.7
Latvia	0.1	1.7	5.9	98.3	6.0
Lithuania	0.1	0.8	11.6	99.2	11.7
Luxemburg	0.1	4.9	1.9	95.1	2.0
Malta	0.2	12.1	1.8	87.9	2.0
Netherlands	2.8	1.5	188.0	98.5	190.9
Norway	0.5	7.3	5.7	92.7	6.2
Poland	1.9	0.3	749.6	99.7	751.6
Portugal	0.8	0.6	135.1	99.4	135.9
Romania	3.3	1.2	262.9	98.8	266.1
Slovakia	0.2	1.7	13.9	98.3	14.1
Slovenia	0.4	6.1	6.7	93.9	7.2
Spain	0.9	0.1	1769.5	99.9	1770.4
Sweden	0.8	7.7	9.5	92.3	10.3
Switzerland	0.7	2.2	31.9	97.8	32.6
United Kindom	14.3	5.8	233.9	94.2	242.2
Total 31 countries	**68.6**	**1.0**	**6634.4**	**99.9**	**6703.0**

2. The gut microbiota

The microbial communities that inhabit the GIT are well known to play a fundamental role in many host processes, and understanding of these complex communities continues to advance at a rapid pace.
Some definitions are necessary to better understand the whole scenario:

- **Microbiota**: "microbial ecosystems living with plants and animals" (Berg, 1996);

- **Microbiome**: "the totality of the microbes, their genetic elements, and the environmental interactions in a particular environment" (Elzinga *et al.*, 2019; Whipps *et al.*, 1988);

- **Superorganism**: "the host and the microorganisms inhabiting it" (Elzinga *et al.*, 2019; Thursby and Juge, 2017);

- **Holobiont**: "hybrid consortia of body cells and microbial communities that together, synergistically and cooperatively, regulate health and disease" (Coleman *et al.*, 2018).

The number of microorganisms inhabiting the human GIT has been estimated to exceed 10^{14}, which encompasses ~10 times more bacterial cells than the number of human cells and over 100 times the amount of genomic content (microbiome) as the human genome (Ley *et al.*, 2005; Luckey, 1972; Savage, 1977). However, a recently revised estimate has suggested that the *ratio* of human:bacterial cells is actually close to 1:1 (Sender *et al.*, 2016a, 2016b). This *ratio* has been changed replacing the old 10:1 or 100:1 values because the number of human and bacterial cells has been recently recalculated (Sender *et al.*, 2016a, 2016b).

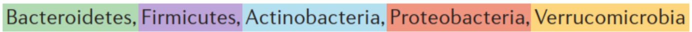
Indeed, microbiota and its effects on host phenotypes have emerged as major factors to be considered in animal science (Estellé, 2019). Understanding the complexity of superorganisms as dynamic ecosystems is essential for interpreting data from pathogen challenge studies of humans and laboratory animals (Foster *et al.*, 2018).

The microbes residing in the gut harvest energy from the food, train our immune system, break down xenobiotics and other foreign products, and release metabolites and hormones important for regulating our physiology (Duvallet *et al.*, 2017; Turnbaugh *et al.*, 2006). Chemical signals from the microbiota can act locally within the gut, and can also have larger systemic effects, such as the case of the “gut-brain axis” (Carabotti *et al.*, 2015; Cryan and O’Mahony, 2011; Duvallet *et al.*, 2017; Mayer *et al.*, 2015; Osadchiy *et al.*, 2019). Due to the physiological interplay between humans and microbial communities, many diseases are hypothesized to be associated with shifts away from a “healthy” gut microbiome. These include metabolic disorders (Dominguez-Bello *et al.*, 2019; Mohammadkhah *et al.*, 2018; Zimmermann *et al.*, 2019), inflammatory and auto-immune diseases (De Luca and Shoenfeld, 2019; Li *et al.*, 2018; Opazo *et al.*, 2018), neurological conditions (Destrez *et al.*, 2019; Griffiths and Mazmanian, 2018; Ma *et al.*, 2019) and cancer (Garrett, 2019, 2017; Helmink *et al.*, 2019; Vivarelli *et al.*, 2019; Wong *et al.*, 2019), among others (Rinninella *et al.*, 2019; Turnbaugh *et al.*, 2006; Wang *et al.*, 2017). Certain gut-related conditions (*e.g.*, obesity and IBD) have been extensively studied in human cohorts and in animal trials, where significant and sometimes causal microbial associations have been shown (Forbes *et al.*, 2018; Zuo and Ng, 2018). In this scenario, the GIT microbiota of mammals has been recognized to take part in the reduction

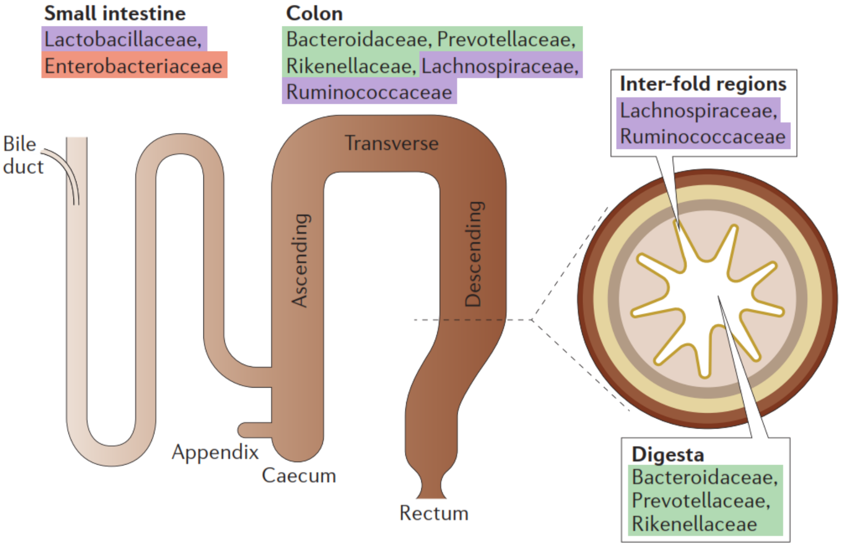
in the incidence of infectious, inflammatory, and other immune diseases (Ding *et al.*, 2019; Round and Mazmanian, 2014; Shreiner *et al.*, 2016; Valdes, 2018; Wang *et al.*, 2017). While the inherited host genome remains almost stable during lifetime, the microbiome is extremely dynamic and can be influenced by a number of factors, among which, age, diet, hormonal cycles, travel, therapies and illness (Argenio and Salvatore, 2015; Fouhy *et al.*, 2019; Kers *et al.*, 2018; Kim *et al.*, 2019; Yieh *et al.*, 2018). Moreover, the gut microbiota varies qualitatively and quantitatively according with the chemical and nutrient gradients, and with the physiological and immune compartmentalisation, from the proximal to the distal part of the GIT, establishing the densest communities in caecum and colon (Figure 2). Furthermore, the differences over the intestinal cross-section axis determine compartments between mucosal folds and also between lumen and intestinal wall, which can represent microhabitats with peculiar microbial communities (Crespo-Piazuelo *et al.*, 2018; Donaldson *et al.*, 2015).

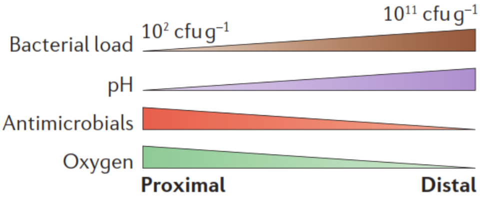
Figure 2: Microbial habitats in the human lower gastrointestinal tract (Donaldson *et al.*, 2015). The dominant bacterial phyla in the gut are *Bacteroidetes*, *Firmicutes*, *Actinobacteria*, *Proteobacteria* and *Verrucomicrobia*. The dominant bacterial families of the small intestine and colon reflect physiological differences along the length of the gut. For example, a gradient of oxygen, antimicrobial peptides (including bile acids, secreted by the bile duct) and pH limits the bacterial density in the small intestinal community, whereas the colon carries high bacterial loads. In the small intestine, the families *Lactobacillaceae* and *Enterobacteriaceae* dominate, whereas the colon is characterized by the presence of species from the families *Bacteroidaceae*, *Prevotellaceae*, *Rikenellaceae*, *Lachnospiraceae* and *Ruminococcaceae* (colours correspond with the relevant phyla). A cross-section of the colon shows the digesta, which is dominated by *Bacteroidaceae*, *Prevotellaceae* and *Rikenellaceae*, and the inter-fold regions of the lumen, which are dominated by *Lachnospiraceae* and *Ruminococcaceae*.

Dominant gut phyla:



Predominant families in the:





a. The gut microbiota in pigs

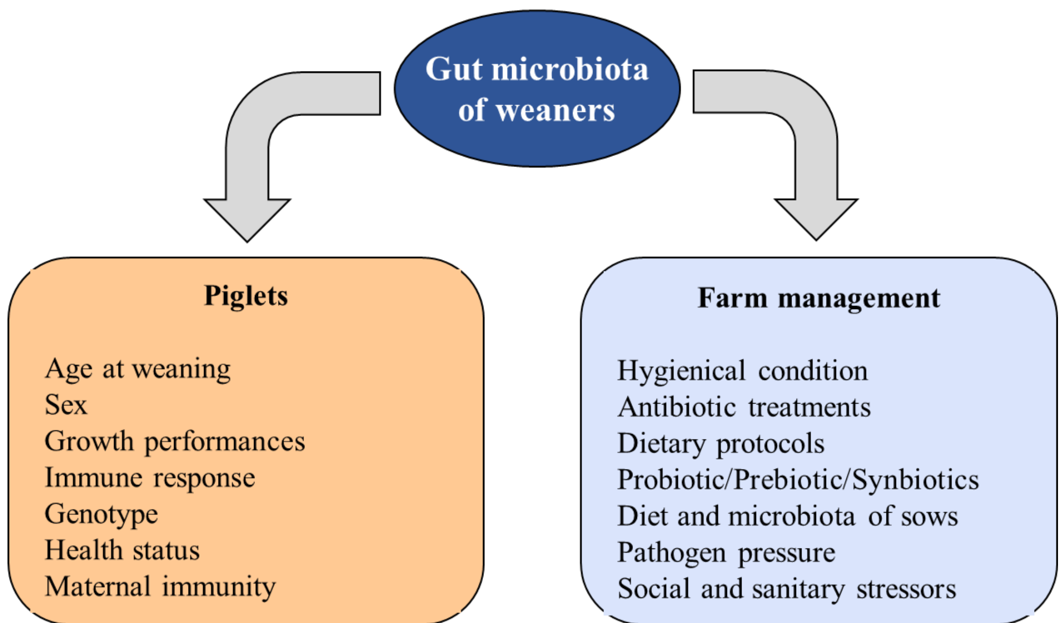
Large-scale studies have investigated the swine gut microbiome. In the faecal metagenome of 287 pigs from France, Denmark, and China 7,685,872 non-redundant genes, representing 719 metagenomic species were identified and constituted a first gene catalogue of the pig gut microbiota (Xiao *et al.*, 2016). Interestingly, 96% of the functional pathways found in the human gene catalogue are present in the swine gut microbiome gene catalogue, confirming the importance of pigs as human biomedical models (Xiao *et al.*, 2016). These data give an idea of the complexity of the gut ecosystem, and intuitively, the plethora of possible functions the gut microbiota can have (Canibe *et al.*, 2019).

The pig gut microbiota imparts specific function in host nutrient metabolism, xenobiotic and drug metabolism, maintenance of structural integrity of the gut mucosal barrier, immunomodulation, and enhance resistance against pathogenic bacteria (Bum and Isaacson, 2015; Guevarra *et al.*, 2019; Jandhyala *et al.*, 2015; Mohajeri *et al.*, 2018). The swine microbial ecosystem is composed of rich and diverse populations that harbour thousands of different microbial species (aerobic, facultative anaerobic, and strictly anaerobic), dwelling in different anatomical biogeographic locations (Crespo-Piazuelo *et al.*, 2018; Holman *et al.*, 2017). Alteration of the swine microbial environment may detrimentally influence the host's health status and inhibit the pathogen colonization and consequently the gut microbiota of animals (Marchesi *et al.*, 2016). The initial colonising bacteria in suckling and weaner piglets largely drive microbiome establishment and development. However, the microbiome is a dynamic system that is changing and influenced by a variety of factors

at weaning (Figure 3). Some of these factors include antibiotic use, stress, diet, age, and the rearing environment (Mach *et al.*, 2015; Nowland *et al.*, 2019). Moreover, several studies have been conducted to evaluate the effect of antibiotics on the pig gut microbiota (Gresse *et al.*, 2017; Luppi, 2017; Schokker *et al.*, 2014; Soler *et al.*, 2018). These studies demonstrate how antibiotics may shape the intestinal microbiota of pigs during the suckling period, and strongly suggest a link between antibiotic supplementation and gut microbiota disruption in early life of pigs (Gresse *et al.*, 2017; Luppi, 2017; Schokker *et al.*, 2014; Soler *et al.*, 2018).

Previous studies in humans have suggested that the most important period for microbial establishment is the first years of life since it is during this time that the microbiome is more dynamic and susceptible to diversification (Koenig *et al.*, 2011; Lallés *et al.*, 2007; Sordillo *et al.*, 2019). Such as for humans, also in pigs, disruption of the gut microbiota during the weaning period results in disease (Dou *et al.*, 2017; Gresse *et al.*, 2017). Thereafter, the microbiome changes toward a more adult-like state where it becomes more stable and resistant to change (Koenig *et al.*, 2011; Sordillo *et al.*, 2019).

Figure 3: factors affecting the gut microbiota in piglets at weaning (adapted from Guevarra *et al.*, 2019; Muirhead and Alexander, 2012; Wang *et al.*, 2019).



b. Gut microbiota and gut health: what is a healthy gut microbiota?

A definition of a healthy gut microbiota in an eubiotic status is still not completely defined. Globally, a healthy gut microbiota is associated with the concept that the microbial community is mainly composed by potentially beneficial species, while pathogenic bacteria are present with a percentage too low to be infectious (Iebba *et al.*, 2016). Moreover, a homogeneous, richer and balanced gut microbiota is widely acknowledged to be beneficial (Rinninella *et al.*, 2019).

In pigs, the "core-healthy" gut microbiota among all gastrointestinal sites is mainly constituted by *Firmicutes* and *Bacteroidetes* phyla, accounted

for nearly 85%, and *Proteobacteria* represent the third more abundant phylum (Holman *et al.*, 2017; Rinninella *et al.*, 2019). The genera *Alloprevotella*, *Blautia*, *Clostridium*, *Lactobacillus*, *Prevotella*, *Roseburia* and *Ruminococcus* are widely recognised to constitute a healthy core microbiota in pigs (Crespo-Piazuelo *et al.*, 2018; Holman *et al.*, 2017).
A healthy host–microorganism balance must be respected in order to optimally perform metabolic and immune functions and prevent disease development. Indeed, disturbances to the delicate host–microbe relationship may negatively affect the development of the immune system, which may in turn result in diseases susceptibility (Patterson *et al.*, 2019).

i. Eubiosis, dysbiosis and symbiosis

The intestinal microbial ecosystem balance, called eubiosis, is a fundamental concept. As early as 400 B.C., Hippocrates said: “death is in the bowels” and “poor digestion is the origin of all evil”. Ali Metchnikoff, who lived from 1845 to 1916, suggested that most diseases begin in the digestive tract when the “good” bacteria are no longer able to control the “bad” ones. He called this condition dysbiosis, meaning an ecosystem where bacteria no longer live together in mutual harmony (Iebba *et al.*, 2016). In case of dysbiosis, “good bacteria” no longer control the “bad bacteria” which take over (Zhang *et al.*, 2015).
The importance of maintaining an eubiotic condition in the intestinal microbial ecosystem is quickly highlighted when we look at some of the deleterious sequelae after antibiotic treatment or pathogenic status (Iebba *et al.*, 2016; Quigley, 2013; Sekirov *et al.*, 2010; Yoon and Yoon, 2018).

Additionally, it should be highlighted that the host response to exogenous infectious agents amplifies/promotes a dysbiosis status. The host responses include inflammation induction, leading to an alteration of the intestinal nutritional environment, and often to a secretory diarrhoea, having strong effects on the microbiota ecosystem. Under an inflammatory condition, we can observe an unexpected decrease in the vitality of the intestinal microbiota, enhancing the availability of ecological niches for pathogen colonization (Iebba *et al.*, 2016; Shreiner *et al.*, 2016).

Historically among scientists, there has been disagreement on the proper use and definition of the term symbiosis, which is derived from the Greek "syn" meaning together and "bios" meaning life ("Symbiosis| Origin and Meaning of Symbiosis by Online Etymology Dictionary," 2017). While Heinrich Anton de Bary is credited with popularizing the term in 1879, it was first used in 1877 by Albert Bernhard Frank in reference to the coexistence of different species (Tipton *et al.*, 2019). Both Frank and de Bary used the term "symbiosis" to refer to all types of interactions between species ranging from parasitism, where one partner benefits without any measurable effect to the other(s), to mutualism – where all partners benefit (Sapp, 2004).

However, the use of the term among microbiome researchers has retained the connotation of mutualism. This extreme mutualism is sometimes simplified as "cross-feeding" but can also take the form of individuals "cheating" mutualisms through adaptive gene loss, as proposed in the "Black Queen Hypothesis"(Morris *et al.*, 2012). Future studies of microbiome will continue to inform and refine our understanding of the breadth of biotic interactions, and may lead to reconsiderations of what constitutes a symbiosis (Tipton *et al.*, 2019).

ii. Probiotics, Prebiotics and Synbiotics

As already said in previous sections, the gut microbiota composition plays an important role in the health of pigs and modulating the population of bacteria in the gut may improve the health of the animals and decrease the risk of diseases (Liu *et al.*, 2018; Patel and Dupont, 2015; Roselli *et al.*, 2017; Tossou *et al.*, 2016; Tran *et al.*, 2018; Van Der Aar *et al.*, 2017).
In fact, during the last years, research has moved on towards the bacterio-therapy that includes 3 different agents (Patel and Dupont, 2015): probiotics, prebiotics and synbiotics, defined as it follows.

- **Probiotics**: "live microorganisms that, when administered in adequate amounts, confer health benefits to the host" (Hill *et al.*, 2014; WHO-FAO, 2001)

- **Prebiotics**: "a selectively fermented ingredient that allows specific changes, both in the composition or activity in the gastrointestinal microflora that confer benefits upon host well-being and health" (Roberfroid, 2007)

- **Synbiotics**: "combination of probiotics and prebiotics" (Yang *et al.*, 2015)

Over the past few decades, probiotics and prebiotics or their combination, have been the subject of many research studies because of their potential therapeutic and preventive health benefits to animals (Yang *et al.*, 2015). Previous reports have shown that probiotics and prebiotics have a broad range of beneficial effects in pigs, including fortification of the intestinal barrier function (Barba-Vidal *et al.*, 2017; Wang *et al.*, 2018a), reduction

of diarrhoea duration and severity (Hancox *et al.*, 2015; Inatomi *et al.*, 2017; Liao and Nyachoti, 2017), inhibition of pathogenic bacteria (Barba-Vidal *et al.*, 2017; Tran *et al.*, 2018) and immunological development (Barba-Vidal *et al.*, 2017; Wang *et al.*, 2018b).

As regards to probiotics, there are three general mechanisms by which probiotics appear to exert their beneficial effects:

i. antimicrobial effects

ii. enhancement of mucosal barrier integrity

iii. immune modulation

Probiotic strains alter the luminal environment, decrease adhesion and cellular invasion, and can produce antibacterial products (*e.g.*, bacteriocins, hydrogen peroxide, and organic acids) that can inhibit the growth of pathogens. This is the case of the genus *Lactobacillus* responsible for producing bacteriocins. The inhibitory action of these bacteriocins varies from inhibiting other lactobacilli to directly inhibiting a wider range of gram-positive, gram-negative bacteria, viruses, and certain fungi (Gaspar *et al.*, 2018; Kenny *et al.*, 2011). Moreover, hydrolytic enzymes produced by some probiotics contribute to the increase of lactic acid, propionic acid, butyric acid, and other SCFAs in the intestinal lumen, reducing the luminal pH. Maintaining a lower pH creates a physiologically restrictive environment that can inhibit the growth and colonization by pathogenic bacteria (Holman and Chénier, 2015; Smiricky-Tjardes *et al.*, 2003). Furthermore, intestinal barrier function is maintained by mucus production, chloride and water secretion, and tight junctions, which bind the apical portions of epithelial cells. Disruption of the epithelial barrier is seen in several conditions including infectious diarrhoea (Luppi, 2017), IBD (Edwards, 2017; Mohajeri *et al.*, 2018;

Rooks *et al.*, 2014), and autoimmune diseases (De Luca and Shoenfeld, 2019; Li *et al.*, 2018). Enhancement of the mucosal barrier may be a crucial mechanism by which probiotic bacteria benefit to the host in these diseases. Moreover, probiotics can alter mucosal immunity considerably as they are able to affect many host cell types involved in the local and systemic immune responses (Roselli *et al.*, 2017; Zhang *et al.*, 2007).

Prebiotics are non-digestible oligosaccharides, such as fructooligosaccharides, galactooligosaccharides, lactulose, and inulin, which have the potential to stimulate growth of selective and beneficial gut bacteria, particularly genera of *Lactobacillus* and *Bifidobacterium* (Bouhnik *et al.*, 2004; Samanta *et al.*, 2013). Because of their composition, prebiotics cannot be digested until they reach the large intestine , where they can be fermented by a specific microbe into SCFAs and lactate (Bouhnik *et al.*, 2004). Recent evidence shows that prebiotics are able to increase the production of SCFAs, which in turn modulate cytokine production within the gut mucosa by altering the gut microbiota composition (Baxter *et al.*, 2019; Beek *et al.*, 2018; Poeker *et al.*, 2018).

The quite recent concept of synbiotics is to combine a probiotic and a prebiotic to facilitate the survival and activity of proven probiotics *in vivo*, as well as stimulating indigenous anaerobic bacteria. Probiotics and prebiotics work synergistically to provide a combined benefit. Several studies have shown positive synergistic effects of synbiotics in humans (İşlek *et al.*, 2014; Markowiak and Śliżewska, 2018; Min *et al.*, 2016).

iii. Next-generation probiotics from microbiota studies: the example of *Faecalibacterium prausnitzii*

Most of the currently commercialized probiotics used to treat and prevent medical conditions are mainly limited to the *Lactobacillus* and *Bifidobacterium* strains (George *et al.*, 2018).

With the development of improved bacterial culturing methodologies, more affordable genome and metagenome sequencing (massive parallel sequencing), and powerful tools able to edit and modify bacterial genomes, we are entering a new era in probiotic research that allows us to develop probiotics that address specific needs and issues for both humans and animals. Information gained from several studies are helping to set a rationale for selection of NGPs such as *Faecalibacterium prausnitzii* (Martín *et al.*, 2017), *Akkermansia muciniphila* (Cani and De Vos, 2017), *Bacteroides uniformis* and *Bacteroides fragilis* (Hage *et al.*, 2017). These NGPs were evaluated in preclinical trials and yielded positive outcomes for inflammatory and metabolic disorders in humans (Patel and Dupont, 2015).

One of the most abundant species to be found in the large intestine of humans is *Faecalibacterium prausnitzii*, which has been reported to be depleted in individuals with IBD (Martín *et al.*, 2017). Therefore, it seems reasonable that if there was a causal link between disease status and the absence of this microorganism, then by simply feeding it to the individual its health promoting features should be restored (Martín *et al.*, 2017). However, there is no evidence, since now, for this bacterium efficacy as a probiotic to be able to reverse the symptoms of IBD when fed to humans. In mice, evidence is available and feeding animals with *F. prausnitzii* does

lead to or associate with induction of anti-inflammatory cytokines or reduction of pro-inflammatory cytokines in induced models of colitis/IBD (Martín *et al.*, 2017; Rossi *et al.*, 2015; Sokol *et al.*, 2008; Zhang *et al.*, 2014). The presence of the anti-inflammatory properties of *F. prausnitzii* also opens the possibility to test them in other animal models to determine further their beneficial effects before testing them in human clinical trials.

3. Health and disease

The term “health” is defined as a state of complete physical, mental and social well-being that allows the pig to exploit its genetic potential for maximising productivity, reproductive performance and lean meat production (Muirhead and Alexander, 2012). Another important definition is animal welfare, which means how an animal is coping with the conditions in which it lives. An animal is in a good state of welfare if (as indicated by scientific evidence) it is healthy, comfortable, well nourished, safe, able to express innate behaviour, and if it is not suffering from unpleasant states such as pain, fear, and distress (OIE, 2010). Good animal welfare requires disease prevention and veterinary treatment, appropriate shelter, management, nutrition, humane handling and humane slaughter/killing. Animal welfare refers to the state of the animal; the treatment that an animal receives is covered by other terms such as animal care, animal husbandry, and humane treatment

The term “disease” means an unhealthy disorder of body and mind, sometimes with pain and unease that is likely to prevent the pig from exploiting its genetic potential resulting in lowered production efficiency and consequently productivity (Muirhead and Alexander, 2012).

Disease can be clinical (*i.e.* the affected pig shows clinical signs) or sub-clinical (the affected pig shows no obvious clinical signs). Any physical or psychological disturbance of immunity may render the pig susceptible to opportunistic pathogens. Good husbandry, meaning good housing, good nutrition and good management, aims to avoid such disturbances in herds (Zimmerman *et al.*, 2012). Good husbandry is the most important factor in preventing disease and maximising health and production.

A recent concept in animal health is the resilience. Resilience takes on different meanings, depending on the context and the field in which it is used. In animal science, it is defined as follows:

> **Resilience**: “the capacity of animals to cope with short-term perturbations in their environment and return rapidly to their pre-challenge status” (Colditz and Hine, 2016).

a. Diseases in pig production

According to Koch’s postulate, the classical concept involves the relationship ‘one microorganism – one disease’. However, after determining that the number of microorganisms (viruses, eukaryotes and bacteria) colonizing animals is extremely large, this concept was shown to be an oversimplification, and that it cannot explain the aetiology of a disease.

The main causes of disease are considered under five infectious and five non-infectious main headings (Muirhead and Alexander, 2012):

Infectious agents	*Non-infectious agents*
Bacteria	Trauma
Viruses	Hereditary and congenital defects
Fungi	Nutritional deficiencies and excesses
Parasites	Toxic agents (poisons)
Prions	Stress

The complete list of the main viral and bacterial diseases of pigs and their main clinical signs are shown in Table 2.

Table 2: main viral and bacterial diseases and their main clinical signs in pigs (adapted from Muirhead and Alexander, 2012).

	Diseases	Pathogen	Clinical signs					
			Lameness	**Diarrhoea**	**Respiratory**	**Nervous**	**Infertility**	**Misc.****
VIRAL	*__Aujeszky's disease / pseudorabies virus (AD/PRV)__	*Suid herpesvirus 1* (SuHV-1)- *Herpesviridae* family						
	*__Classical swine fever (CSF), African swine fever (ASF)__	*Pestivirus- Flaviviridae* family, *African swine fever virus- Asfarviridae* family						
	Cytomegalovirus (CMV)	*Cytomegalovirus- Herpesviridae* family						
	Encephalomyocarditis (EMC)	*Cardiovirus- Picornaviridae* family						
	*Foot-and-mouth-disease (FMD)	*Aphthovirus- Picornaviridae* family						
	Porcine circovirus associated disease (PCVAD)	*Porcine circovirus 2* (PCV-2)- *Circoviridae* family						
	Porcine epidemic diarrhoea (PED)	*Coronavirus- Coronoviridae* family						
	Porcine parvovirus (PPV)	*Parvovirus- Parvoviridae* family						
	Porcine respiratory circovirus (PRCV)	*Coronavirus- Coronoviridae* family						
	*__Porcine reproductive and respiratory syndrome (PRRSV)__	*Arterivirus- Arteriviridae* family						
	Rotavirus	*Rotavirus*						
	Swine influenza virus (SIV)	*Swine influenza virus- Orthomyxoviridae* family						
	Swine pox	*Swine pox virus*						
	*Swine vesicular disease (SVD)	*Enterovirus- Picornaviridae* family						
	*Teschovirus	*Teschovirus- Picornaviridae* family						
	*__Transmissible gastroenteritis (TGE)__	*Coronavirus- Coronoviridae* family						
	Vomiting waste disease (HEV)	*Coronavirus- Coronoviridae* family						

	Diseases	Pathogen	Clinical signs					
			Lameness	Diarrhoea	Respiratory	Nervous	Infertility	Misc.**
BACTERIAL	Actinobacillus pleuropneumonia	*Actinobacillus pleuropneumoniae*			■			
	Anthrax	*Bacillus anthracis*		■				
	Progressive atrophic rhinitis	Toxigenic *Pasteurella multocida*			■			
	Bordetellosis	*Bordetella bronchiseptica*			■			
	Brucellosis	*Brucella suis*	■					
	Clostridial dysentery	*Clostridium perfringens*		■				
	Cystitis/nephritis	*Actinobaculum suis*						■
	Mycoplasma suis	*Mycoplasma suis*					■	■
	*Erysipelas	*Erysipelothrix rhusiopathie*	■				■	■
	***E. coli* enteritidis**	*Escherichia coli*		■				
	Mycoplasma pneumonia	*Mycoplasma hyopneumoniae*			■			
	Exudative epidermitis	*Staphilococcus hyicus*						■
	Glasser's disease	*Haemophilus parasuis*	■		■	■		
	Ileitis	*Lawsonia intracellularis*		■				
	*Leptospirosis	*Leptospira* spp.					■	
	Mycoplasma arthritis	*Mycoplasma hyosynoviae*	■					
	Oedema disease	*Escherichia coli*				■		
	Pasteurellosis	*Pasteurella multocida*			■			
	Salmonellosis	*Salmonella* spp.	■	■	■	■	■	■
	Spirochaetal diarrhoea	*Brachyspira pilosicoli*		■				
	Streptococcal infection	*Streptococcus suis*	■		■	■		
	Sudden death in sows	*Clostridium novyii*						■
	Swine dysentery	*Brachyspira hyodysenteriae*		■				
	Tetanus	*Clostridium tetani*				■		
	Tuberculosis	*Mycobacterium bovis*						■

*Notifiable in most countries.
Bold=important at farm level.
Underlined=zoonotic disease
**Miscellaneous- urinary, mastitis, skin, heart, sudden death, etc.

b. Weaning enteric diseases

Enteric infections are gastrointestinal disorders among the most common and economically significant diseases affecting swine production worldwide (Zimmerman *et al.*, 2012). Clinical signs of these infections include diarrhoea, reduced growth rates, weight loss, and death of pre- and post-weaned piglets. The most common causes include bacterial and viral etiological agents, such as *Enterotoxigenic Escherichia coli*, *Salmonella* spp., type A *Clostridium perfringens*, *Coronavirus* (responsible of PED) and *Rotavirus* (Argüello *et al.*, 2019, 2018a; Theuns *et al.*, 2014; Zimmerman *et al.*, 2012).

Control measures for enteric diseases should focus on elimination of environmental risk factors through cleaning and improved biosecurity. Moving medicated animals to a clean, segregated environment is often successful in elimination efforts. Extensive environmental clean-up with removal of all contaminated faecal material is essential for infected facilities, and a protocol of pressure washing, disinfection, and application of concentrated lime solution to environmental surfaces has been reported to be effective (Muirhead and Alexander, 2012).

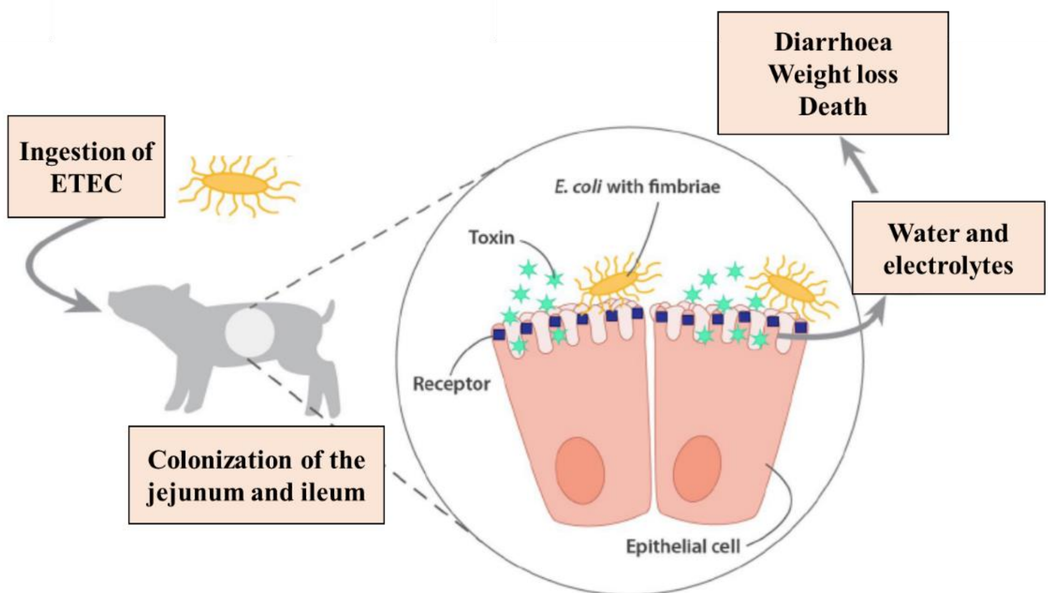
i. The Enterotoxigenic *Escherichia coli* infection in piglets

During weaning, maternal separation, change of environment, mixing with non-litter mates, transportation, change in temperature, new sources of feed and water, handling and administration of vaccines can all coincide and put piglets under considerable stress. This is important because stress

at weaning has been shown to reduce growth rates, and even cause dysfunction in the intestines that often open the way to pathogen colonization, such as the colibacillosis infections (Gresse *et al.*, 2017).
Accumulating evidence suggests that a strong shift in the microbial state may be mainly attributed to the transition from a primarily liquid milk diet to one that relies on solid food (Lallés *et al.*, 2007).
Therefore, during the weaning, piglets are subjected to the ETEC infection and the microbiota may have an important effect on the development of the disease. In Italy and in France, ETEC are among the main enteric pathogen affecting the piglets during the weaning (ANSES, 2015; Bin *et al.*, 2018; Gresse *et al.*, 2017; Luppi, 2017; Murphy *et al.*, 2017; Trevisi *et al.*, 2015). *Escherichia coli* is a gram negative peritrichously flagellated bacteria belonging to the family *Enterobacteriaceae* and is the etiological agent of a wide range of diseases in pigs, including neonatal diarrhoea and PWD, which are important causes of death occurring worldwide in suckling and weaned pigs respectively (Fairbrother and Gyles, 2012). Two main pathotypes are involved in enteric colibacillosis: ETEC and EPEC. Between those, ETEC is the most important pathotype in swine (Fairbrother and Gyles, 2012). The ETEC strains possess fimbrial adhesins, identified as F4 or F18, that mediate microbial attachment to the intestinal epithelium (Luppi, 2017). Briefly, pathogenic bacteria contaminating the environment are ingested by susceptible animals and enter the intestinal tract. The fimbriae allow the ETEC to adhere to specific receptors on the brush borders of the small intestine enterocytes (Fairbrother and Gyles, 2012). Resulting bacterial colonization is found mostly on the jejunal and or ileal mucosa. The adherent bacteria produce enterotoxins, which stimulate water and electrolyte loss into the intestinal

lumen, leading to dehydration and possibly death, and a decreased weight gain in surviving animals (Figure 4) (Sun and Woo, 2017). The degree of colonization and proliferation determines whether the disease results from an infection. PWD is commonly observed 2–3 weeks after weaning and although not exceptionally, it can be recorded at 6–8 weeks after weaning (Luppi, 2017).

Figure 4: pathogenesis of ETEC infection in piglets (adapted from Rhouma *et al.*, 2017).

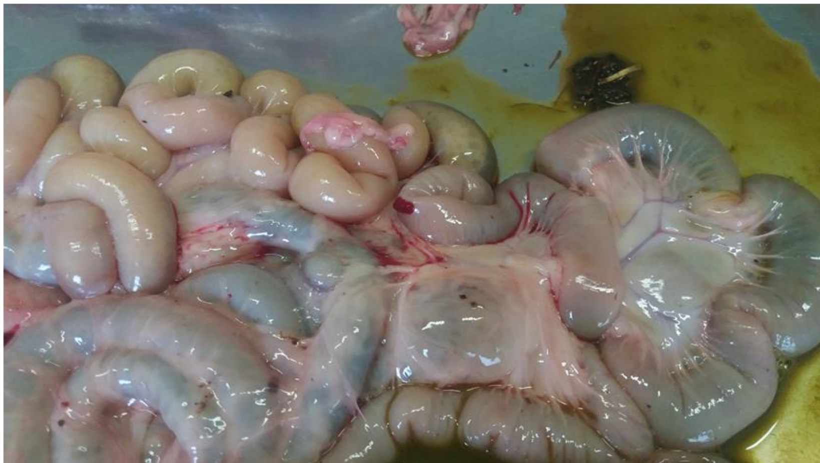


The cases of post-weaning colibacillosis due to ETEC are usually characterized by yellowish, grey or slightly pink watery diarrhoea with a characteristic smell, generally lasting one week (Figure 5). The effect of diarrhoea in piglets affected by enteric colibacillosis is a loss of liquids, consequently animals become dehydrated and die rapidly (Luppi, 2017).

Figure 5: post-weaned piglets in intensive herd (A). Intestine of a piglet affected by post-weaning diarrhoea. The picture shows dilatation of the small intestine and colon filled with liquid intestinal content (B).

A

B



Zinc oxide and antimicrobials are the main choices in the treatment of PWD. Feed containing between 2400 and 3000 ppm of zinc oxide reduce diarrhoea, mortality and improve growth (Roselli *et al.*, 2003). Antimicrobial therapy is required in many cases of enteric colibacillosis, besides using approaches to avoid infectious agents and clinical diseases.

Antimicrobial therapy must be selected which reaches therapeutic concentrations in the intestinal lumen, as observed for different classes of antibiotics: β- lactam antibiotics, cephalosporins, aminoglucosides, aminocyclitols, sulphonamide combined with trimethoprim, fluorochinolones, quinolones and polymyxins (Fairbrother and Gyles, 2012; Giguère and Prescott, 2013). Antimicrobial resistance to several antibiotics such as apramycin, neomycin, trimethoprim-sulphametoxazole and colistin has been increasingly observed in ETEC strains causing PWD (Magistrali *et al.*, 2018; Zhang, 2014). The development of resistance to a wide range of antimicrobial drugs, as well as the demonstrated trend of resistance in ETEC strains to the antibiotics used for the treatment of colibacillosis in pigs, is nowadays a concern (Aarestrup *et al.*, 2008).
Among the physiological and GIT factors impacted by the weaning transition, microbiota disruption in the GIT has likely a key influence leading to PWD. Most studies conducted during the weaning transition have reported a decrease in bacteria of the *Lactobacillus* spp. group and a loss of microbial diversity, whereas *Clostridium* spp., *Prevotella* spp. or facultative anaerobes such as *Proteobacteriaceae*, including *E. coli*, were increased (Dou *et al.*, 2017; Gresse *et al.*, 2017). Furthermore, in-feed and (or) in-water antibiotics also cause differences in the GIT microbiota at weaning due to their wide spectrum of activity and thus their potential ability to kill or prevent the growth of both pathogenic and beneficial microbes (Gresse *et al.*, 2017).

ii. The *MUC4* and *FUT1* candidate genes in piglets

According to their genetics, piglets are not equally susceptible to ETEC infection. Susceptibility to ETEC F4 has been associated to 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).

The 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).

OBJECTIVES

Section II

The global objective of this thesis was to increase the knowledge on determinants affecting the post-weaning diarrhoea and the faecal microbiota in piglets, through the study of management and husbandry practises, the ETEC infection and host genetics.

This global objective was addressed with the following specific objectives of the two studies performed:

1. To characterize the gut microbiota dynamics in antibiotic-free piglets weaned at different ages and describe the faecal microbiota differences between early and late weaning (Section III- Paper I);

2. To explore the effect of the host genotypes for *MUC4* and *FUT1* and different routes of amoxicillin administration on the development of post-weaning diarrhoea and the faecal microbiota composition in weaned piglets during a natural infection by Enterotoxigenic *Escherichia coli* (Section III- Paper II).

SCIENTIFIC PAPERS

Section III

Animal Microbiome

Late weaning is associated with increased microbial diversity and *Faecalibacterium prausnitzii* abundance in the fecal microbiota of piglets.

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1. Abstract

Background
In pig production systems, weaning is a crucial period characterized by nutritional, environmental, and social stresses. Piglets transition from a milk-based diet to a solid, more complex plant-based diet, and their gut physiology must adapt accordingly. It is well established that piglets weaned later display improved health, better wean-to-finish growth performance, and lower mortality rates. The aim of this study was to evaluate the impact of weaning age on fecal microbiota diversity and composition in piglets. Forty-eight Large White piglets were divided into 4 groups of 12 animals that were weaned at different ages: 14 days (early weaning), 21 days (a common weaning age in intensive pig farming), 28 days (idem), and 42 days (late weaning). Microbiota composition was assessed in each group by sequencing the 16S rRNA gene using fecal samples taken on the day of weaning, 7 days later, and at 60 days of age.
Results
In each group, there were significant differences in fecal microbiota composition before and after weaning ($p < 0.05$), confirming that weaning can drastically change the gut microbiota. Microbiota diversity was positively correlated with weaning age: microbial alpha diversity and richness were higher in piglets weaned at 42 days of age both on the day of weaning and 7 days later. The abundance of *Faecalibacterium prausnitzii* operational taxonomic units (OTUs) was also higher in piglets weaned at 42 days of age.
Conclusions
Overall, these results show that late weaning increased gut microbiota diversity and the abundance of *F. prausnitzii*, a microorganism with positive effects in humans. Piglets might thus derive a competitive advantage from later weaning because they have more time to accumulate a higher diversity of potentially beneficial microbes prior to the stressful and risky weaning period.

Keywords: piglet, gut microbiota, age, weaning, diversity, *F. prausnitzii*

2. Introduction

Weaning is one of the most important life transitions experienced by pigs raised for commercial meat production, and piglets go through post-weaning transient anorexia, which results in undernutrition and weight loss (Lallés *et al.*, 2007). Indeed, it has been estimated that only 50% of piglets consume their first meal within 24 hours of weaning, and 10% still have not eaten 48 hours later (Brooks *et al.*, 2001). However, piglets generally return to their pre-weaning level of energy intake 8–14 days after weaning (Le Dividich and Seve, 2000). In modern pig production systems, weaning usually occurs between the third and fourth week of life (Colson *et al.*, 2006), and piglets are forced to switch from a highly digestible milk-based diet to a more complex, less digestible, and solid plant-based diet (Lallés *et al.*, 2007). During this period, piglets may be afflicted with diarrhea due to gut dysbiosis and/or the colonization of the gut by enteric pathogens (Gresse *et al.*, 2017; Lallés *et al.*, 2007). In addition, piglets experience social stresses, such as being moved to the post-weaning building, being separated from their mothers, and being forced to live with piglets that are not their littermates (Colson *et al.*, 2012; Lallés *et al.*, 2007).

The swine gut microbiota comprises a large and diverse community of bacteria that play a significant role in pig health. Many recent studies have used high-throughput sequencing of the 16S rRNA gene to characterize the composition and structure of this community. In pigs, as in other mammals, the microbiota establishment begins at birth (Katouli *et al.*, 1997; Thompson *et al.*, 2008). From birth until weaning and then during the post-weaning period, the gut microbiota is dynamic and undergoes major compositional changes driven by age, exposure to microbes,

environmental conditions, and diet (Mach *et al.*, 2015). Pigs bred under free-range conditions have been reported to wean between 11 and 12.5 weeks of age (Bøe, 1991; Stolba and Wood-Gush, 1984) and in some cases even later (i.e., after 17 weeks (Jensen and Recén, 1989)). Studies comparing piglet weaning ages have found that later weaning can improve health, boost wean-to-finish growth performance, and reduce mortality during the post-weaning period (Davis *et al.*, 2006; Main *et al.*, 2004). Delaying the age at weaning in production farms has been proposed as a possible strategy for modulating and limiting the effects of weaning-associated problems (Früh, 2011). However, few studies have examined how weaning age affects the early-life establishment of the pig gut microbiota and individual susceptibility to weaning-related health issues. Hence, the overall aim of this study was to characterize gut microbiota dynamics in piglets fed antibiotic-free diets and weaned at different ages.

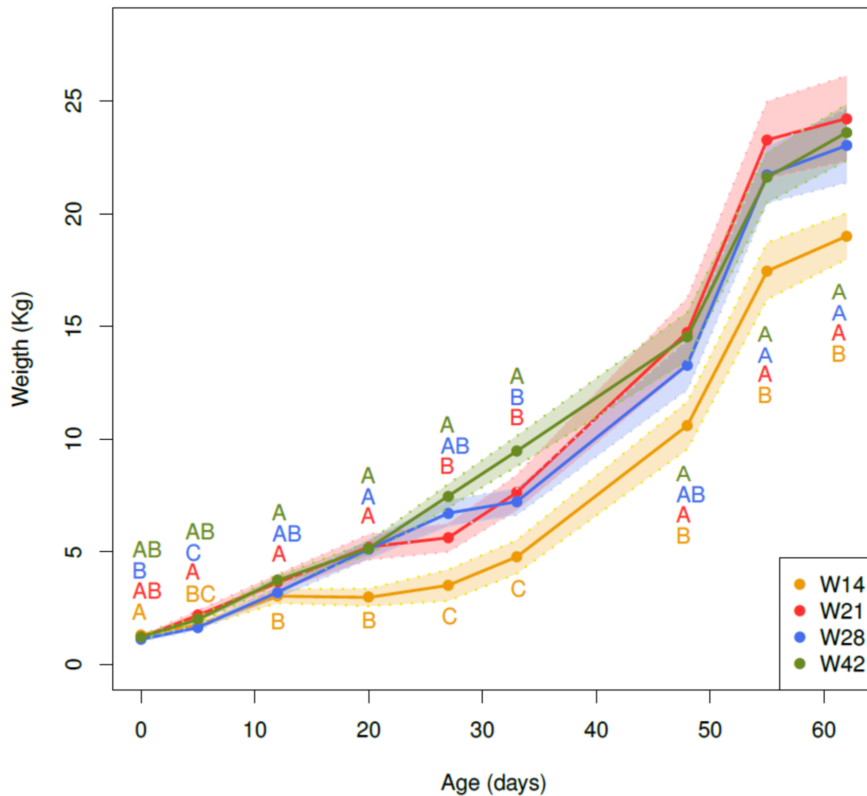
3. Results

Effect of weaning age on piglet weight and occurrence of diarrhea

Forty-eight Large White piglets (23 females and 25 males) were divided into four groups of 12 animals that were weaned at different ages: 14 days (W14), 21 days (W21), 28 days (W29), and 42 days (W42). These groups are hereafter referred to as the weaning groups. Animals presenting diarrhea were unevenly distributed across groups, with a strong reduction in the proportion of affected animals in the groups W28 and W42: 3/10 (30%) in the W14 group, 5/12 (41%) in the W21 group, 1/12 (8%) in the W28 group and 0/11 (0%) in the W42 group. A Chi-square test confirmed that these differences were significant ($p<0.05$).

To characterize piglet growth, we monitored the weight of pigs in each weaning group from birth (day 0) to 62 days of age (weight was measured at 5, 12, 20, 27, 33, 48, 55, and 62 days of age). Using ANOVAs, we found that the weaning groups differed in weight across time and that patterns of differences varied (Table S1). In general, after weaning, the mean weight for the W14 group was consistently lower than the mean weights for the other groups (Figure 1). In addition, piglets in the groups W14 (at day 20), W21 (at day 27), and W28 (at day 33) lost weight immediately after weaning. Indeed, three animals from the W14 group were euthanized because they were lethargic and failed to grow (decision made in accordance with the project's established ethical guidelines). On day 62, the mean weights for the groups W21, W28, and W42 were statistically similar to each other, and they all differed from the weight for the W14 group ($p<0.05$).

Figure 1: Growth curves for piglets weaned at 14 days of age (W14), 21 days of age (W21), 28 days of age (W28), and 42 days of age (W42). The solid and dashed lines show each group's mean and standard deviation, respectively. The initial sample sizes for each group were as follows: W14: 10 animals, W21: 12 animals, W28: 12 animals, and W42: 10 animals. The samples sizes for each group after weaning were as follows: W14: 4 animals, W21: 6 animals, W28: 6 animals, and W42: 5 animals. Any statistical differences between groups are indicated by different letters in each time point, and further details can be found in Table S1.

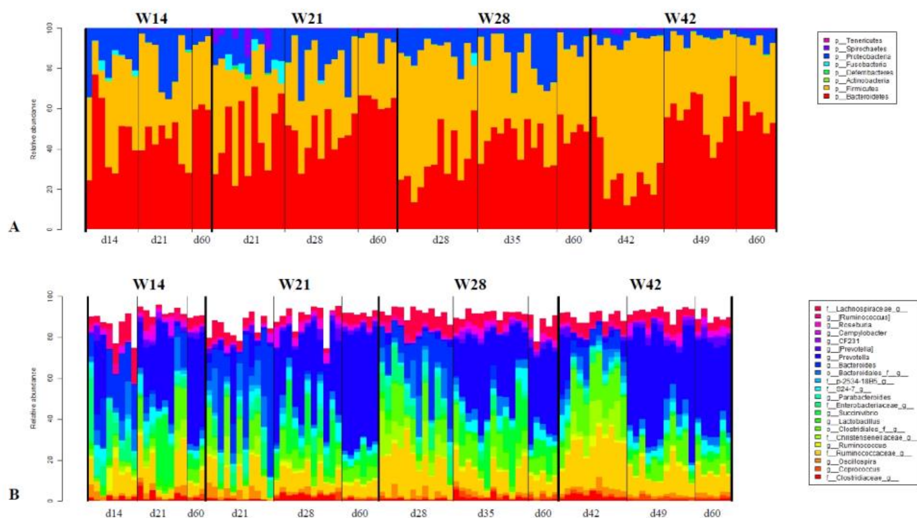


Fecal microbiota sequencing, OTU identification and annotation

The piglets' fecal microbiota were analysed by sequencing the bacterial 16S rRNA gene using an Illumina MiSeq Sequencer. Samples with fewer than 10,000 reads following quality control procedures were removed from the analysis, resulting in sample sizes of 3–12 piglets per sampling point (see the Methods section). After performing quality control, a mean

of 63,716 reads were available for each sample. Sequences from the whole sample set were successfully clustered into 1,121 operational taxonomic units (OTUs), and only 0.26% of the OTUs could not be assigned to a given phylum. Overall, 539 of the 1,121 OTUs (48%) were assigned to a genus. The phyla *Firmicutes* (700/1,121) and *Bacteroidetes* (340/1,121) represented 62% and 30% of the OTUs, respectively. Within the phylum *Firmicutes*, 95% (665/700) of the OTUs were assigned to the order *Clostridiales*, 40% (265/665) to the family *Ruminococcaceae*, and 23% (153/665) to the family *Lachnospiraceae*. Within the phylum *Bacteroidetes*, 53% (179/340) were assigned to the genus *Prevotella*. Other phyla were also represented, but they were less common (e.g., *Proteobacteria*: 5%, *Spirochaetes*: 0.45%, *Fusobacteria*: 0.45%, *Actinobacteria*: 0.35%, *Deferribacteres*: 0.27%, and *Tenericutes*: 0.01%; Figure 2A). At the phylum (Figure 2A) and genus (Figure 2B) levels, the overall abundance of diverse OTUs varied based on weaning age and among sampling points within weaning groups (see the following sections). When we examined the 75% most prevalent taxa in each group at the three sampling points, we found that, out of the 1,121 OTUs observed overall, 760 OTUs were present in the W14 group, 807 OTUs were present in the W21 group, 882 OTUs were present in the W28 group, and 933 OTUs were present in the W42 group. This result illustrates that OTU richness increased with age at weaning.

Figure 2: Relative abundance of the different microbial phyla (A) and genera (B) at each sampling point for every individual pig in each weaning group. Only genera present in at least 20% of the piglets are shown.



Effect of weaning age on fecal microbiota diversity and composition before and after weaning

Alpha diversity, beta diversity, and richness were calculated using the rarefied OTU counts for each group and then compared among weaning groups and sampling points (Figure 3). ANOVAs and Tukey's honest significant difference (HSD) tests were used to assess any resulting differences (Table S2). Overall, there were significant differences ($p<0.05$) in alpha diversity and richness among sampling points within all the weaning groups except W42. In the W42 group, only beta diversity differed significantly among sampling points. The results for alpha diversity and richness reflect the diversification that takes place in the gut microbiota during and after weaning. The results for beta diversity fit with the idea that microbiota heterogeneity declines as animals grow older. The Tukey’s HSD tests highlighted that the significant differences mainly originated from differences in diversity and richness between the pre- and

post-weaning sampling points. Moreover, we observed that beta diversity declined between 7 days post weaning and 60 days of age, except in the W14 group (Figure 3B).

Non-metric multidimensional scaling (NMDS) analyses were carried out using Bray-Curtis dissimilarity values quantifying overall differences in gut microbiota composition between samples collected before weaning, 7 days after weaning, and at 60 days of age for piglets in each weaning group (Figure 4). For the groups W14, W21, and W28, there were clear differences between the results for the three sampling points. For the group W42, in contrast, the centroid for the pre-weaning data was distinct from the centroids for the data from 7 days post weaning and 60 days of age, which overlapped.

We used the metagenomeSeq package in R to identify differentially abundant (DA) OTUs within the full dataset (1,121 OTUs) for each weaning group; we specifically compared the pre-weaning data and the data obtained 7 days after weaning. In the W14 group, there were 224 DA OTUs (Table S3). In the W21 group, this number increased to 484 (Table S4). In W28 and W42, there were 395 DA OTUs (Table S5) and 461 OTUs (Table S6), respectively. There was some degree of overlap among the DA OTUs (Figure S1), although there were unique OTUs in all the weaning groups (W14: 44, W21: 106, W28: 71, and W42: 107). Overall, *Bacteroides*, *Ruminococcus*, *Oscillospira*, and *Clostridium* were more abundant before weaning and *Succinivibrio*, *Prevotella*, and *Campylobacter* were more abundant 7 days after weaning. Interestingly, *Faecalibacterium prausnitzii* was found to be highly abundant after weaning in all the weaning groups.

Figure 3: Boxplots for alpha diversity (A), beta diversity (B) and richness (C) for each sampling time point in animals weaned groups at 14 days of age (W14), 21 days of age (W21), 28 days of age (W28) and 42 days of age (W42). Statistical differences are included in the figure. Significative values are reported as follows: * (p<0.05); ** (p<0.01); *** (p<0.001).

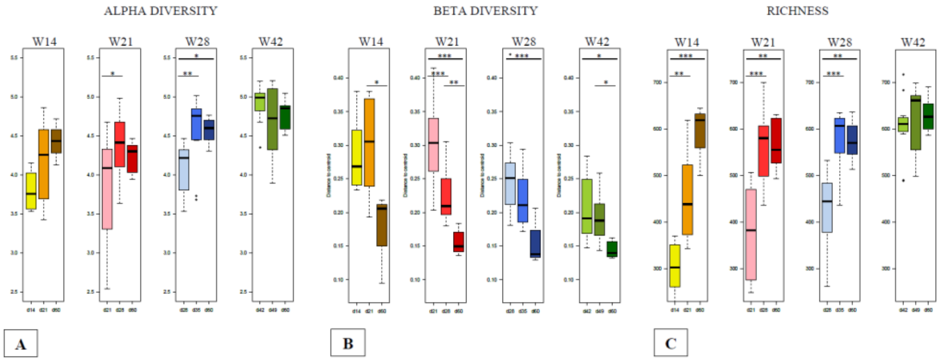
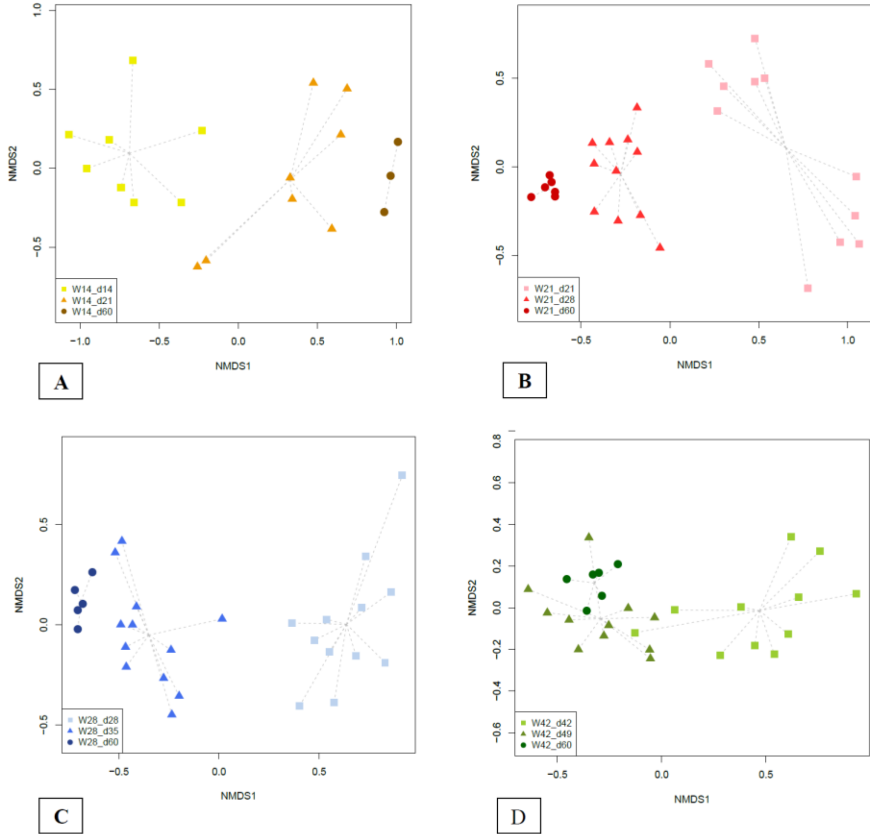


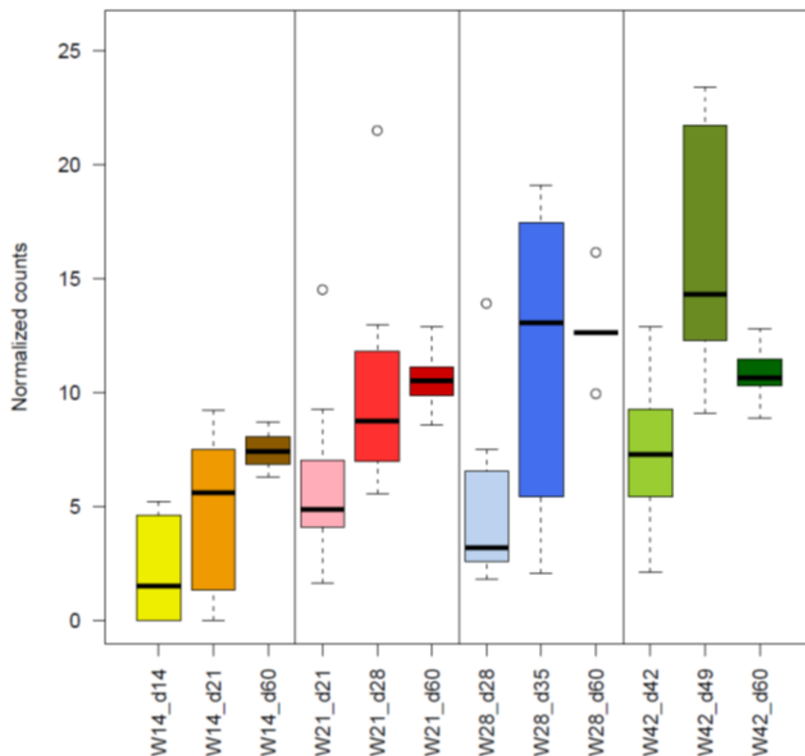
Figure 4: NMDS ordination for microbiota composition at each time point for every weaned group (A: piglets weaned at 14 days of age; B: piglets weaned at 21 days of age; C: piglets weaned at 28 days of age; D: piglets weaned at 42 days of age).



Effect of weaning age on *F. prausnitzii* abundance before and after weaning

In the full dataset, three OTUs were annotated as *F. prausnitzii* (OTU IDs 851865, 350121, and 525215). Since at least one of these OTUs was DA in most comparisons, we decided to explore the overall abundance of *F. prausnitzii* by summing the abundances of the three OTUs for each sample. We had previously normalized these data by log scaling the cumulative sum scaling (CSS) values obtained in metagenomeSeq. For each weaning group, there was a clear increase in *F. prausnitzii* abundance over time, and the highest abundances were observed in the W42 group (Figure 5). In the groups W14 and W21, there was a marked increase in abundance between weaning and 60 days of age; in the groups W28 and W42, abundance tended to be more stable 7 days post weaning. At weaning, *F. prausnitzii* was most abundant in the W42 group, equivalently abundant at lower levels in the W21 and W28 groups, and least abundant in the W14 group. There were significant differences among the four weaning groups (ANOVA: $p<0.05$), and *F. prausnitzii* was more abundant before weaning in piglets weaned at a later age (Table S7). Indeed, piglets weaned at 14 days of age had the lowest abundance of *F. prausnitzii* before weaning, a pattern that persisted until 60 days of age. Post-hoc analysis found differences in the abundance of *F. prausnitzii* between the groups W14 and W42 before weaning and between various combinations of the weaning groups at 7 days post weaning and 60 days of age (Table S8).

Figure 5: Abundance of *F. prausnitzii* at each sampling point for piglets weaned at 14 days of age (W14), 21 days of age (W21), 28 days of age (W28), and 42 days of age (W42). The normalized abundances of the three OTUs annotated as *F. prausnitzii* (OTU IDs 851865, 350121, and 25215) were summed for each individual sample.



Effect of weaning age on fecal microbiota diversity and composition before weaning

Before weaning, alpha diversity was significantly higher in the W42 group than in the other three groups (Tukey's HSD: $p<0.05$) (Table S9; W42 versus W14, W42 versus W21, and W42 versus W28). The same pattern was seen for richness, with an additional significant difference between the groups W14 and W28 (Table S9). Beta diversity was only significantly different between the W42 group and the groups W14 and W21 (Table

S9). In the NMDS analysis, there were significant associations with litter and weaning group ($p<0.05$) (Figure S2A).

Furthermore, before weaning, there were 165 DA OTUs for the four weaning groups combined (Table S10). These OTUs belonged to the phyla Firmicutes, Bacteroidetes, and Proteobacteria and the genera Bacteroides, Ruminococcus, and Prevotella. There was some overlap among groups: 44 of the DA OTUs were shared (Figure S3).

Among the weaning groups, there was differential abundance of the phyla *Tenericutes*, *Spirochaetes*, *Deferribacteres*, and *Fusobacteria* (Table S11) and the genera *Paludibacter*, *Comamonas*, *Helicobacter*, *Peptostreptococcus*, *Streptococcus*, *Treponema*, *Catenibacterium*, and *Dorea* (Table S12).

Effect of weaning age on fecal microbiota diversity and composition at seven days post weaning

Seven days after weaning, there was no difference in alpha diversity and richness among the four weaning groups (Table S9). Beta diversity was significantly higher in the W14 group than in the other three groups, and the W42 group had the lowest beta diversity. The NMDS analysis found no differences among the groups (Figure S2B). There were a total of 165 DA OTUs (Table S13) that mainly belonged to the phyla Firmicutes, *Bacteroidetes*, and *Proteobacteria* and the genera *Prevotella*, *Ruminococcus*, *Bacteroides*, and *Oscillospira*. One of the *F. prausnitzii* OTUs was more abundant in the groups W28 and W42. The weaning groups shared 25 OTUs (Figure S4), which were more heterogeneous than the OTUs shared by the groups prior to weaning; they belonged to the orders *Clostridiales* and *Bacteroidales*. In the analyses at the phylum and

genus levels, only the genera *Actinobacillus*, *Peptostreptococcus*, and *Klebsiella* were differently abundant among the weaning groups (Table S14).

Effect of weaning age on fecal microbiota diversity and composition at 60 days of age

When the piglets were 60 days old, alpha diversity was significantly different between the groups W21 and W42 ($p<0.05$); richness and beta diversity did not vary based on weaning age (Table S9). Similarly, the NMDS analysis found no differences among weaning groups (Figure S2C). There were 54 DA OTUs (Table S15) that belonged to phyla *Firmicutes*, *Bacteroidetes* and *Proteobacteria* and, for the most part, the genera *Prevotella*, *Ruminococcus*, and *Bacteroides*.

4. Discussion

To the best of our knowledge, this study presents the first thorough comparison of fecal microbiota composition in piglets weaned at different ages, from 14 days (very early weaning) to 42 days (organic-like weaning). We characterized patterns of microbiota diversity and composition from just before weaning to 60 days of age and showed that piglets weaned later had time to accumulate more diverse microbial communities, which contained higher abundances of potentially beneficial bacteria like *F. prausnitzii*, before facing the difficult transition that is weaning.

Indeed, the *F. prausnitzii* OTUs were present in all the groups, regardless of weaning age, and they were significantly more abundant after weaning, when the gut microbiota diversified and matured. The abundance of the *F.*

prausnitzii OTUs tracked overall alpha diversity and richness. The W14 group had the lowest abundance of *F. prausnitzii* at all the sampling points, and the W28 and W42 groups had the highest abundance after weaning. The W42 group also had the highest abundance of *F. prausnitzii* before weaning. Since we saw no signs of diarrhea in the W42 group after weaning, it might be hypothesized that *F. prausnitzii* contributes to the resilience of weaned piglets. Indeed, based on the results for the pre-weaning period, it appeared that the later-weaned piglets (W42) had a higher abundance of *F. prausnitzii* than did earlier-weaned piglets (W14). The W14 group still had the lowest levels of *F. prausnitzii* at 60 days of age, indicating that very early weaning could have long-term effects on the abundance of this potentially beneficial species. Indeed, *F. prausnitzii* is considered to be one of the most promising next-generation probiotics (NGP) in humans because it improves gut health, notably by helping to treat inflammation-related diseases (Sokol *et al.*, 2008). It has also been proposed that *F. prausnitzii* serves as an indicator of human intestinal health (Miquel *et al.*, 2013) because declines in its abundance have been correlated with various diseases and disorders resulting from dysbiosis (Cao *et al.*, 2014; Dave *et al.*, 2011; Lopez-Siles *et al.*, 2018; Martín *et al.*, 2017; Miquel *et al.*, 2016, 2013; Sitkin and Pokrotnieks, 2018). Levels of *F. prausnitzii* are lower in patients suffering from intestinal and metabolic disorders such as inflammatory bowel disease, irritable bowel syndrome, colorectal cancer, obesity, and celiac disease, among others (Balamurugan *et al.*, 2008; Furet *et al.*, 2010; Neish, 2009; Sokol *et al.*, 2008). *F. prausnitzii* has also been shown to have anti-inflammatory and protective effects in preclinical models of colitis (Martín *et al.*, 2015). Overall, these findings agree with the hypothesis that piglets could benefit from having a

higher abundance of *F. prausnitzii* in their gut microbiota prior to weaning because it could provide protection against post-weaning dysbiosis and help the gut microbiota transition to a new state of gut homeostasis. To confirm this hypothesis, it will be necessary to conduct further research where sample sizes are larger at each sampling point, and also to examine a broader diversity of environmental conditions and production systems. In addition, because there are limitations associated with 16S rRNA gene sequencing and OTU assignments might not always be precise, it would be fruitful to use qPCR to quantify absolute levels of *F. prausnitzii* as well as to perform whole-metagenome sequencing to identify individual species strains.

Expanding our focus beyond *F. prausnitzii*, it has generally been shown that gut microbiota diversity and richness is positively correlated with gut health. In humans and pigs, enteric diseases, poor intestinal health, and intestinal inflammation are often associated with lower bacterial richness in the gut (Chang *et al.*, 2008; Dou *et al.*, 2017; Lozupone *et al.*, 2013; McCann, 2000; Willing *et al.*, 2010). Interestingly, our results showed that piglets in the W42 group had higher alpha diversity before weaning than did piglets in the other groups, and they also had higher alpha diversity at 60 days of age than did piglets in the W14 group. Such diversity might help additionally protect gut homeostasis at weaning. Beta diversity was the lowest in the W42 group before weaning, after weaning, and at 60 days of age, meaning that piglets in this group had more homogenous gut microbiota, even early on.

Our results confirm findings from previous studies that compared the gut microbiota of piglets before and after weaning (Clemente *et al.*, 2012; Costa *et al.*, 2014; Faith *et al.*, 2013; Kim *et al.*, 2011; Mach *et al.*, 2015;

Schokker *et al.*, 2014; Turnbaugh *et al.*, 2006). Notably, we also observed that the phyla *Bacteroidetes* and *Firmicutes* were dominant in the fecal microbiota of weaning pigs. These two taxa accounted for more than 90% of all the sequences obtained, like in prior studies examining the ileal, cecal, and fecal microbiota of weaning and weaned pigs (Kim *et al.*, 2011; Mach *et al.*, 2015; McCormack *et al.*, 2017; Schokker *et al.*, 2014; Yang *et al.*, 2017). In piglets, the gut microbiota diversifies after weaning, and a new equilibrium of the microbiota ecosystem is established that is based on rich and stable microbial communities (Katouli *et al.*, 1997; Thompson *et al.*, 2008). The NMDS analysis confirmed that piglets differed in their fecal microbiota before and after weaning, which concurs with results from past research showing that weaning is associated with drastic changes in the gut microbiota that have a general impact on the intestinal ecosystem (Dou *et al.*, 2017; Mach *et al.*, 2015).

We analyzed growth performance in the four weaning groups. Although there was an initial imbalance in mean birth weights among groups (animals were heavier in the W14 group), we found that weaning age affected growth: piglets in the groups W14, W21, and W28 lost weight after weaning. Post-weaning weights for the W42 group were not obtained until day 48, but its overall growth curve declined less dramatically than did the curves for the other three groups. Our results concur with those of previous studies in which weight loss was seen immediately after weaning (Al *et al.*, 2017; Han *et al.*, 2017). Our study showed that, even at 60 days of age, piglets in the W14 group had lower body weight than piglets in the other groups, suggesting very early weaning might have long-term effects on growth performance. In addition, the W14 group (but not the other groups) displayed morbidity after weaning, resulting in the euthanasia of

three animals in accordance with the study's ethical guidelines. Piglets in the W21, W28, and W42 groups all had more similar weights at 60 days of age, highlighting that the impact of weaning age on growth seems to be more limited after 21 days of age. Moreover, studies comparing two different weaning ages (14 days and 21 days) found that weaning age affected growth performance in a wean-to-finish facility, as well as behavioural and immunological responses to weaning and new social conditions after the nursery phase (Davis *et al.*, 2006). In our study, some piglets in all the groups except W42 had diarrhea, confirming that late weaning could provide protection against intestinal issues. We thus confirmed that piglets appear to be more sensitive to diarrhea when they are weaned at an earlier age (Gresse *et al.*, 2017; Lallés *et al.*, 2007), and our results also sustain organic farming practices that promote late weaning to reduce the incidence of diarrhea (Jensen and Recén, 1989; Stolba and Wood-Gush, 1984).

5. Conclusions

In conclusion, our results suggest that piglet gut health could be enhanced by late weaning (i.e., at 42 days of age), as it would give the gut microbiota more time to diversity prior to weaning. Even though we looked at a relatively small number of animals from a single farm, our results fit with what has been seen in response to organic farming practices, where piglets are weaned at older ages (Bøe, 1991; Jensen and Recén, 1989; Stolba and Wood-Gush, 1984). Implementing late weaning in conventional production systems would be challenging since pig farms are structured to wean animals at 21 or 28 days of age. However, it may be possible to

obtain the benefits of late weaning by using nutritional strategies and/or probiotics to increase microbial diversity before weaning. Indeed, our results indicate that *F. prausnitzii* could be a promising probiotic for preventing health issues related to weaning dysbiosis, and the economic loss associated to a reduced growth yield. Our results also underscore that weaning piglets are a valuable model for studying how *F. prausnitzii* might affect intestinal health in humans.

6. Methods

Study animals and phenotypes

In our study, we used 48 Large White piglets (23 females and 25 males) from 6 different litters that were bred on INRAE's experimental farm at the PAO Experimental Unit in Nouzilly (France). The piglets were randomly assigned to four groups that were weaned at different ages: 14 days (W14), 21 days (W21), 28 days (W28), and 42 days (W42). Each group included animals from two different litters to minimize block effects. At weaning, piglets were transferred into four different pens based on their litter of origin; the pens had fully slatted floors, used a flat deck system, and were temperature controlled. Six piglets from each group were euthanized seven days after weaning to take tissue samples for a complementary study, while the others were followed until they reached 62 days of age. The quality of environmental conditions, and housing conditions were monitored throughout the study. Animals were kept in the same pen during the entire post-weaning period, and no new piglets were introduced. After weaning, piglets were fed an ad libitum standard diet of grain-based pellets, which was formulated to exceed the animals'

nutritional requirements. None of the piglets were treated with antibiotics during the experiment. Pigs were free of major pathogens and of enterotoxigenic E. coli, whose presence/absence was tested via PCR (Casey and Bosworth, 2009) performed on the fecal samples.

The piglets were weighed at birth and at 5, 12, 20, 27, 33, 48, 55, and 62 days of age. At the beginning of the experiment, sample sizes for each group were as follows: W14: 10 animals, W21: 12 animals; W28: 12 animals, and W42: 10 animals. After weaning, three animals in the W14 group were lethargic and failed to grow; they were therefore euthanized in accordance with the study's ethical guidelines. Furthermore, half of the animals in each group were euthanized seven days after weaning to collect tissues for a complementary study. On day 60, the sample sizes for each group were as follows: W14: 4 animals, W21: 6 animals, W28: 6 animals, and W42: 5 animals. During the period from weaning to seven days after weaning, we visually scored the animals' feces for the presence/absence of diarrhea (0 = normal feces; 1 = liquid diarrhea) (W14: 3 cases of diarrhea out of 18 observations; W21: 6/33; W28: 1/15; and W42: 0/19).

Fecal DNA extraction and quality control

Fecal samples were collected directly from the piglets' rectums at three different sampling points: the day of weaning, 7 days after weaning (day 21 for W14; day 28 for W28; day 35 for W28; and day 49 for W42), and at 60 days of age. Samples could only be collected from half of the animals at 60 days of age because of the earlier tissue sampling. Furthermore, in the W14 group, three piglets had been euthanized, leaving just 3 piglets to reach the age of 60 days. All the fecal samples were directly frozen in liquid nitrogen and further stored at −80°C until use.

A modified version of the protocol developed by Godon *et al.* (Godon *et al.*, 1997) was used for DNA extraction. The method was adapted as follows to be compatible with the chemagic STAR nucleic acid workstation (Hamilton, Perkin Elmer, USA). For each sample, 200 mg of frozen fecal matter was placed in a tube and suspended in a mixture of 250 µl of guanidine thiocyanate buffer (4 M guanidine thiocyanate–0.1 M Tris [pH 7.5]), 40 µl of 10% N-lauroyl sarcosine–0.1 M phosphate buffer (pH 8.0), and 500 µl of 5% N-lauroyl sarcosine. These samples were then incubated at 70°C for 1 h. Afterwards, a 750-µl volume of 0.1-mm-diameter silica beads (Sigma-Aldrich, Germany) was added, and the samples were shaken for 10 minutes at 25 agitations per second in a MM301 Mixer Mill (Retsch, Germany). The samples were subsequently centrifuged at 14,000 rpm and 4°C for 5 min, the supernatant was collected, and 30 µl of Proteinase K (chemagic STAR DNA BTS Kit, Perkin Elmer, USA) was added. The samples were then incubated with shaking (MultiTherm Vortexer, Benchmark Scientific, USA) at 250 rpm and 70°C; there was a final 5-min heating step at 95°C for enzyme inactivation. Finally, the samples were again centrifuged at 14,000 rpm and 4°C for 5 min, and the supernatant was transferred into deep-well plates for further extraction using the chemagic STAR DNA BTS Kit (Perkin Elmer, USA), in accordance with the manufacturer's instructions (starting at the Protease K incubation step). A NanoDrop spectrophotometer (Thermo Scientific, USA) was used to assess the quality of the DNA extracts.

Fecal DNA sequencing and bioinformatic data processing

Microbial profiling was performed via the high-throughput sequencing of the V3-V4 hypervariable region of the 16S rRNA gene (2x250 bp paired-end reads) using an Illumina MiSeq Sequencer (Illumina, USA). We employed the standard Illumina protocol and the primers PCR1F_343 (5'-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3') and PCR1R_784 (5'-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAA TCCT-3'). Quality control was performed on the resulting FastQ files using FastQC software (https://www.bioinformatics.babraham.ac.uk/projects/fastqc); the files were then analyzed using QIIME software (v. 1.9.1) (Caporaso *et al.*, 2010) by using the subsampled open-reference OTU picking approach (Rideout *et al.*, 2014). Singleton OTUs and OTUs representing less than 0.005% of the total number of sequences were removed from the dataset as suggested by the software developers (Bokulich *et al.*, 2012). Chimeric sequences were identified using the BLAST algorithm and removed using QIIME. Samples with fewer than 10,000 reads after quality control procedures were eliminated from the study. On the day of weaning, 7 days after weaning, and at 60 days of age, the sample sizes were (respectively) as follows: W14: 8, 8, and 3 animals; W21: 11, 11, and 6 animals; W28: 12, 12, and 5 animals; and W42: 11, 11, and 6 animals.

Biostatistical analyses

All our statistical analyses were performed in R (v. 3.5.1) (TeamCore, 2018). We analyzed piglet weight using ANOVAs (aov function), and we assessed the frequency of piglets with diarrhea using a Chi-square test

(prop.trend.test function). To examine microbiota diversity and composition, the biom OTU table was imported into R using the Phyloseq package (v. 1.24.2) (McMurdie and Holmes, 2013). The vegan (v. 2.5-2) package (Oksanen, 2015) was used to perform rarefaction analyses of the OTUs in each weaning group at each taxonomic level. Richness and diversity analyses were performed at the OTU level. Alpha diversity and beta diversity were calculated using the Shannon index and Whittaker's index, respectively. Richness was defined as the total number of OTUs present in each sample. Alpha diversity, beta diversity, and log-transformed richness were then analyzed using ANOVAs (aov function); post-hoc comparisons were performed with Tukey's HSD tests. We also used the vegan package to perform non-metric multidimensional scaling (NMDS): we calculated Bray-Curtis dissimilarity values and used the metaMDS function, which standardizes scaling, to assess differences in the overall diversity of fecal microbiota among samples. The env_fit function was used evaluate the statistical significance of the study variables within NMDS ordination space. These variables were sex, litter ID, piglet ID, and sampling point or weaning group. In addition, permutational multivariate analyses of variance were performed using distance matrices and the adonis function. The alpha level was $p < 0.05$.

OTU differential abundance testing was carried out with the metagenomeSeq package (Paulson *et al.*, 2013). OTU counts were normalized using the cumulative sum scaling (CSS) method, and a zero-inflated Gaussian distribution mixture model (fitZig function) was employed to assess differences in relative OTU abundance; the significance level was set to a false discovery rate (FDR) lower than 0.05. The model accounted for the different sampling points for each weaning

group, and litter effect was included as a cofactor. The overall abundance of *F. prausnitzii* was estimated by summing the log-scaled CSS normalized abundances of the three *F. prausnitzii* OTUs (OTU IDs 851865, 350121 and 525215) for each sample. Differences in abundance were then evaluated using ANOVAs (aov function) and post-hoc comparisons were performed with Tukey's HSD tests.

7. Declarations

Ethics approval and consent to participate
All animal procedures were performed according to the guidelines for the care and use of experimental animals established by INRAE and the French authorities (Ability for animal experimentation to E. Guettier: R-45GRETA-F1-04; agreement for experimentation of INRAE's Experimental Unit of Animal Physiology of Orfrasiere: F37-175-2; protocol approved by the French Ministry of Research with authorization ID APAFIS#328-2015031616056915 v5 after the review of ethics committee n°019).
Consent for publication
All authors accepted the final version of the manuscript.
Availability of data and materials
The raw sequencing data has been submitted to NCBI's Sequence Read Archive (SRA) repository (BioProject: PRJNA540598; accessions SAMN11547623 to SAMN11547734).
Competing interests
The authors declare that they have no competing interests.
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Authors' contributions
JE, MB, MJM, JD, PL and CRG designed the research. EG and MB were responsible of the animal production and phenotyping at INRAE's UE PAO farm. GL and JE sampled microbiota and FB managed sampling processing. DJ and FRM did faecal DNA extractions and MNR performed the 16S rRNA sequencing. FRM analysed all data under the supervision

of JE. FRM interpreted the results and wrote the first draft of the manuscript under the supervision of CRG and JE. All authors read and approved the final manuscript.

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9. Supplementary information

Additional supporting information accompanies this paper at the following link https://doi.org/10.1186/s42523-020-0020-4

Supplementary figures

Figure S1: Venn diagram showing the overlap in the differentially abundant OTUs before and after weaning for each weaning group.

Figure S2: NMDS plot of microbiota composition before weaning (A), after weaning (B), and at 60 days of age (C); samples from all the weaning groups were combined.

Figure S3: Venn diagram showing the overlap in the differentially abundant OTUs before and after weaning for each weaning group.

Figure S4: Venn diagram showing the overlap in the differentially abundant OTUs that were more abundant after weaning in each weaning group.

Supplementary tables

Table S1: Differences in mean weight among weaning groups and sampling points. General differences were determined using ANOVAs, and Tukey's HSD tests were employed for post-hoc comparisons. Significant p-values are in bold.

Table S2: Differences in alpha diversity, beta diversity, and richness among sampling points for each weaning group. General differences within each group were determined using ANOVAs, and Tukey's HSD tests were employed to carry out post-hoc comparisons between sampling points. Significant p-values are in bold.

Table S3: Differentially abundant OTUs before and after weaning in the W14 group.

Table S4: Differentially abundant OTUs before and after weaning in the W21 group.

Table S5: Differentially abundant OTUs before and after weaning in the W28 group.

Table S6: Differentially abundant OTUs before and after weaning in the W42 group.

Table S7: Differences in normalized *F. prausnitzii* abundances among weaning groups across all sampling points. The existence of a general difference among the groups was determined using an ANOVA, and Tukey's HSD tests were employed to carry out post-hoc comparisons between all the groups at all the sampling points. Significant p-values are in bold.

Table S8: Differences in normalized *F. prausnitzii* abundances among sampling points for the four weaning groups. General differences were determined using ANOVAs, and Tukey's HSD tests were employed to compare *F. prausnitzii* abundances between weaning groups for each sampling point: before weaning, after weaning, and at 60 days of age. Significant p-values are in bold.

Table S9: Differences in alpha diversity, beta diversity, and richness among sampling points. General differences were determined using ANOVAs, and Tukey's HSD tests were employed for the post-hoc comparisons. Significant p-values are in bold.

Table S10: Differentially abundant OTUs before weaning for the weaning groups.

Table S11: Differentially abundant phyla before weaning for the weaning groups.

Table S12: Differentially abundant genera before weaning for the weaning groups.

Table S13: Differentially abundant OTUs after weaning for the weaning groups.

Table S14: Differentially abundant genera after weaning for the weaning groups.

Table S15: Differentially abundant OTUs at 60 days of age for the weaning groups.

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Host genotype and amoxicillin administration affect the incidence of diarrhoea and faecal microbiota of weaned piglets during a natural multi-resistant ETEC infection.

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1. Abstract

Enterotoxigenic *Escherichia coli* (ETEC) is the etiological agent of post-weaning 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 multi-resistant 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, 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 multi-resistant ETEC infection. Therefore, alternative control measures, able to maintain a healthy faecal microbiota in weaners are recommended.

Keywords: Antibiotic-resistance, *Escherichia coli*, *FUT1*, gut microbiota, *MUC4*, swine.

2. Introduction

Weaning is considered the main critical period for pigs raised in intensive farms (Lallés *et al.*, 2007). This phase may be associated with the onset of gastrointestinal disorders with post-weaning diarrhoea (PWD), caused by Enterotoxigenic *Escherichia coli* (ETEC) that play a major role (Baker *et al.*, 1997; Luppi, 2017). PWD leads to pig morbidity and mortality causing considerable economic losses to farmers worldwide (Fairbrother and 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 and Gyles, 2012). Beside adhesion, ETEC strains secrete enterotoxins able to impair enterocyte functions by increasing cell cation exchanges and reducing water absorption (Sun and Woo, 2017), finally resulting in a severe diarrhoea.

Piglets are not equally susceptible to ETEC infection. Susceptibility to ETEC F4 has been associated to 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 a 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 of *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 *et al.*, 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 and 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 *et al.*, 2018; Stanisavljevi *et al.*, 2019; Zhang *et al.*, 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 of extended-spectrum beta-lactamase (ESBL) *E. coli* in pigs (Cameron-Veas *et al.*, 2015). Of greater concern is the spread of multi-drug 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.

3. Materials and Methods

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 n°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, 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 (Longocillina 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 hours interval. The group O received 12-20 mg/kg bodyweight of the suspension orally twice a day, approximately 7:00 am and 7:00 pm 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.

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, Milan, Italy), 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, Bagno a Ripoli, FI, Italia), 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, Cambridge, UK), according to the EUCAST guidelines (The European Committee on Antimicrobial Susceptibility Testing, 2017). *E. coli* ATCC 25922 was used as control strain. The following antimicrobial discs (Oxoid Ltd, Cambridge, UK) 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, 2017) 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.

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, ThermoFisher Scientific).

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 and Bosworth, 2009).

Microbial profiling was performed using high-throughput sequencing of the V3-V4 hypervariable region of the 16S rRNA gene (2x250 bp paired-end reads) on an Illumina MiSeq platform following the standard Illumina sequencing protocol and by using primers PCR1F_343 (5’-CTTTCCCTACACGACGCTCTTCCGATCTACGGRAGGCAGCAG-3’) and PCR1R_784 (5’-GGAGTTCAGACGTGTGCTCTTCCGATCTTACCAGGGTATCTAATCCT-3’). The generated FastQ files were first quality checked though 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 sub-sampled 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 post-quality control reads were removed from the analysis, which resulted in eliminating only one sample (pig number 622 sampled at T2).

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 and 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 $p < 0.05$.

The differential abundance analysis was performed using the function "fitZig" in the metagenomeSeq (v.1.26.0) package at the OTU level (Paulson *et al.*, 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).

4. Results

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 7 piglets were positive for both; 8 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 multi-resistant, 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.

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 (6 were *MUC4*^{GG}, *FUT1*^{CC}; 6 were *MUC4*^{GG}, *FUT1*^{CT}; 9 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 7 had *MUC4*^{CG}, *FUT1*^{TT}). In addition, 17 pigs were resistant for ETEC F4 and susceptible for ETEC F18 (7 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.

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 ($p > 0.05$). Moreover, the sex of the animals and the presence/absence of diarrhoea did not affect the weight of the animals ($p > 0.05$). Using the ANOVA analysis, the weight was different between the two ages of the piglets at the three time points (T0, $p = 0.003$; T1, $p = 0.0005$; T2, $p = 0.0004$) and consequently by litter of origin (T0, $p = 0.002$; T1, $p = 0.0001$; T2, $p = 0.0003$). 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=0.01). The *post-hoc* test showed differences between *FUT1*CC and *FUT1*CT genotypes (Tukey's HSD, p= 0.01), but did not show differences between the comparison of *FUT1*CC *vs.* *FUT1*^{TT} and between *FUT1*^{CT} *vs.* *FUT1*^{TT} (Tukey's HSD, p>0.05). *MUC4* genotypes and the presence of ETEC F18 did not affect the weight of animals (p >0.05).

Moreover, at T2 we described that the weight was influenced by the *FUT1* gene (ANOVA, p=0.02) which were referred to *FUT1*^{CC} and *FUT1*^{CT} (Tukey's HSD, p= 0.04) and not to *FUT1*^{CC} *vs.* *FUT1*^{TT} or *FUT1*^{CT} *vs.* *FUT1*^{TT} (Tukey's HSD, p>0.05). *MUC4* genotypes, the presence of ETEC F4 and ETEC F18 did not affect the weight of animals (p >0.05).

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 category 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=0.003) and the occurrence of diarrhoea (categories 0, 1= negative for diarrhoea; category 2= positive for diarrhoea) (p=0.01). 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=0.01) but not with the faecal scores (p>0.05) 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>0.05) at each time point.

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	**0.01**
		R	10	9	
	FUT1	S	45	13	*0.49*
		R	9	4	
T1	*MUC4*	S	34	18	*1*
		R	12	7	
	FUT1	S	38	20	*0.94*
		R	8	5	
T2	*MUC4*	S	45	7	*1*
		R	16	3	
	FUT1	S	49	9	*0.76*
		R	12	1	

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>0.05). Conversely, antibiotic administration showed a significant association with the status of ETEC

F18 at T1 (p=0.017), 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 in the three groups (p=0.004): 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=0.009) and at T2 (p=0.02), with more animals showing diarrhoea in the group O compared to the other two groups (Figure S2, Table 2).

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	0.61
	O	17	7	
	C	20	4	
T1	P	11	10	*0.009*
	O	14	10	
	C	21	3	
T2	P	19	4	*0.02*
	O	18	6	
	C	24	0	

Faecal microbiota sequencing, identification and annotation of OTUs

After quality control, a mean of 36706 reads were available for each sample. OTU counts per sample and OTU taxonomical assignments are available in supplementary Table S4. Sequences across the whole sample sets were successfully clustered into 1080 OTUs and only (10/1080) 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=0.004; Figure S4).

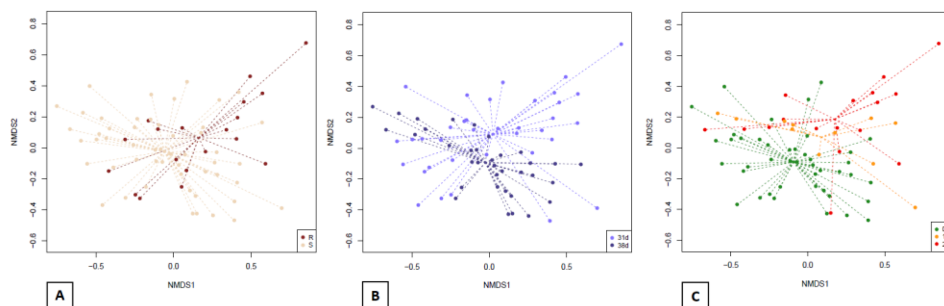
Differences in the faecal microbiota at T0 in piglets

The overall composition of the microbiota at T0 (NMDS, Figure 1) was mainly driven by *MUC4* gene (Adonis test, $p = 0.004$), the age of the piglets (Adonis test, $p = 0.001$) and the faecal score (Adonis test, $p = 0.001$), whereas *FUT1* genotype and the presence of ETEC F4 and ETEC F18 had no influence (Adonis test, $p>0.05$). The beta diversity was different only between the class of ages of the piglets (ANOVA test, $p=0.001$; 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 distributed in groups P, C and O (Figure S1B). In the NMDS plot, the *MUC4* genotypes (envfit test, $p=0.018$; Figure 1A), the age of piglets (envfit test, $p=0.039$; Figure 1B) and the faecal score (envfit test, $p=0.0004$; 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, $p>0.05$; Figure S5A, S5C), but the co-factor age of the piglets revealed differences (ANOVA test, $p=0.002$; Figure S5B), showing the 38 days-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, $p>0.05$; Figure S5A, S5C) and the age of piglets (ANOVA test, $p=0.001$; 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) 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.

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).



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, $p = 0.0009$), whereas *MUC4* and *FUT1* genotypes, ages, faecal score and the status of ETEC F4 and ETEC F18 had no influence (Adonis test, $p>0.05$). The beta diversity was not different between the antimicrobial treatment groups (ANOVA test, $p>0.05$; 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, $p=0.02$; Figure 2A). The alpha diversity at OTU level was different between the antimicrobial groups (ANOVA test, $p=0.03$; 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, $p>0.05$; 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 the group C and in the group P comparing to the group O (Figure 3A). Accordingly, ANOVA analyses showed significant differences (p=8.56 x 10-5) among the three groups at the OTUs level. In addition, the *post-hoc* test showed differences between the O *vs.* C group (Tukey's HSD, p=0.0001), P *vs.* O group (Tukey's HSD, p=0.01) and did not show a significant p-value among C *vs.* P group (Tukey's HSD, p>0.05). When comparing two groups, we have described 144 DA OTUs in the comparison P *vs.* O, 127 O *vs.* C and 65 by comparing P *vs.* C (Tables S8, S9 and S10, respectively). In the Venn diagram, the overlapping DA OTUs between the two by two groups comparison is showed (Figure 2C).

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 are coloured by comparisons: control *vs.* amoxicillin parenteral-administered (C *vs.* P, yellow), control *vs.* amoxicillin oral-administered (C *vs.* O, blue) and amoxicillin oral-administered *vs.* amoxicillin parenteral-administered (O *vs.* P, green).

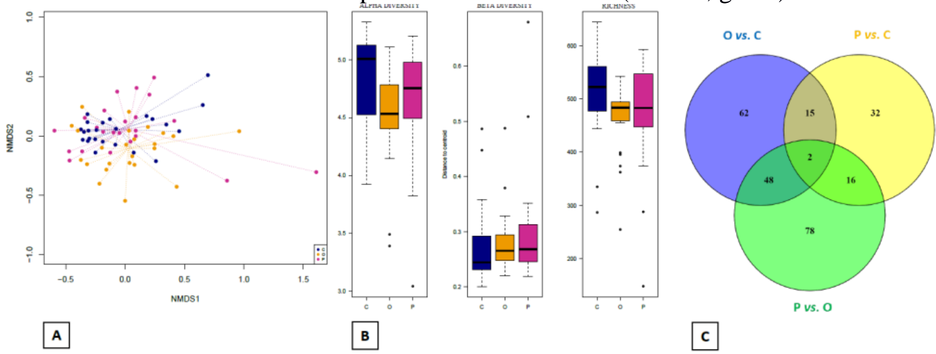
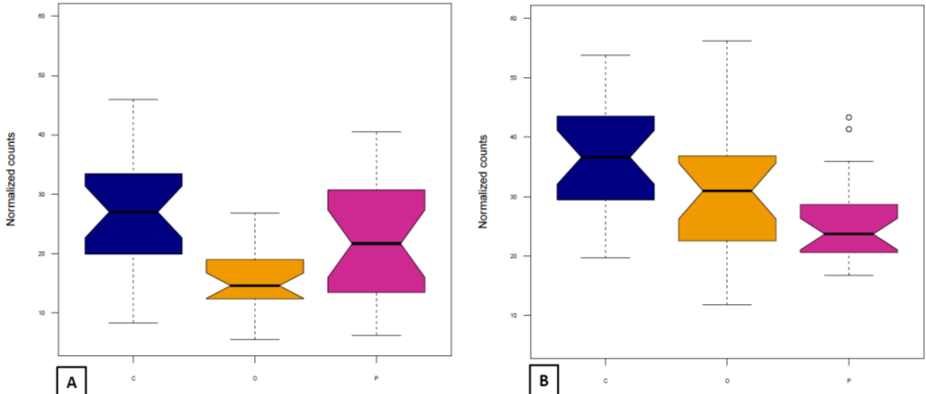


Figure 3: Abundances of *Lactobacillus* spp. at T1 (A) and T2 (B) among the experimental groups. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral-administered (O, orange) and amoxicillin parenteral-administered (P, purple). Abundances were calculated as the addition of normalized for OTUs annotated as *Lactobacillus* spp. in the whole dataset (MetagenomeSeq R package). The notched boxplots displays the confidence interval around the median. If two boxes' notches do not overlap there is 'strong evidence' (95% confidence) their medians differ and consequently the difference is described as "statistically significant at the .05 level".

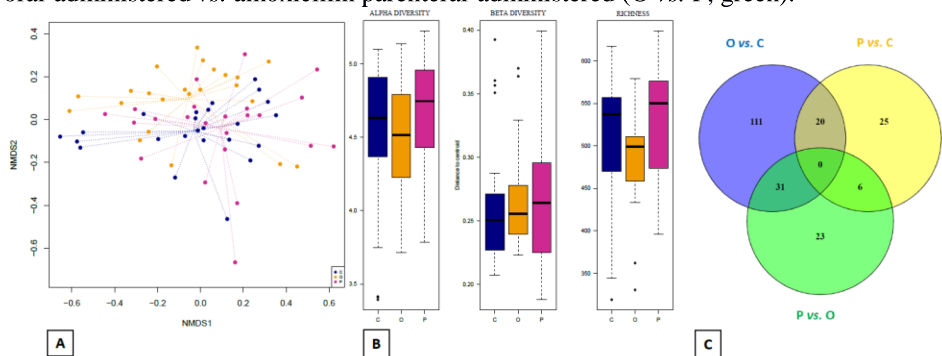


Differences in the faecal microbiota at T2 in piglets

The overall composition of the microbiota at T2 (NMDS, Figure 4) was mainly linked to the antibiotic treatment (Adonis test, $p = 0.0001$) and the faecal score (Adonis test, $p = 0.0002$), whereas *MUC4*, *FUT1* genotypes, the age and the presence of ETEC F4 and ETEC F18 had no influence (Adonis test, $p>0.05$). The beta diversity was not significantly different across the antimicrobial treatment groups (ANOVA test, $p>0.05$). In the NMDS plot, the centroids of the group O appeared separated from the P and the C group, resulting in a significant value (envfit test, $p=0.03$; Figure 4A). The alpha diversity at OTU level and the observed microbial richness did not show differences among the groups (ANOVA test, $p>0.05$; 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 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 the group C (Figure 3B). ANOVA analyses showed significant differences ($p=0.001$) between the experimental groups. In addition, the *post-hoc* test showed significant differences between P *vs.* C group (Tukey's HSD, $p= 0.0009$) and a significant trend between the O *vs.* C group (Tukey's HSD, $p= 0.055$). No differences were described between O and P group (Tukey's HSD, $p>0.05$). When comparing two groups, we have described 162 DA OTUs in the comparison O *vs.* C, 61 P *vs.* O and 51 when comparing P *vs.* 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 *vs.* C comparison, we have described *Prevotella copri*, *Ruminococcus* and *Lactobacillus* to be more abundant in the C than in the O group.

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 are coloured by comparisons: control *vs.* amoxicillin parenteral-administered (C *vs.* P, yellow), control *vs.* amoxicillin oral-administered (C *vs.* O, blue) and amoxicillin oral-administered *vs.* amoxicillin parenteral-administered (O *vs.* P, green).



5. 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 post-weaning period is associated with multiple stressors, causing a faecal microbiota dysbiosis, which is among the leading causes

of post-weaning 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 have 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 *et al.*, 2019), thus confirming its beneficial role in porcine gut health. Contrary to what reported by Messori *et al.*, (2013), we did not described *Clostridium barlettii* in the resistant *MUC4* piglets, in accordance to 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 *et al.*, 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* is in accordance with what already reported in 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.*, Submitted; 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.*, Submitted).
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 *et al.*, 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 and Sperling, 2018), which may affect the abundance of Gram-positive commensals, such as *Lactobacillus* species. Moreover, it has been described that the abrupt decrease of *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 and Sperling, 2018; Haas and Grenier, 2016; Waack and 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 multi-drug 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 practises, 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 multi-resistant ETEC infection and this effect is stronger when the antibiotic is orally administered than parenterally. Alternative control measures, such as selection of resistant genotypes or vaccination, should be included in farm management practices to preserve a balanced and stable gut microbiota in weaners.

6. Declarations

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Availability of data and materials
The raw sequencing data has been submitted to NCBI's Sequence Read Archive (SRA) repository (BioProject: PRJNA543556; Biosample: SUB5638166, accessions 11771978 to 11772198).
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Conflict of Interest Statement
The authors declared that they had no conflict of interests with respect to their authorship on the publication of this article.

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8. Supplementary information

Additional supporting information may be found online in the Supporting Information section at the end of the article. (https://onlinelibrary.wiley.com/doi/10.1111/jbg.12432)

Supplementary figures

Figure S1: Description of our cohort. Distribution of animals in the experimental groups (C=control, P= parenteral administrated, O=oral administrated).
(A) Bar plot of sex represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each sex: female (pink), and male (blue); (B) Bar plot of age at weaning represented in each experimental group. For each group, the bar plot represents the number of individuals ascribed to each age: 31 days-old (31d, orange) and 38 days-old (38d, grey); (C) Bar plot of litter of origin represented in each experimental group. For each group, the bar plot represents the number of individuals ascribed to each litter number: 14N178 (red), 153 (blue), 156 (green), 159159 (purple), 169099 (orange), 16T115 (yellow), 174 (brown) and 177053 (pink); (D) Bar plot of *MUC4* genotypes represented in each experimental group. For group, the bar plot represents the number of individuals ascribed to each *MUC4* genotypes: $MUC4^{CC}$ (red), $MUC4^{CG}$ (grey) and $MUC4^{GG}$ (beige); (E) Bar plot of *FUT1* genotypes represented in each experimental group. For group, the bar plot represents the number of individuals ascribed to each *FUT1* genotypes: $FUT1^{CC}$ (red), $FUT1^{CT}$ (grey) and $FUT1^{TT}$ (beige).

Figure S2: Description of health status of our cohort. Distribution of animals in the experimental groups (C=control, P= parenteral administrated, O=oral administrated).
(A) Bar plot of ETEC F4 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T0: negative (green) and positive (red); (B) Bar plot of ETEC F4 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T1: negative (green) and positive (red); (C) Bar plot of ETEC F4 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T2: negative (green) and positive (red); (D) Bar plot of ETEC F18 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T0: negative (green) and positive (red); (E) Bar plot of ETEC F18 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T1: negative (green) and positive (red); (F) Bar plot of ETEC F18 represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T2: negative (green) and positive (red); (G) Bar plot of ETEC F4 represented in each of the *MUC4* genotypes identified as resistant (R) and susceptible (S). For each *MUC4* genotype, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T0: negative (green) and positive (red); (H) Bar plot of ETEC F4 represented in each of the *MUC4* genotypes identified as resistant (R) and susceptible (S). For each *MUC4* genotype, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T1: negative (green) and positive (red); (I) Bar plot of ETEC F4 represented in each of the *MUC4* genotypes identified as resistant (R) and susceptible (S). For each *MUC4* genotype, the bar plot represents the number of individuals ascribed to each ETEC F4 status at T2: negative (green) and positive (red); (L) Bar plot of ETEC F18 represented in each of the *FUT1* genotypes identified as resistant (R) and susceptible (S). For each *FUT1* genotype, the bar plot represents the number of individuals ascribed to each ETEC F18 status at T0: negative (green) and positive (red); (M) Bar plot of ETEC F18 represented in each of the *FUT1* genotypes identified as resistant (R) and susceptible (S). For each *FUT1* genotype, the bar plot represents the number of individuals ascribed to each ETEC F18 status at T1: negative (green) and positive (red); (N) Bar plot of ETEC F18 represented in each of the *FUT1* genotypes identified as resistant (R) and susceptible (S). For each *FUT1* genotype, the bar plot represents the number of individuals ascribed to each ETEC F18 status at T2: negative (green) and positive (red); (O) Bar plot of diarrhoea status represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each diarrhoea status at T0: score 0 (green), score 1 (orange) and positive (red); (P) Bar plot of diarrhoea status represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each diarrhoea status at T1: score 0 (green), score 1 (orange) and positive (red); (Q) Bar plot of diarrhoea status represented in each of the experimental groups. For each group, the bar plot represents the number of individuals ascribed to each diarrhoea status at T2: score 0 (green), score 1 (orange) and positive (red).

Figure S3: Relative abundance of the Phyla (A) and Genera (B) in each time point for every individual belonging to each experimental group (C=control, P= parenteral

administrated, O=oral administrated). Only Genera present in at least 20% of the individuals are shown.

Figure S4: Plots include all the samples obtained at T0, T1 and T2. 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 time point: T0 (blue), T1 (purple) and T2 (yellow).

Figure S5: Box plots include only the samples obtained from T0. (A) 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 *MUC4* genotype. Samples are coloured by *MUC4* genotypes: *MUC4*^{CC} (red), *MUC4*^{CG} (grey) and *MUC4*^{GG} (beige); (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 age at weaning. Samples are coloured by age: 31 days-old (31d, light blue) and 38 days-old (38d, blue); (C) 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 faecal score. Samples are coloured by diarrhoea status: score 0 (green), score 1 (orange) and positive (red).

Figure S6: Heat maps illustrating the abundances of differentially abundant (DA) OTUs. (A) Heat map of the OTUs differentially expressed at T0 among the susceptible (light pink) and the resistant (red) *MUC4* genotypes; (B) Heat map of the OTUs differentially expressed at T0 among the non-diarrhoeic (green) and diarrhoeic (red) animals; (C) Heat map of the OTUs differentially expressed at T1 among the experimental groups. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral-administered (O, orange) and amoxicillin parenteral-administered (P, purple); (D) Heat map of the OTUs differentially expressed at T2 among the experimental groups. Samples are coloured by experimental groups: control (C, blue), amoxicillin oral-administered (O, orange) and amoxicillin parenteral-administered (P, purple).

Supplementary tables

Table S1: Ingredient and chemical composition of the concentrates of post-weaning pigs.

Table S2: Table summarizing the phenotypic traits and genotypes of piglets.

Table S3: Number of pigs belonging to the experimental groups (C=control, P= parenteral administrated, O=oral administrated) along their distribution on the genotypes for *MUC4* and *FUT1*.

Table S4: The OTU taxonomical assignments and OTU counts in each individual and time point of the whole dataset are showed.

Table S5: Differentially abundant OTUs when comparing the *MUC4* genotypes and the age categories at T0.

Table S6: Differentially abundant OTUs when comparing the non-diarrhoeic and diarrhoeic animals at T0.

Table S7: Differentially abundant OTUs when comparing the experimental groups at T1.

Table S8: Differentially abundant (DA) OTUs when comparing P (parenteral administrated) *vs.* C (control) group at T1. DA OTUs were used to be plotted in the Venn diagram (Figure 2).

Table S9: Differentially abundant (DA) OTUs when comparing O (oral administrated) *vs.* C (control) group at T1.DA OTUs were used to be plotted in the Venn diagram (Figure 2).

Table S10: Differentially abundant (DA) OTUs when comparing P (parenteral administrated) *vs.* O (oral administrated) group at T1. DA OTUs were used to be plotted in the Venn diagram (Figure 2).

Table S11: Differentially abundant OTUs when comparing the experimental groups at T2.

Table S12: Differentially abundant (DA) OTUs when comparing P (parenteral administrated) *vs.* C (control) group at T2. DA OTUs were used to be plotted in the Venn diagram (Figure 4).

Table S13: Differentially abundant (DA) OTUs when comparing O (oral administrated) *vs.* C (control) group at T2. DA OTUs were used to be plotted in the Venn diagram (Figure 4).

Table S14: Differentially abundant (DA) OTUs when comparing P (parenteral administrated) *vs.* O (oral administrated) group at T2. DA OTUs were used to be plotted in the Venn diagram (Figure 4).

GENERAL DISCUSSION

Section IV

Weaning can be considered as the period that causes stress to the pigs during rearing. Among the stresses, it includes a sudden shift from the milk diet to a cereal-based diet, the abrupt separation from the sows, and other environmental changes like the mixing of several litters in a single production slot (Pluske *et al.*, 2018, 1997). All this occurs when the immune system is still not mature and the thermoregulation and the digestive capacity are not well managed by the young piglets. Beyond this, the gut microbiota composition suffers also from drastic shifts that can result in an increased risk to pathologies, especially to enteric diseases.

In the present thesis, enteric disorders in piglets throughout the weaning period have been investigated, analysing the gut microbiota modifications related to different weaning ages and amoxicillin administrations during the colibacillosis. In this section, the main results of the PhD project will be discussed from biological and technical perspectives and, then a summary of challenges and opportunities for the prevention of piglet weaning diarrhoea will be proposed.

1. On microbiota's role in the post-weaning diarrhoea of piglets

In intensive farming systems, piglets are weaned much earlier than in a natural environment and several studies comparing different weaning strategies have shown that increasing weaning age improved both health and growth. Delaying the age at weaning in production farms has been proposed as a possible strategy to modulate and decrease the weaning-associated problems (Früh, 2011), as a measure to cope with the future necessary parsimonious use of antibiotics. However, only few studies have been performed investigating how early life management affects the early-

life establishment of the pig gut microbiota together with the occurrence of enteric disorders.

In our first study, different practises of weaning have been investigated, describing the gut microbiota composition in piglets weaned at different ages (Figure 6), ranging from ultra-early weaning (14 days old), main weaning ages in pig intensive farms (21 and 28 days old) and to organic-like weaning (42 days of age). This study was carried out in antibiotic-free and pathogen-free conditions, allowing us to study the interaction of the gut microbiota composition and the weaning ages without other affecting determinants. In contrast, in our second study, we investigated antibiotic treatment alternatives in a context of natural infection with Enterotoxigenic *E. coli*.

Figure 6: summary of the design of the study (Paper I)

N. piglets	Weaned at	Birth (d0)	d14	d21	d28	d35	d42	d49	d60
12	14 days	⟶	Feces (12pigs)	Feces (12pigs)	- - -	- - -	- - -	- - - →	Feces (6pigs)
12	21 days	⟶	⟶	Feces (12pigs)	Feces (12pigs)	- - -	- - -	- - - →	Feces (6pigs)
12	28 days	⟶	⟶	⟶	Feces (12pigs)	Feces (12pigs)	- - -	- - - →	Feces (6pigs)
12	42 days	⟶	⟶	⟶	⟶	⟶	Feces (12pigs)	Feces (12pigs)	Feces (6pigs)

Interestingly, the study comparing different weaning ages showed that animals weaned late (at 42 days of age) presented no diarrhoea and an increased gut microbiota diversity. These results could suggest that late weaning provides a competitive advantage to piglets accumulating a

higher diversity of potentially beneficial microbes prior to the stressful and risky weaning transition. Since no diarrhoea was observed for the piglets weaned at 42 days of age, we assumed that weaning at early age constitutes a risk factor for having animals less robust against enteric disorders. Since our study was carried out on a small number of animals, a wider investigation evaluating if a late weaning could be protective to pathogen colonization, could represent an excellent starting point to confirm our findings and provide to the farmers consistent data on possible health benefits deriving from a modulation of existing weaning practices. Rethinking the age at weaning should be considered in the design of new intensive herds as a "good practice" to improve robustness and health status of animals, even if it could be a difficult issue to overstep by farmers having existing facilities, as it will be analysed later in the discussion.

Late-weaned animals were characterized by higher richness at pre-weaning, enhancing a protective effect on the gut homeostasis. In fact, several studies stated that a stable gut microbiota composition is correlated with a higher richness compared to enteric diseases and unhealthy or inflammatory states often related with a lower richness of bacteria (Chang *et al.*, 2008; Dou *et al.*, 2017; Lozupone *et al.*, 2013; Willing *et al.*, 2010). The most practically relevant finding obtained in the study was the detection of an increase of *F. prausnitzii* relative abundance in the piglets weaned at 28 and 42 days old. We hypothesize that *F. prausnitzii* and the absence of diarrhoea status in this group of animals could represent a key factor for the increase of piglets' resilience. *F. prausnitzii* abundance is correlated with the establishment of primo-colonizing bacteria that create an adequate environment in a strictly anaerobic condition (Hopkins *et al.*, 2005). Moreover, it should be taken into account that the presence of *F.*

prausnitzii along the GIT may also result from a combination of environmental factors such as other commensal species, redox mediators, oxygen concentration, mucus layer as well as bile salt concentrations and pH (Lopez-Siles *et al.*, 2012).

In literature, indeed, it is well-established that *F. prausnitzii* plays an important role in GIT homeostasis and appears as less abundant in enteric pathological status, which makes it a gut health biomarker (Miquel *et al.*, 2013). In fact, *F. prausnitzii* depletion is correlated with CD, IBD, CRC and IBS (Cao *et al.*, 2014; Dave *et al.*, 2011; Lopez-Siles *et al.*, 2018, 2017, 2015, 2014; Martín *et al.*, 2017, 2015; Miquel *et al.*, 2016, 2013; Sitkin and Pokrotnieks, 2018; Sokol *et al.*, 2009, 2008).

Considering their beneficial effects on GIT, *F. prausnitzii* together with *Akkermansia municiphila*, *Bacteroides* spp. and *Clostridium butyricum* are now being studied as NGP, both in animals and humans (Chang *et al.*, 2019; Langella *et al.*, 2019). While most used probiotics are generally recognised as safe and some of them show beneficial effects in the homeostasis of gut microbiota, results obtained about the prevention or even the treatment of specific diseases remain marginal (Chang *et al.*, 2019). Based on these findings, identification and characterization of novel and disease-specific NGP are urgently needed (Chang *et al.*, 2019). However, it has to be considered that the inclusion in the Qualified Presumption of Safety (QPS) Microorganisms list of EFSA, for NGPs, will be a crucial stage and it might be difficult considering the lack of data about their safety if used in both animal and humans (Brodmann *et al.*, 2017; Saarela, 2019). Overall, while technical and bureaucratic issues are being tackled, it would be interesting to perform new studies aiming at evaluating the use of *F. prausnitzii* as a tool for enhancing the gut health

of livestock and whether it is able to prevent the outcomes of enteric diseases. Application at large scale of new probiotic strategies promoting gut eubiosis could represent a valuable approach even to achieve the reduction of drug use in farms, fighting antimicrobial resistance and costs for farmers.

In a complementary approach, our second article investigated a common situation occurring in commercial pig herds during the weaning period: antibiotic administration and colibacillosis caused by MDR ETEC strains at weaning (Figure 7). PWD is mainly caused by ETEC F4 and ETEC F18 (Baker *et al.*, 1997; Luppi, 2017) being responsible of pig morbidity and mortality, causing considerable economic losses to farmers worldwide (Fairbrother and Gyles, 2012). Different questions were investigated: *i)* the host genotype *versus* ETEC F4 and ETEC F18 infection *ii)* the outbreak of colibacillosis against which antibiotics are usually administered to weaning piglets and *iii)* the recent spread of MDR bacteria focused the attention on antibiotic resistance, one of the world's most pressing public health issue.

Figure 7: summary of the design of the study (Paper II)

Group	T0 (d4)	T1 (d9)	T2 (d16)
P	Amoxicillin		Withdrawal period
O	Amoxicillin		
C	Placebo		

A first question to assess during the study design concerned the choice of the antibiotic to be used in the study. In 2005, WHO published a regularly updated list of all antimicrobials currently used in humans, mostly prescribed in veterinary medicine also, grouped into three categories based on their importance to human medicine (WHO, 2017):

i) Critically Important;
ii) Highly Important;
iii) Important.

Antibiotics belonging to the first category are not allowed for the veterinary usage. In June 2017, the EU Commission adopted the new One Health Action Plan against Antimicrobial Resistance and the theme 'reduce, replace and re-think' has been created by the EMA and EFSA for a new responsible antibiotic usage in livestock production (Murphy *et al.*, 2017).

The main aims of these plans are:

i) reduce antimicrobial consumption;
ii) reduce the usage of antimicrobials in animals with alternative measures;
iii) use critically important antimicrobials for human medicine in animals only as a last resort;
iv) re-think the livestock system implementing farming practices to prevent the introduction and spread of disease.

All these measures are essential for the future of animal and public health. For this reason, management practises should be implemented at farm levels to limit the spread of MDR bacteria and infections when the use of antibiotic is still essential. Thus, in the second study, we decided to include amoxicillin administration, one of the most prescribed antibiotic in commercial pig herds at weaning for treatment and control of severe and systemic infections (Burch and Sperling, 2018; Haas and Grenier, 2016;

Waack and Nicholson, 2018). Since amoxicillin is not included in the CIA list, it remains available for veterinary medicine. Nevertheless, it is recommended that its use takes place under responsible use considerations, such as after laboratory diagnosis, culture and sensitivity testing (WHO, 2017). Oral administration is by far the most common route of administration for antimicrobials in pigs (Callens *et al.*, 2018, 2012; Merle *et al.*, 2012). Several studies reported that oral administration of antimicrobials increases the risk of AMR (Burow *et al.*, 2014; Zhang *et al.*, 2013). The oral route is usually associated with a suboptimal administration, meaning a non-correct usage and dosage of the molecule, often resulting in an overuse of antibiotics in healthy pigs. In fact, sick animals usually do not assume the normal ratio of daily feed and, when the medication is supplied in pig feed, it is more difficult to achieve the correct dose of antibiotics.

In intensive herds, amoxicillin is mainly prescribed to treat streptococcosis by oral route, but it is common to find in the same herd two contemporary infections in which streptococcosis coexists with colibacillosis.

Considering what is described above, the second article is based on a common situation frequently occurring in commercial pig herds during the weaning period: when animals are naturally infected by ETEC strains and treated with amoxicillin. Since amoxicillin is administrated by oral route, our hypothesis was that a different administration route, such as the parenteral one, could differently affect the enteric disorders during a natural infection of ETEC. Different studies have been carried out on laboratory animals investigating different amoxicillin administration routes (Aguilar *et al.*, 2004; Marx *et al.*, 2014; Zhang *et al.*, 2013).

To the best of our knowledge, this is the first paper in pigs, in which the oral antibiotic treatment was compared with the parenteral administration. The results showed a major impact on the health status of animals, with oral antibiotics being correlated with a higher risk of diarrhoea and a higher shedding of ETEC F18, if compared to parenteral administration (note that the ETEC strains present in the animals were resistant to amoxicillin). Moreover, the alpha diversity, which weighs both microbial community richness (number of different species) and evenness (equitability), was found significantly lower in animals treated by oral route by comparison to the parenteral administration and the control group. This finding is coherent with the direct effect on the gut microbiota composition exerted by the oral administration. Alike, a decreased abundance of the commensal *Lactobacillus* was described in the group treated by oral route. This finding is in accordance with a previous study (Connelly *et al.*, 2018) in which a lower abundance of *Lactobacillus* was associated with the oral administration of amoxicillin, even though parental administration was not included for comparison. Amoxicillin, according to its pharmacological activity, affects the abundance of Gram-positive commensal bacteria (Burch and Sperling, 2018) and this effect is thus positively effective when the disease is caused by a Gram-positive bacterium. However, different Gram-positive bacteria, such as the *Lactobacillus* genus, are considered essential bacteria for maintaining the intestinal eubiosis and play a major role in disease prevention. The decrease of *Lactobacillus* spp. is frequently associated with an increased risk of enteritis at weaning (Konstantinov *et al.*, 2006). The same trend of the oral treatment was not recorded after a parenteral administration and the faecal microbiota of parenteral administrated piglets was comparable to the control group. After the

withdrawal period of amoxicillin (7 days), the control group showed a higher abundance of OTUs belonging to the *Lactobacillus* genus compared to both amoxicillin treated groups, proving that even the parenteral administration exerts a long-term effect on the abundance gut bacteria. Beyond *Lactobacillus*, comparing the gut microbiota compositions among groups, we described how control and parenteral administered group showed a higher abundance of *Prevotella copri* and *Ruminococcus* spp. compared to the orally treated group, in accordance with previous published data (Connelly *et al.*, 2018; Konstantinov *et al.*, 2006). Globally, our results highlight that the faecal microbiota composition of piglets is highly affected by the oral administration of the amoxicillin.

It is important to consider that even in absence of an antimicrobial administration other factors can influence microbiota diversification. Enteric disorders, also caused by colibacillosis, are differently affected by the host genotype, and it is known that animals are not equally susceptible to this infection. For these reasons, the interactions among the host genetics, the phenotype traits, such as the presence of diarrhoea and the faecal microbiota composition in animals naturally infected with ETEC F4 and ETEC F18, were also investigated. Genetic difference exists for susceptibility to ETEC F4 and ETEC F18 infection in piglets and *MUC4* and *FUT1* represent the best generally accepted marker genes (Jørgensen *et al.*, 2004; Luise *et al.*, 2019; Muñoz *et al.*, 2018; Rampoldi *et al.*, 2011; Wang *et al.*, 2012). In our study, the association of the *MUC4* and *FUT1* genes with the shedding of ETEC F4 and ETEC F18 agreed with reported studies (Joller *et al.*, 2009; Jørgensen *et al.*, 2004; Luise *et al.*, 2019; Muñoz *et al.*, 2018; Rampoldi *et al.*, 2011; Wang *et al.*, 2012). Nevertheless, in contrast to data reported in literature, we described an

association among the *MUC4* resistant genotype and the presence of diarrhoea. Jørgensen *et al.*, (2004) reported the possibility to have resistant genotypes with susceptible phenotypes, which would explain our findings. Likewise, the presence of diarrhoea was not associated with the susceptible *FUT1* genotype, contrary to previous results (Luise *et al.*, 2019; Wang *et al.*, 2012). We hypothesize that in our case the fact of concomitant ETEC F4 and F8 infections, *plus* the resistance to amoxicillin showed by these strains, could interfere to the expected results. In addition, the variability of gut microbiota composition before the infection could have had a role in the outcome of diarrhoea and infection susceptibility. Despite the lack of results of ETEC in the literature, in the case of *Salmonella* (Argüello *et al.*, 2019, 2018b; E Barba-Vidal *et al.*, 2017; Drumo *et al.*, 2016) and *Clostridium difficile* (Grzeskowiak *et al.*, 2019, 2018; Jurburg *et al.*, 2019) infections, the correlation between the infection and the gut microbiota composition is well defined.

In this experiment, we enrolled animals, which were naturally affected by ETEC infection, meaning that bacterial load was not homogeneous among piglets. This is a limit of our investigation, since it may have increased the variation of the parameters within the experimental groups, thus reducing the power of our study. At the same time, compared to an experimental infection, the enrollment of naturally infected piglets better reflects the conditions occurring on the field during weaning. In our study, both ETEC F4 and ETEC F18 were classified as MDR which is a common feature of ETEC strains Worldwide (Hedegaard *et al.*, 2017; Jiang *et al.*, 2019; Luppi *et al.*, 2015; Magistrali *et al.*, 2018; Rosager *et al.*, 2017; Smith *et al.*, 2010). In this scenario, the use of amoxicillin could take over the ecological niche and exert a selective pressure on the resistant pathogen

strains. Administering amoxicillin through oral route could exert a selective pressure amending the gut microbiota and open the gate to a higher risk of pathogen colonization. Consistently with the previous study, even in the second study, animals were weaned at different ages and the beneficial role of a late weaning age was confirmed. A more homogeneous and richer microbiota composition in the late weaned piglets was described.

2. On technical choices for the study of gut microbiota in our work

For a better understanding of the results obtained in the two trials performed in this PhD project, it is useful to take into account the two approaches to metagenomic analysis of microbial communities that are available nowadays: *i*) Whole (meta-)Genome Sequencing (WGS) and *ii*) 16S rRNA gene fragment analysis.

We applied the 16S amplicon approach, the most commonly employed method to analyse gut microbiomes, and that presents several important advantages (Ranjan *et al.*, 2017):

i. it is cost effective;
ii. data analysis can be performed by established pipelines without using large computing infrastructures;
iii. there is a large body of archived data for reference.

Indeed, considering that the 16S technique is the most used by livestock researchers, it allows to compare results more effectively.

However, there are multiple substantial advantages of the WGS approach such as the accuracy of taxa at the species level and a direct access to the global microbial genes present in the sample. The biggest disadvantage remains still that WGS is more expensive, requires more specialized and

extensive data analysis and may be necessary to sequence the microbial genomes with high coverage and increased cost for studying low-abundant microbes (Sims *et al.*, 2014). In our case, the 16S rRNA sequencing remained the first choice to analyse the microbiota composition, essentially due to a cost-effectiveness issue.

Another important issue to be considered is that intestinal microbiota can be deeply modified across the different intestinal tracts, both in terms of composition and abundance (Crespo-Piazuelo *et al.*, 2018; Dieterich *et al.*, 2018; Zhao *et al.*, 2015). Moreover, other factors could influence the biogeography of bacteria within the gut, including diet, antimicrobials, mucus, adherence and the host immune system (Donaldson *et al.*, 2015). In our investigations, we only considered the modification occurring at the faecal microbiota level to ensure consistency between experimental trials in environmentally controlled facilities and in field studies. This allow us to sample the same animals at different time points. Nevertheless, it would be interesting to confirm that the observed results are coherent with the microbial composition at different intestine sections.

3. Summary of challenges and opportunities for the prevention of diarrhoea at weaning

In commercial herds, a robust, balanced and already well-diversified and gut microbiota able to limit the occurrence of enteric disorders should be expected in piglets at weaning. In our studies, we achieved a good correlation between relevant phenotypic traits and abundance of beneficial microbes on late weaned animals, and about the impact of antibiotics on the gut microbiota composition during a natural ETEC infection. From a practical point of view, once the results are confirmed on a larger scale, it

will be interesting to disseminate these scientific results to farmers. In fact, applied science as intended in this PhD thesis should be carried out with a continuous focus on its practical effectiveness in herds. For a long time, several studies have been carried out in order to prevent the problem of diarrhoea at weaning improving managing practises in herds (Zimmerman *et al.*, 2012). In previous years, studies were mainly based on phenotypical data collection, histological analysis, vaccination and feed supplementation (Alexa *et al.*, 1995; Baranyiova and Holub, 1993; Boudry *et al.*, 2002; Ciosek *et al.*, 1983; Driesen *et al.*, 1993; Hampson *et al.*, 1988; Kyriakis *et al.*, 1997; Lecce, 1983; Melin *et al.*, 2000; Nabuurs *et al.*, 1986; Nabuurs, 1998; Schone *et al.*, 1988; Shu *et al.*, 2001; Svensmark *et al.*, 1989). Nonetheless, thanks to the invention of new techniques like high-throughput sequencing, new studies focusing on the prevention of diarrhoea should allow achieving new results and make closer the possibility of interventions. In my view, the coupling of the gut microbiota analysis with the modern 'omics technologies, such as transcriptomics and metabolomics in addition with managing practises, could help on finding feasible alternatives for fighting the diarrhoea at weaning in production herds.

At the production farm level, and in order to optimize production efficiency and animal welfare, producers should be aware of economic consequences of different protocols for preventing diarrhoea and be able to choose the solution that fits into their productive reality. A cost-benefit analysis should be applied to estimate the strengths and weaknesses of alternative control measures for limiting diarrhoea at weaning. However, literature delineating the economic costs associated with this critical period is relatively scarce. A European study carried out in 504 herds over a 3-

year period (2014-2016), reported that *E. coli* was the cause of PWD in 83.3%, of which 45.8% were ETEC F4 and 37.5% ETEC F18 (Vangroenweghe and Luppi, 2019). This relevant result showed a high prevalence of infected farms. Moreover, depending on the severity of the disease, the cost of PWD was estimated to range from €40 to €314 per sow per year (Sjölund *et al.*, 2014). Likewise, farmers should add to these estimations the cost of antibiotics usage at weaning, although it could be negligible given the low price of some antibiotic formulations.

Since the antibiotic molecules are becoming less and less effective due to the increase of antibiotic resistances, the incidence of diseases is not decreasing. For this reason, alternative molecules with an antimicrobial effect should be taken into consideration. This is the case of the Zinc oxide; in fact, after the recent restriction use of colistin (EMA, 2016b), Zinc oxide became the first choice for treatment of colibacillosis and it is common used in a therapeutic dosage for the prevention of PWD (Hedegaard *et al.*, 2017; Heo *et al.*, 2013; Pluske, 2013; W. Wang *et al.*, 2019).

The use of pharmacological dose of Zinc can prevents diarrhoea and colibacillosis, doses of Zinc oxide in piglet weaning diets stabilises intestinal microbiota and prevents adhesion of pathogenic bacteria to the intestinal villi, which prevent many problems associated with post-weaning diarrhoea (Roselli *et al.*, 2003; W. Wang *et al.*, 2019). However, recent findings highlighted its negative effects. A study conducted in 2015, suggested that the use of high doses of dietary zinc beyond 2 weeks after weaning should be avoided in pigs due to the possible increase of antibiotic resistance in Gram-negative bacteria (Vahjen *et al.*, 2015). The Agency's Committee for Medicinal Products for Veterinary Use (CVMP) concluded that the benefits of zinc oxide for the prevention of diarrhoea in pigs do

not outweigh the risks for the environment (EMA, 2017). The committee recommended a refusal on future authorisations for medicinal products containing Zinc, and a withdrawal of approvals for existing products. From 21 June 2017, the EU gave all its member states up to five years to phase out ZnO at medicinal levels in piglet feeds (EMA, 2017).

Another important point to take into account is that, though still a niche in the food market especially in the US and Europe, increasing consumer awareness is paving the way for a growing market in organic and/or antibiotic-free foods. In fact, in a survey carried out in 2008 in the US, consumers already displayed a strong attitude against the use of antibiotics in animal husbandry (Brewer *et al.*, 2008). Interestingly, about one third of the respondents declared that they were unwilling to purchase food from antibiotic-treated animals and almost one quarter claimed that they had reduced the intake of meat for the same reason (Brewer *et al.*, 2008). This attitude increased over the last ten years, with 43% of the respondents in a consumer survey declaring that they often or always look for meat with a ‘raised without antibiotics’ claim (Consumer reports, 2018). To face consumers demand for a healthier and welfare friendly food, farmers raise animals that are not only highly productive and healthy but also treated without antibiotics or following the organic system.

A recently published estimation stated that more that 10 million people would be expected to die every year by antibiotic-resistant infections (O’Neill, 2016). This type of studies has contributed to an increased awareness of the consumers, which reacts by asking for organic or antibiotic free farms and for better health and welfare conditions of animals. Specific national and international guidelines are available to discriminate these two production systems. It is important to highlight that

the organic philosophy grown and matured in Europe from the 1920s and the establishment of international groups, such as the International Federation of Organic Agriculture Movements (IFOAM) occurred in the 1970s. Key requirements for organic poultry, cattle and pigs are (IFOAM and FiBL, 2019):

i) Must be raised organically on certified organic land;
ii) Must be fed certified organic feed;
iii) No added growth hormones are allowed;
iv) Only one cycle of antibiotic treatment is allowed throughout the productive cycle of animals;
v) Must have outdoor access.

The animals' organic feed cannot contain animal by-products, antibiotics or genetically engineered grains and cannot be grown using persistent pesticides or chemical fertilizers. However, according to the welfare rules, animals on an organic farm can be treated with antibiotics if they are sick but must be clearly identified and sold separately into the non-organic market.

In the last 4-5 years, the label "Raised without Antibiotics" is being found more frequently on packaging. This indicates that the animals were grown without any antibiotics used for animal health maintenance, treatment or prevention of diseases. This definition may be misleading since meat should be always free of antibiotic residues, and then be defined as "antibiotic-free." In fact, the withdrawal period ensures that there is enough time for the animal's body to clear the antibiotic and the related residues from tissues and organs before slaughter.

Supermarkets and private companies have started to develop policies and positions on the use of antibiotics in meat-producing animals. Some are already offering organic or antibiotic-free meat to customers; others

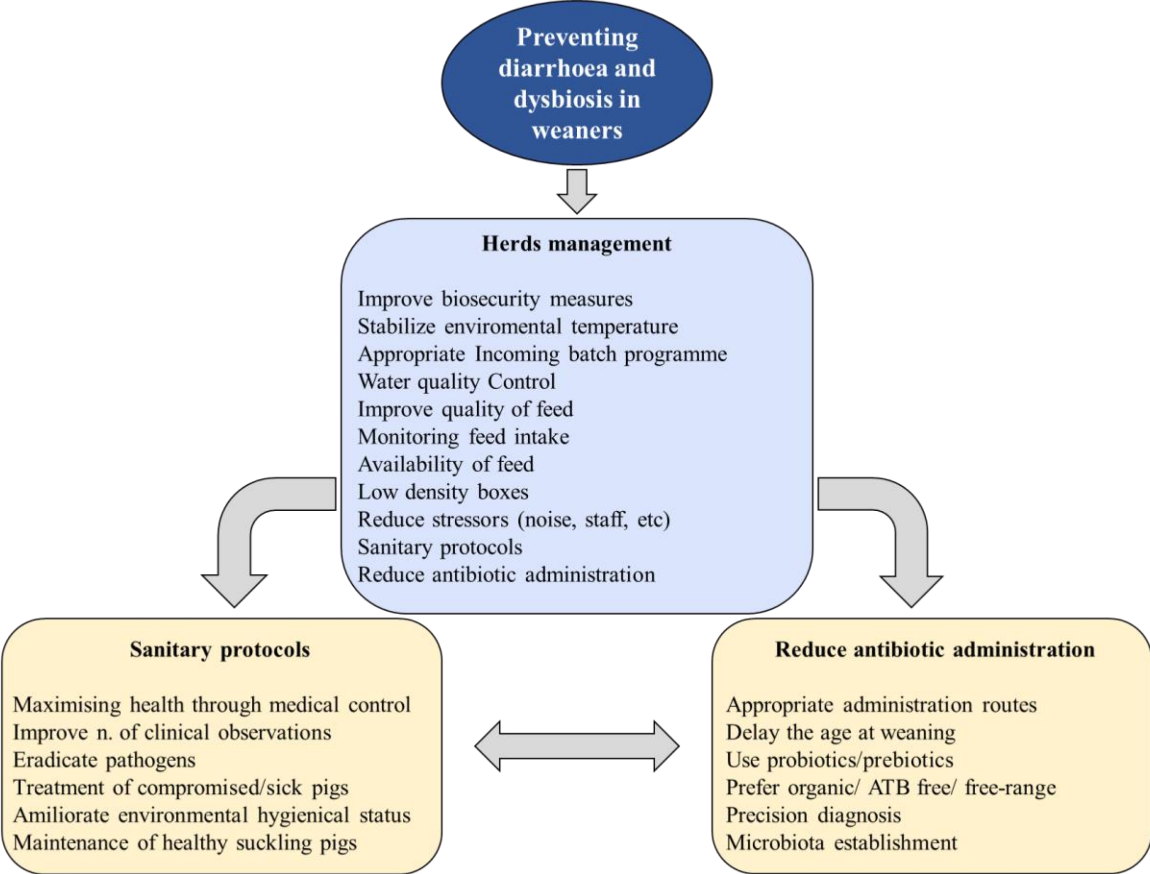
possess clear position statements while others are discussing internally on this rising issue. The consumption of organic food is reported to increase 10.5% each year (IFOAM and FiBL, 2019). However, both the consumer and the health industry have directed the evolution of antibiotic-free and organic pork production. Over the last 20 years, discussions on antibiotic -resistant organisms in human medicine have evolved into the livestock industry to help control potential bacterial resistance over time across all species. In addition, the consumers requesting to eat products of animals raised in organic or antibiotic-free productive systems are motivated by personal health, taste, quality and environmental concerns.

In that scenario, according to our results, the enteric disorders occurring at the weaning moment could be potentially limited and prevented by applying different measures on management practices at the farm levels. A global list of the main procedures to apply at the farm level in order to prevent the diarrhoea at weaning are shown in Figure 8.

In my view, farmers, who need to face the enteric disorders and the colibacillosis at weaning without using antimicrobials, should consider the benefits of late weaning in terms of diarrhoea prevention and an associated richness and composition of the gut microbiota. Furthermore, even during a colibacillosis infection, the strong effect exerted by amoxicillin treatment on the gut microbiota, in a context where the use of antibiotics and Zinc oxide will be limited should pave the way for the introduction of alternative control measures. However, a multi-disciplinary approach, evaluating fattening and finishing periods of pig herds weaned at different ages should be performed in order to have a widen view of performances, health conditions and meat quality, achieving the consumer demand for an healthier and welfare friendly food. Meanwhile, enforcing biosecurity

management practises, selecting resistant genotypes to ETEC infection and making available the inclusion of next-generation probiotics supplementation in feed, should be considered in a holistic strategy aimed at containing enteric disorders in weaners. In this last scenario, our results highlighting *F. prausnitzii* as a relevant candidate to be considered in porcine production need to be confirmed by performing specific probiotic trials in controlled environment and, when legislation makes it possible, in real-life production farms.

Figure 8: procedures to apply at the farm level in order to prevent the diarrhoea and the enteric dysbiosis in piglets at weaning.



CONCLUSIONS

Section V

I. Comparing different weaning ages, our study showed that animals weaned late at 42 days of age presented an increase of microbial diversity pre-weaning and did not show post-weaning diarrhoea. Thus, late weaning could be involved in an enhancement of gut health in piglets by promoting a more mature gut microbial ecosystem.

II. An increased relative abundance of *F. prausnitzii* concomitant with the overall increased microbial richness was described in the group weaned at 42 days old. Since *F. prausnitzii* is considered a next-generation probiotic and it is positively correlated in stable and balanced guts, it could be an important probiotic to consider for the prevention of disorders linked to weaning dysbiosis in pigs.

III. Studying the impact of the host genotype during a natural outbreak of colibacillosis, we confirmed that the *MUC4* and *FUT1* genotypes as genetic markers for the susceptibility to ETEC F4 and F18 infection, respectively.

IV. Amoxicillin, commonly used at weaning for the treatment of streptococcosis occurring in the same timeframe as colibacillosis, showed adverse outcomes on pig gut health during a multi-resistant ETEC infection and this effect was stronger through the oral compared to the parenteral route.

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ANNEXES

Section VII

1. Awards

Bando da Vinci 2018 at the Università Italo Francese (CHAPTER 2 - Mobility contributions for shared PhD project).

Travel grant in order to participate to the “Challenges and new concepts in antibiotics research” Conference at the Institut Pasteur, Paris (France) March 19th -21st, 2018.

Short Mission Scientific Mission of PiGutNet - COST Action FA1401. “Influence of intestinal microbiota composition on piglet robustness at weaning”. INRAE, Département de Génétique Animale, UMR1313 (GABI), Equipe Génétique Immunité Santé (GIS) Jouy-en-Josas, (France) March 1st - 31st 2017.

PiGutNet Training School COST Action FA1401. “Analysis of porcine metagenomic datasets.” INRAE, Jouy-en-Josas, (France) February 13th-17th 2017.

2. Participation at congresses and meetings

- 70th Annual Meeting of the European Federation of Animal Science, Ghent (Belgium), 26th – 30th Aug 2019 - *oral presentation* "Piglets infected with ETEC F4 and F18: effect of *MUC4* and *FUT1* genotypes."
- 37th International Society for Animal Genetics Conference, 7th-12nd July 2019, Lleida Spain – poster presentation.
- Plant & Animal Genome Conference (PAG), January 12-16, 2019 - San Diego, CA, USA – poster presentation.
- Modelling the Mammalian- Microbiota Host Superorganism conference, Institute Pasteur, Paris (France). October 15th -16th, 2018.
- 69th Annual Meeting of the European Federation of Animal Science page 498. Dubrovnik, Croatia, 27th- 31st August 2018 – *oral presentation* "Impact of weaning age on gut microbiota composition in piglets."
- Challenges and new concepts in antibiotics research Conference. Institut Pasteur, Paris (France). March 19th -21st, 2018 – poster presentation.
- The swine gut microbiota: current methodologies and new perspectives. Istituto Zooprofilattico Sperimentale Umbria e Marche "Togo Rosati", Perugia (Italy), 14th March 2018 – *speaker*
- PiGutNet- COST ACTION meeting: Exchange meeting between WG1 and WG2 partners. Wageninghen University. 4th-7th December 2017
- 7th International Conference on Colonic Spirochaetal Infections in Animals and Humans. 6th-7th Oct, 2016, Hannover – *oral presentation* "A longitudinal study on the epidemiology of *B. hyodysenteriae* infection in fattening pigs."
- National congress of Società Italiana di Patologia ed Allevamento dei Suini (SIPAS), March 2016, Brescia – *oral presentation* "Dinamica dell'infezione da *Brachyspira hyodysenteriae* in un allevamento di suini da ingrasso endemicamente infetto."
- National congress of Società Italiana di Patologia ed Allevamento dei Suini (SIPAS), March 2015, Brescia – *oral presentation* "Contaminazione da *Salmonella* spp. in due mattatoi del centro Italia: un approccio quantitativo."

3. Congress proceedings

- Revilla M., Lemonnier G., Leplat J.J, **Massacci F.R.**, Jardet D., Rossignol M.N., Blanc F., Mercat M.J., Ravon L., Munoz-Tamayo R., Friggens N.C., Le Floch N., Zemb O., Lepage P., Rogel-Gaillard C. and Estellé J. "The pre-weaning gut microbiota composition in piglets and its links with post-weaning robustness." Plant & Animal Genome Conference (PAG), January 11-15, 2020 - San Diego, CA, USA. PE0412
- **Massacci F.R.,** Tofani S., Tentellini M., Orsini S., Lovito C., Forte C., Luise D., Bevilacqua C., Marchi L., Bertocchi M., Rogel-Gaillard C., Pezzotti G., Estellé J., Trevisi P., Magistrali C.F. "Piglets infected with ETEC F4 and F18: effect of *MUC4* and *FUT1* genotypes." 70th Annual Meeting of the European Federation of Animal Science, Ghent (Belgium), 26 - 30 Aug 2019. Pag307
- García-Casco J. M., Muñoz M., Lemonnier G., Babilliot J. M., Bouchez O., Fernández A. I., **Massacci F. R.**, Fernández-Barroso M. A., López-García A., Caraballo C., Óvilo C., and Estellé J. "The gut microbiota composition at slaughter as a potential certification tool for the Iberian pig traditional farming system." 37th International Society for Animal Genetics Conference, 7th-12nd July 2019, Lleida Spain. OP209
- **Massacci F.R.**, Tofani S., Tentellini M., Lovito C., Orsini S., Forte C., Marchi L., Rogel-Gaillard C., Pezzotti G., Trevisi P., Estellé J., Magistrali C.F. "Effect of *MUC4* and *FUT1* genotypes on piglets infected with enterotoxigenic *Escherichia coli* F4 and F18." 37th International Society for Animal Genetics Conference, 7th-12nd July 2019, Lleida Spain. (Poster P358)
- **Massacci F.R.**, Berri M., Olivier M., Savoie J., Lemonnier G., Jardet D., Rossignol M.N., Blanc F., Revilla M., Mercat M.J., Doré J., Lepage P., Rogel-Gaillard C. and Estellé J., on behalf of the PIGLETBIOTA consortium. "Weaning age influences the gut microbiota dynamics in piglets." Plant & Animal Genome Conference (PAG), January 12-16, 2019 - San Diego, CA, USA. (Poster)

- Scoccia E., Ferroni L., Pesciaroli M., Orsini S., Marchi L., **Massacci F.R.**, Tofani S., Pezzotti G., Magistrali C.F. and Maresca C. "Antimicrobico-resistenza nei polli da carne: linee produttive a confronto." XLII Convegno AIE, Lecce (Italy) – 24- 26 Ottobre 2018.
- Magistrali C.F., Blasi F., Lovito C., Tofani S., Orsini S., **Massacci F.R.**, Epifanio M.E., Forte C., Bano L., Drigo I., Pezzotti G. "Prevalenza di *Clostridium difficile* nei vitelli di allevamenti da latte e da carne in Umbria: ribotipi e profili di sensibilitá agli antibiotici." XVIII Congresso Nazionale S.I.Di.L.V. Perugia (Italy), 7 - 9 Novembre 2018. (Poster)
- Marchi L., Orsini S., **Massacci F.R.**, Crotti S., Cruciani D., Pesciaroli M., Dettori A., Felici A, Pezzotti G., Magistrali C.F. "Studio della contaminazione microbica in oche allevate in vigna nell'ambito di un progetto di agroforestry." XVIII Congresso Nazionale S.I.Di.L.V. Perugia (Italy), 7 - 9 Novembre 2018. (Poster)
- Pesciaroli M., Orsini S., Marchi L., **Massacci F.R.**, Tofani S., Maresca C., Scoccia E., Filippini G., Magistrali C.F., Pezzotti G. "L'impatto del sistema di allevamento sull'antibiotico resistenza dei batteri commensali dell'intestino del pollo da carne." XVIII Congresso Nazionale S.I.Di.L.V. Perugia (Italy), 7 - 9 Novembre 2018.
- **F.R. Massacci**, M. Berri, M. Olivier, J. Savoie, G. Lemonnier, D. Jardet, M.N. Rossignol, F. Blanc, M. Revilla, M.J. Mercat, J. Doré, P. Lepage, C. Rogel-Gaillard and J. Estellé. "Impact of weaning age on gut microbiota composition in piglets." 69th Annual Meeting of the European Federation of Animal Science page 549. Dubrovnik, Croatia, 27th- 31st August 2018.
- Muñoz M., García-Casco J.M., Lemonnier G., Jardet D., Bouchez O., Fernández-Barroso M.A., **Massacci F.R.**, Fernández A.I., López-García A., Caraballo C., González-Sánchez E., Óvilo C. and Estellé J. "Gut microbiota composition in Iberian pigs fed with olive oil by-products during the growing period." 69th Annual Meeting of the European Federation of Animal Science page 498. Dubrovnik, Croatia, 27th- 31st August 2018. (Poster)

- Estellé J., **Massacci F.R.**, Esquerré D., Jardet D., Lemonnier G., Óvilo C., Skrlep M., Poklukar K. and Čandek-Potokar M. "Gut microbiota composition in Krškopolje pigs under conventional and organic production systems." 69th Annual Meeting of the European Federation of Animal Science page 499. Dubrovnik, Croatia, 27th- 31st August 2018. (Poster)
- Estellé J., **Massacci F.R.**, Esquerré D., Jardet D., Lemonnier G., Óvilo C., Čandek-Potokar M., Salajpal K. and Karolyi D. "Gut microbiota composition of Turopolje pigs in outdoor production and acorn supplementation." 69th Annual Meeting of the European Federation of Animal Science page 500. Dubrovnik, Croatia, 27th- 31st August 2018. (Poster)
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