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FEEDING THE GUT MICROBIOME AND IMMUNE MATURATION TO MANAGE WEANED PIGLETS

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Table of Contents

Abstract

This thesis reports five studies that may contribute to understand how weaning affects the immune and intestinal microbiota maturation of the piglet and proposes some possible nutritional strategies to attenuate its negative effects. The first study showed that weaning is associated in Payer's patches with the activation of MHC response against class I antigens and that related to the stimulation to IFN-γ and showed, for the first time, that their blood at weaning remains dominated by immature blood cells. In the second study we tested if the use of a live vaccine against a conditionally but also genetically based intestinal disease, like PWD, could have an impact on the growth performance of pigs and their intestinal microbiota and if it could provide a model to test the response to nutritional strategies under conditions of an immune and intestinal stimulation for animals susceptible to ETEC type. In this study, we demonstrated how a vaccinal strain of F4/F18 *E. coli* can affect the gut microbial composition of piglets, regardless of their genetic susceptibility to ETEC infection. In the third study we evidenced how a nucleotide supplementation can favor the proliferation of jejunal Peyer patches and anticipate the maturation of the fecal microbiota. In the fourth study we reported how xylanase can favor the proliferation of *Lactobacillus reuteri*. Finally, we showed some first results on the muscles fiber development in fast- and slow-growing suckling pigs and the relationship with the intestinal microbiota. Taken together, the results presented in this thesis provide new insight about the interplay between the host-genetics, gut microbial composition, and host physiological status. Furthermore, it provides confirmation that the use of known genetic markers for ETEC F4 and F18 could represent a potential tool to stratify the animals in the trials both in healthy or challenge-based protocols.

List of original manuscripts included in the thesis

1. Correa, F., Luise, D., Bosi, P., Trevisi, P., 2022. Weaning differentially affects the maturation of piglet peripheral blood and jejunal Peyer's patches. Scientific Reports 12, 1604. https://doi.org/10.1038/s41598-022-05707-9

2. Luise, D., Spinelli, E., Correa, F., Salvarani, C., Bosi, P., Trevisi, P., 2020. Effects of E. coli bivalent vaccine and of host genetic susceptibility to *E. coli* on the growth performance and faecal microbial profile of weaned pigs. Livestock Science 241, 104247. https://doi.org/10.1016/j.livsci.2020.104247

3. Correa, F., Luise, D., Archetti, I., Bosi, P., Trevisi, P., 2021. Investigation of Early Supplementation of Nucleotides on the Intestinal Maturation of Weaned Piglets. Animals 11, 1489. https://doi.org/10.3390/ani11061489

4. Luise, D., Motta, V., Boudry, C., Salvarani, C., Correa, F., Mazzoni, M., Bosi, P., Trevisi, P., 2020. The supplementation of a corn/barley-based diet with bacterial xylanase did not prevent diarrhoea of ETEC susceptible piglets, but favoured the persistence of *Lactobacillus reuteri* in the gut. Livestock Science 240, 104161. https://doi.org/10.1016/j.livsci.2020.104161

5. Effect of the growth of suckling pigs on the gut microbiota and on the pattern of muscular fiber types. First results of an experimental trial performed during my period abroad at Agrscope, Switzerland under the supervision of Dr. Marion Girard and Dr. Giuseppe Bee.

Background

In modern pig industry, weaning occurs abruptly and earlier than in natural conditions. Piglets are weaned earlier in order to increase the number of reproductive cycles per sow per year and thus, to increase the number of piglets produced. In nature weaning is a gradual process that generally occurs between 10 and 12 weeks of age and coincides with the complete development of the intestinal mucosa, whereas in farming practices it occurs between 3 and 4 weeks (Moeser et al., 2017) and involves the complete separation of piglets from the sow, resulting in a drastic change in diet from primarily or totally sow's milk to solid feed. In addition, weaning usually involves piglets handling and transporting to a new habitat with a bigger group of piglets, resulting in the formation of a new social order among non-littermate piglets. These stressor leads to a reduction of the feed ingestion in the first days post weaning, which can have detrimental effects on the correct morphological and functional development of the intestinal mucosa, followed by the proliferation of pathogenic bacteria and the emergence of post-weaning diarrhea (PWD) (Gresse et al., 2017). Moreover, these factors are amplified in larger litter with a higher proportion of low-birth-weight piglets, as these animals can be less mature in terms of immune and digestive system development, to face the stress of weaning. In the past, PWD was prevented by using antibiotics as growth promoters or pharmaceuticals or by dietary pharmacological levels of zinc oxide (ZnO). The emergence of antibiotic resistance led to the ban of the use of antibiotics as growth promoter in 2006 in Europe and to restrictions to their in-feed pharmaceutical use. Furthermore, due to the environmental impact in pig excreta, dietary zinc at pharmaceutical levels will be banned from June 2022 (European Medicines Agency, 2018). This stimulated studies on dietary alternatives but also a revision of the knowledge on piglet response to pre- and post-weaning condition. In the following paragraph we will highlight the changes that occur in the gastrointestinal physiology, microbiology, and immunology during the pre- and post-weaning phase and the nutritional strategies that can be adopted to prepare the animals for weaning and to attenuate the negatives effects related with it.

1 Gut microbiota eubiosis and dysbiosis

'Microbiota' is defined as all microbes, including their genomes and extra-chromosomal elements, present in and on the host animal, and their interactions within the gastrointestinal tract (GIT), skin, and genital environments. The term 'Microbiome', instead, refers to all the microbial genes (Ursell et al., 2012). The GIT represents the largest place of interplay between host and external environment, and it also contains the largest and diverse microbial community, composed by commensal, symbiotic and pathogenic microorganisms, including bacteria, viruses, parasites, fungi, archaea, and protists (Wang et al., 2016). The co-evolution of the porcine host with gut microbes led to the acquisition or adaptation of several functions including digestion, nutritional utilization, toxin elimination, pathogen defense, and endocrine and immune system control. A gut microbiota in a eubiotic status is characterized by a preponderance of potentially beneficial species, belonging mainly to Firmicutes and Bacteroidetes phylum, while potentially pathogenic species, such as that belonging to Proteobacteria phylum (Enterobacteriaceae) are present, but in a very low percentage (Zheng et al., 2015). An alteration of this equilibrium is defined as "dysbiosis". Dysbiosis can be for example characterized by a loss of bacterial diversity with a decrease in specific Firmicutes and a concomitant increase in *Bacteroidetes* and facultative anaerobes such as Enterobacteriaceae (Carding et al., 2015).

In addition, the gut microbiota composition varies along the digestive tract, because of the different environmental and functional characteristic of each segment, and it's also influenced by other factors such as age, sex, breed, litter of origin, diet, use of antibiotics and the sanitary condition of the farm (Xiao et al., 2016).

1.1 Evolution of the microbiota composition

The first colonization of the GIT is a crucial step for the maturation of the intestinal function and immune system (Chung et al., 2012; Hooper et al., 2001). In pigs, a first colonization of the GIT tract occurs at birth via the passage through the birth canal (Dominguez-Bello et al., 2010), although recent studies have hypothesized that this colonization can occur also earlier, in the intra-uterine life, based on bacteria found in meconium (Wang et al., 2019) or umbilical cord blood (Leblois et al., 2017).

Although this aspect remains quite controversial (Goffau et al., 2021), the bacterial load found in the pre-birth gut remains very low. Instead, at birth, the piglet is exposed to a higher bacterial load that comes first from the birth canal, feces, skin, and mucosa of the mother and from by the environment (Chen et al., 2017). These bacteria represent the first colonizer of the GI tract, which at birth, is an oxy environment. So, the first GI population is characterized by facilitative anaerobic bacteria like *Staphylococcus*, *Streptococcus* and *Enterococcus* subsequently their metabolizing activity will result in oxygen depletion, favoring the proliferation of strictly anaerobic bacteria like Bifidobacteria (Jost et al., 2012). During lactation, the lactose present in the milk favor the proliferation of *Lactobacillus* especially in the stomach and the foregut were they utilize lactose and produce lactic acid and acetate, this mechanism represents the principal source of stomach acidity that activate endogenous enzymes and provide a barrier against pathogenic bacteria (Bach Knudsen, 2012). Beside Lactobacilli, Bifodabacteria becomes one of the most predominant bacteria, because of their ability to utilize highly sialylated carbohydrates present in colostrum and milk, this glycans are indigestible to the host and arrive in the distal part of the GI tract untouched. In these first days of life, colostrum exploits its modulation activity on the microbiota, directly through the presence of antimicrobial substances like lactoperoxidase, lysozyme, defensins and lactoferrin, and indirectly via the immune activation provided by the immunoglobulins. All the nursing period remains dominated by a so-called milk-oriented microbiome; a community whose reflects its focus on milk consumption, besides Bifodobacteria, the main bacteria in this phase are *Enterobacteriaceae* and *Bacteroidaceae* and in particular *Bacteroides*, whose ability to harvest milk glycans is well characterized (Frese et al., 2015; Mach et al., 2015; Marcobal et al., 2011).

1.2 Mature microbiota

During the suckling period, the microbial biodiversity increases and becomes quite stable in its composition (Wang et al., 2019a), until the weaning phase where there is a reduction of the microbial variability, promoting the proliferation of opportunistic pathogens, which can lead to PWD (Gresse et al., 2017). The weaning transition has been associated to drastic reduction of Bacteroidaceae and Enterobacteriaceae, and an increase of *Prevotella* (Motta et al., 2019) that remains the dominant genus

throughout most of the rearing phases. The microbiota composition achieves quite stable levels at 10 days post-weaning, and it is characterized in the hindgut by higher levels of *Prevotella*, *Acetivibrio*, *Dialister*, *Oribacterium*, *Succinivibrio* (L. Chen et al., 2017; Mach et al., 2015). This is mainly due to the capacity of these bacteria to degrade complex polysaccharides of the cereals in the diet through their xylanases, mannanases, β-glucanases enzymes. In the following stages the microbiota composition remains quite stable, even if there is some degree of evolution in this composition. The stabilizing stabilization in the small intestine is less documented, but it is presumed to be determined also by rapidly fermentable substrates present, such as non-structural carbohydrates and soluble fiber, depending also on the specific section (Hoogeveen et al., 2021). In **Figure 1** there is a summarized representation of the appearance pattern of gut microbes in the growing stages of the pigs.

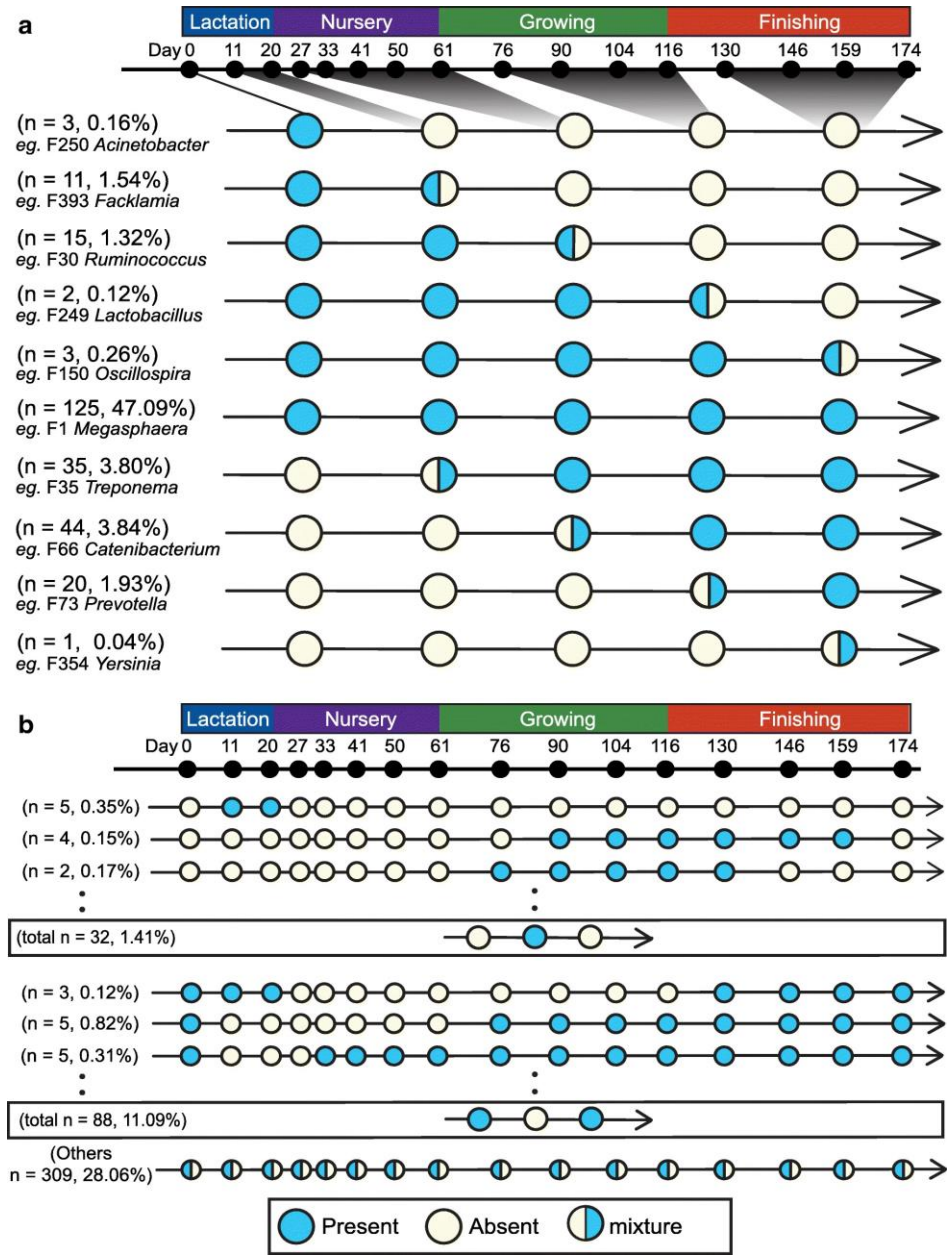

Figure 1. a, b Longitudinal occurrence patterns of the swine gut microbiomes. Top 700 features based on averaged relative abundance on each day were used to summarize the occurrence patterns. Blue circle indicates the presence of a bacterial taxon while a yellow circle shows the absence. Mixed color circles mean transition between "presence" and "absence" during each stage or time period. From Wang et al. (2019a) 'Longitudinal investigation of the swine gut microbiome from birth to market reveals stage and growth performance associated bacteria'Microbiome (2019) 7:109

It has been well established that the bacterial composition changes all along the GI tract, adapting to the different environmental conditions, and increasing in its density and diversity due to more favorable conditions, i.e. slower flow of digesta and higher pH. Furthermore, the microorganisms in the small intestine (duodenum, jejunum, and ileum) are competing with the host for the digestion and use of breakdown products of nutrients (e.g. most of the proteins, lipids, amino acids, monosaccharides, and some oligosaccharides). Microorganisms in the large intestine (colon and cecum), on the other hand, are primarily involved in the breakdown of nutrients that are indigestible: for instance, insoluble cellulose (resistant starch and lignin). Specifically, *Lactobacillus*, *Streptococcus*, *Clostridium*, *Escherichia*, *Helicobacter*, *Anaerobacter*, *Turicibacter*, *Acinetobacter* are the main genera identified in small intestine and *Prevotella*, Ruminococcaceae, and Clostridiales are the dominant taxa reported in the large intestine of pigs (Looft et al., 2014; Mann et al., 2014; Zhao et al., 2015). In addition to the microbiota variation in longitudinal locations, there are also differences in the radial locations. In fact, microbial communities of the gut lumen and mucosa are significantly different. Oxygen exposure, pH, and substrate availability appear to affect the microbial distribution in the gut. In the small intestine mucosa, where there is a higher oxygen level, anaerobes such as *E. coli* are dominant, whereas their abundance is reduced in the lumen (Mu et al., 2017).

2 Physiological changes of the GIT related to weaning

The stressor effect related with weaning causes a reduction of the feed ingestion in the first days after weaning, that can have detrimental effects on the correct morphological and functional development of GIT. Most of these changes occur in the first 5 days post weaning, defined as the acute phase; then from day 5 to 15 after weaning the adaptation to the weaning diet starts to be more evident (Montagne et al., 2007). At stomach level, weaning is associated with an increase of the luminal pH mostly linked to the lack of lactose of the maternal milk that provides a good substrate to produce lactic acid and the transitory need of an adaptation of the oxyntic mucosa. The higher pH can impair the stomach functions because a lower pH is required for conversion of the gastric zymogens into active enzymes (Khan et al., 1999). In addition, the low stomach pH provides a barrier function against pathogenic bacteria sensible to low pH values including *E. coli* (Modler et al., 1990; Prohászka and Baron, 1980).

In the small intestine, the anorexia related to weaning has strong effect on the morphological structure of the mucosa, causing villi atrophy and crypt hyperplasia. These changes have been also related to the reduced activity of the brush-border enzymes lactase, sucrase and maltase (Boudry et al., 2004; Chen et al., 2017a) and alteration in electrolyte and fluid balance leading to a reduction in the net absorption of fluid and electrolytes, and malabsorption of nutrients in the small intestine.

The structural changes observed in the first few days post-weaning can induce a decrease in nutrient absorption and a breakdown of the intestinal mucosa integrity, which can open the door to pathogenic bacteria in the *lamina propria* causing inflammation and diarrhea (Jayaraman and Nyachoti, 2017). Among several pathogens that can develop and colonize the GIT of weaners, *E. coli* is considered one of the most common. In fact, the inflammatory response caused by the post-weaning stress, produces a nitrate-rich environment that could represent an advantage for *E. coli*, thanks to its ability to utilize nitrate (Winter et al., 2013). In addition, the increased blood flow caused by the inflammation process rises the oxygen presence in the gut mucosa, favoring the proliferation of facultative anaerobic bacteria such as Enterobacteriaceae instead of the obligate anaerobic bacteria like those belonging to Bacteroidia and Clostridia classes (Gresse et al., 2017). Moreover, the administration of antibiotics can promote the inflammatory response by reducing the variability of the bacterial community (F**igure 2**). In addition, bacteria that come in close contact with the mucus layer, like *E. coli*, produce lipopolysaccharides (LPS) and flagella, that are recognized by the Toll-like receptors 4 and 5, respectively, and can regulate the mucosal immune development (Cullender et al., 2013).

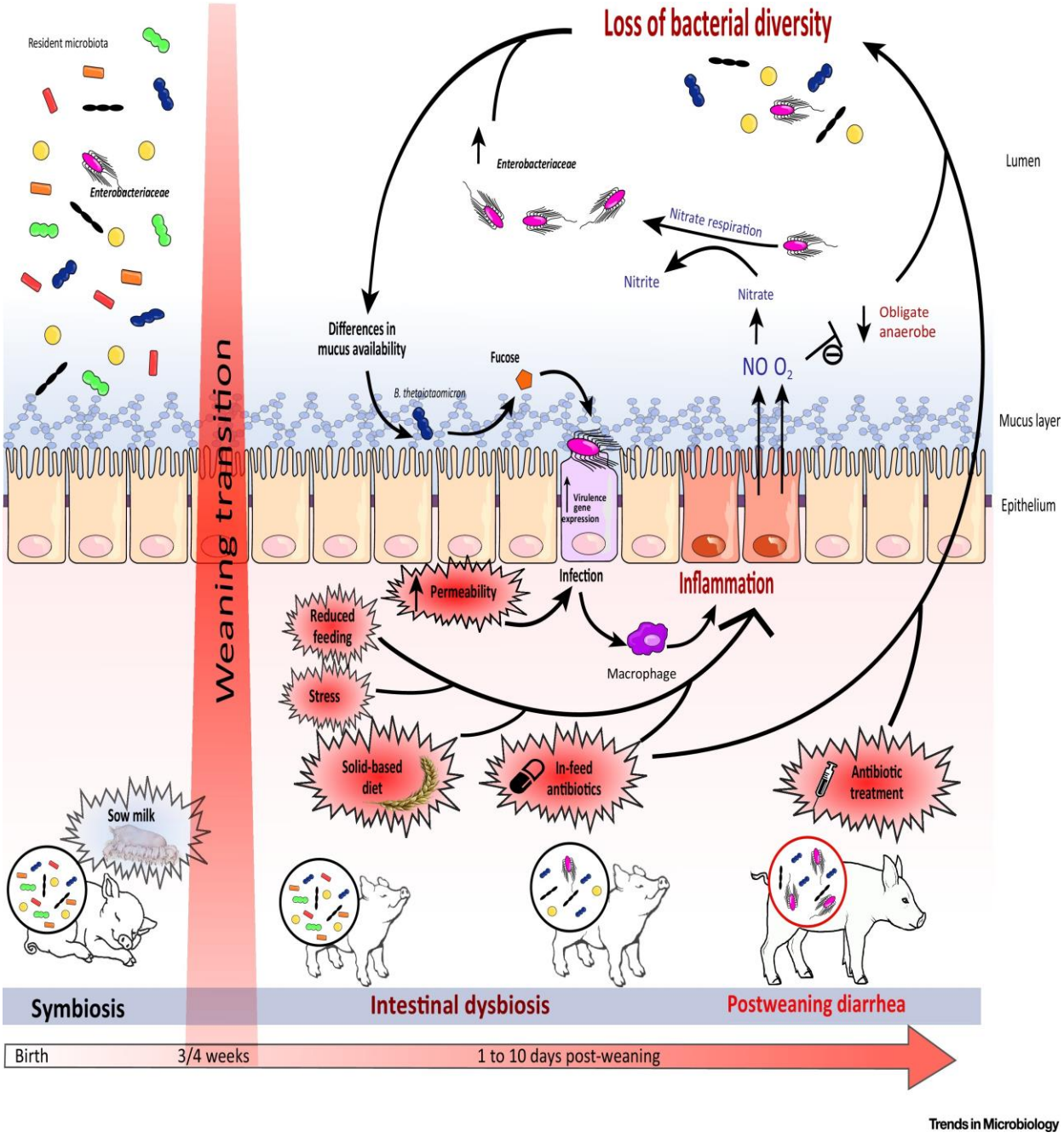

Figure 2. Impact of Weaning Transition on Piglet Gut Microbiota and Expansion of Infectious Agents. From Gresse et al. (2017) 'Gut Microbiota Dysbiosis in Postweaning Piglets: Understanding the Keys to Health' Trends in microbiology Volume 25, Issue 10, p851–873, October 2017. Elsevier and Copyright Clearance Center, Licence number 5147101337406

3 GI mucosal immunity

The mucosal immune system associated with the gastrointestinal tract is necessary for the defense against enteric infection, as well as many other physiological functions required for the proper gut health and development. The initial development of the immune system is influenced by several factors including the maternally derived antigens and antibodies, the genetic background, the dietary elements and the interaction with gut microbiota (Bauer et al., 2006). In particular, microbiota is recognized to be one of the major factors that drives the initial development of the immune system (Ferret-Bernard and Huërou-Luron, 2020). A large amount of studies on germ-free (GF) animals, gave us a lot of insight on this relationship, research on GF mice revealed that the lack of commensal microorganisms is linked to severe reduction of the gut lymphoid tissue architecture and immune function abnormalities (Bauer et al., 1963). Another example is represented by the adhesion of segmented filamentous bacteria to the intestinal epithelium, that enables the differentiation of IL-17$^+$ CD4$^+$ T cells, which represent a class of potent immunomodulatory effector cells (Ivanov et al., 2008). In the following paragraphs, the development of piglet mucosal immune system and the intestinal barrier function will be briefly highlighted.

3.1 Innate mucosal immunity

From birth, the mucosal immune system is immediately exposed to the external environment and stimulated by commensal and potentially pathogenic bacteria, dietary antigens, and virus. The basic mechanism of the mucosal immunity is represented by the non-specific innate immunity, composed by natural killer cells (NK) and macrophages, but also by soluble mediators such as cytokines and the complement system (Beutler, 2004). The intestinal epithelium is the first line of defense from pathogens and antigens, it provides physical barrier restricting the passage of small molecules (2 kDa) (Madara, 1998). The tight junction proteins are essential for maintaining the intestinal integrity. The epithelium is also important for the production of antimicrobial substances like inorganic disinfectants (e.g. hydrogen peroxide and nitric oxide), antimicrobial proteins (e.g. lysozyme and lactoferrin), or antimicrobial peptides (e.g. defensins). Defensins can be divided in α and β defensins, produced by Paneth cells and epithelial cells respectively, their antimicrobial activity is linked to the disruption of

structural elements of the cell membranes, and to the permeabilization of the bacterial envelope (Zasloff, 2002; Zhang et al., 2000). For pigs, β defensin is the only subfamily of defensins which has been characterized so far (Choi et al., 2012). An additional protection is also provided by the mucus layer that cover the intestinal epithelium; mucus glycoprotein capture bacteria and viruses, preventing them to get in contact with the epithelium, and further expelling them by the peristaltic movement of the gut. Nevertheless, some bacteria possess glycosyl hydrolases, that can degrade mucins for their foraging (Lee et al., 2021) and there is recent evidence that this mechanism is also involved in the modulation of porcine hindgut microbiota (Xia et al., 2021)

3.2 Gut-associated lymphoid tissue (GALT)

The GALT is divided in organized lymphoid tissue (inductive site) and diffuse lymphoid tissue (effector site). The inductive sites, represented mainly by the Peyer's patches (PPs), are aggregates of lymphoid tissue, that in pigs form isolated follicles in jejunum and upper ileum or a unique and continuous structure in the terminal ileum. Currently there are evidence that Ileal PPs initiate their development prenatally and accelerate it after birth (Furukawa et al., 2020), being more involved in undiversified IgA production (Butler et al., 2016), while jejunal PPs develop postnatally, being more responsible of initiating the production of diversified IgA. PPs surface is covered by enterocytes and by a specialized epithelial cell type, known as M cells (Brandtzaeg, 2003). On the surface they present a series of microfold with the function of capture antigens and transport them to the underlying GALT tissue; here specialized antigens presenting cells (APCs) like dendritic cells (DCs) and macrophages, present them to the immature T and B cells. After activation, T cells will differentiate in $CD4^+$ or $CD8^+$. The $CD4^+$ helper cells, supported by DCs and the secretion of cytokines, (like transforming growth factor (TGF)-and interleukin (IL)-10)), induce the differentiation of B cells to IgA-committed plasmablasts (Brandtzaeg et al., 1999). B cells blast migrate and continue their proliferation in the mesenteric lymph nodes and then migrate to the effector site trough the blood stream, represented for example by the *lamina propria*; here through a process called "homing", they become IgA producing plasma cells (Kraehenbuhl and Neutra, 1992). On the other hand, $CD8^+$ cells have a direct cytotoxicity on infected cells causing cellular apoptosis by the production of perforin and granzymes. In addition,

DCs can samples antigens directly into the intestinal lumen by extending their dendritic-like process through tight junctions between the epithelial cells (Rescigno et al., 2001). When DCs capture some antigens, they migrate to the mesenteric lymph nodes (MLNs) were they display antigen-derived peptides on their major histocompatibility complex (MHC) molecules, selecting antigen-specific T cells (Burkey et al., 2009). DCs in the intestinal epithelium can also take up commensal bacteria and stimulate T cells to produce IgA locally limiting the penetration of commensal bacteria and avoiding systemic reaction but allowing the persistence of commensal bacteria in the lumen (Macpherson and Harris, 2004). IgA are secreted in the epithelium, via the polymeric immunoglobulin receptor (pIgR), in their secretory form (sIgA) constituted by a dimer of two monomeric IgA molecules with an attached secretory component (SC). The SC is a fragment of the pIgR that remain attached to sIgA after its transport to the apical surface of the epithelial cells (Macpherson et al., 2001).

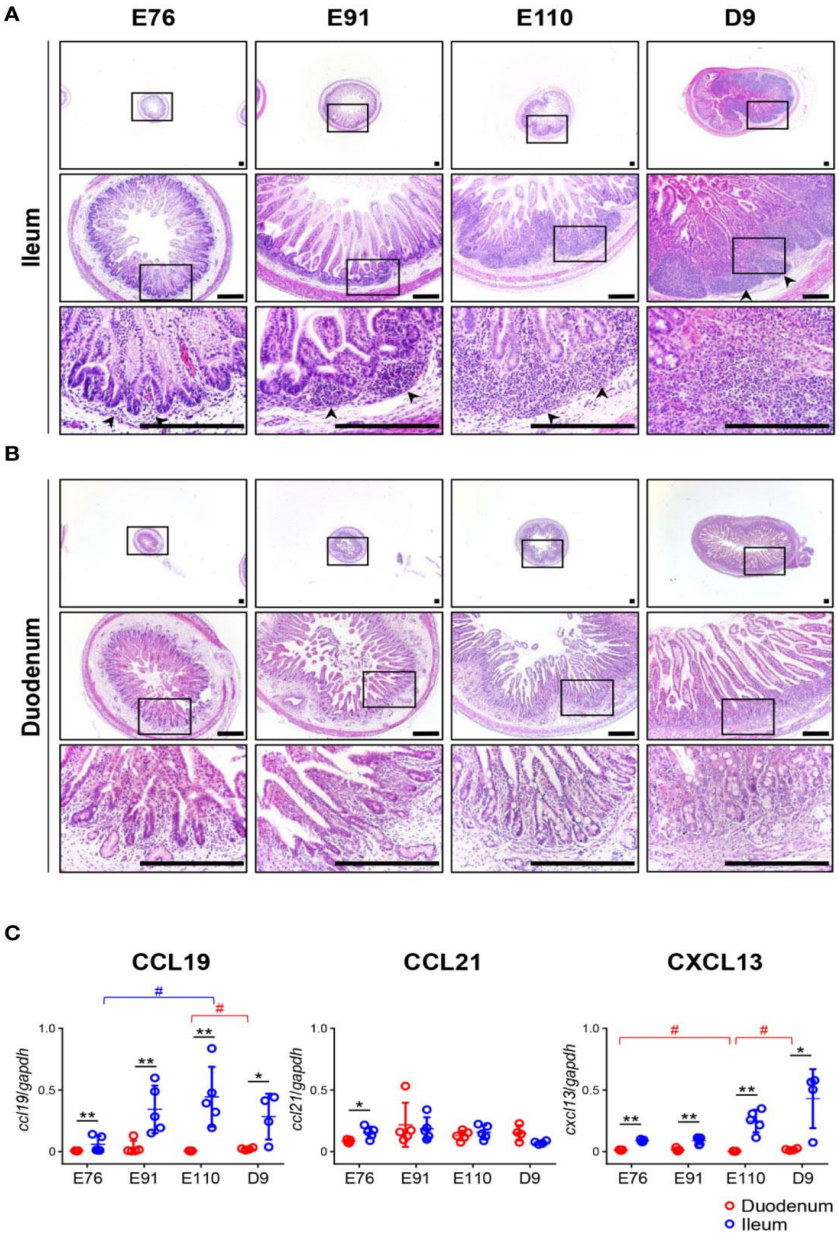

Figure 3. Organogenesis of Peyer's patches (PPs) was initiated prenatally in the ileum and not the duodenum. (A) In the ileum, the formation of lymphoid aggregates was initiated between E76 and E91, and their size increased between the foetal and neonatal stages. (B) In the duodenum, there was no accumulation of infiltrated cells throughout the experiment. (C) Quantitative RT-PCR analyses demonstrated that the expression of ccl19 and cxcl13 mRNAs in the ileum were consistently higher than those in the duodenum. From @Furukawa2020 'Organogenesis of Ileal Peyer's Patches Is Initiated Prenatally and Accelerated Postnatally With Comprehensive Proliferation of B Cells in Pigs'. Front. Immunol. 11:604674

3.3 Pathogen recognition by the host

Cells of the innate immunity, like DCs and macrophages, can protect the organism against novel encountered microorganism. The first step for the activation of innate immunity, is the recognition of microbial molecules called pathogen-associated molecular patterns (PAMPs), this are for example lipopolysaccharides (LPS) for Gram-negative bacteria, and peptidoglycan and lipoteichoic acids (LTA) for Gram-positive bacteria (Barton and Medzhitov, 2002). Both monocytes and DCs are able to recognize PAMPs through pattern-recognition receptors expressed on cell surface, these receptors compose the Toll-like receptors (TLR) family. Monocytes and DCs can express all the TLR receptors known (TL1 to TL10). The activation of these receptors leads to the production of proinflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1 and IL-6. These cytokines activate B and T cells, inducing an adaptive immune response. TLR receptors are also important for commensal microbiota tolerance, for instance, in a study by Round et al. (2011) showed how *Bacteroies fragilis* use the TLR-2 signaling, to persist on human epithelium, producing a surface polysaccharide called PSA, the interaction between PSA and TLR-2 suppress the immune response.

3.4 Maturation of the intestinal immunity

The epitheliochorial nature of the pig placenta prevents the transmission of macromolecules such as antibody or foreign antigen, from sow to fetus, making the newborn piglet immunologically immature. At birth, indeed, low numbers of macrophage and granulocytes are present in villi and crypt regions and small cluster of lymphocytes are present in the mucosa, in areas that will subsequently develop into PPs (Barman et al., 1996). T cells appear in the first week of life, only expressing the CD2 surface marker but not CD4 or CD8 (Vega-López et al., 1995), in this phase PP begin to organize into a relatively ‘adult architecture’. CD4 will colonize the mucosa during the second and third week of life, CD8 will appear only later (after the third week of life), followed by IgA+ plasma cells, which have been observed to show up in large numbers as late as 3–6 weeks (Bailey and Haverson, 2006). By 7 weeks of ages, the intestinal mucosa immunity is comparable to that of the mature animal. In the context of weaning, this indicate that piglets can have an active immune repose against pathogens from

3 weeks of age, but the quantity and quality of the response in quite different from the immune response of an adult animal.

4 ETEC infection

E. coli is considered one of major pathogens that can develop and colonize the pigs' GIT around weaning. This Gram- bacteria are generally present as commensal in the pig gut microbiota, but when the host immunity is compromised, like during weaning, they can take over and proliferate.

The taxonomy differentiation of *E. coli* is based on its antigens, capsule, fimbriae, and flagella. Fimbriae are an important virulence factor because they allow the bacteria to adhere to specific receptor present on the intestinal mucosa (Karlsson, 1989), they're also responsible for the hemagglutinating action. For porcine typical types, F4 and F18 fimbriae are the most studied since they are common in the Enterotoxigenic *Escherichia coli* (ETEC) infection linked to PWD worldwide (Chen et al., 2004; Luppi, 2017; Zhang et al., 2007), while *E. coli* strains expressing K99 (F5), 987P (F6), or F41 (F7), are associated with diarrhea in the neonatal period (Wilson and Francis, 1986). This resistance or susceptibility at ETEC adhesion seems to be related to the fact that specific receptors are expressed in relationships with age (Nagy and Fekete, 2005). For the F4 fimbriae 3 antigenic variants have been identified, specifically F4ab, F4ac and F4ad, and among these, the F4ac is the more prevalent in pigs (Luppi, 2017). Despite these differences, a common major fimbria subunit FaeG, shared by all the three fimbriae variants, has been identified.

For the adhesion on the intestinal epithelium, the F4 seems to bind to carbohydrates composing glycoproteins found in the intestinal epithelium on the mucus layer. For the F4ac, it seems to bind to two intestinal mucin-type sialoglycoproteins (IMTGP-1 and IMTGP-2) (Francis et al., 1999). However, several additional putative receptors with a glycosphingolipids nature such as lactosylceramide, gangliotriaosylceramide, gangliotetraosylceramide, globotriaosylceramide, lactotetraosylceramide, and lactotetraosylceramide have been described and characterized (Coddens et al., 2011; Grange et al., 2002, 1999).

The F18 fimbria is characterized by multiple copy of the FadA major subunit and by the minor subunits FedE and FedF, the latter seems to bind to the glycoproteins on the microvilli of the small intestine (Nagy and Fekete, 2005). For the F18 fimbriae two antigenic variants have been identified: F18ab and F18ac (Rippinger et al., 1995). In pigs, two putative receptors for ETEC F18 (F18R) have been recognized, H-2 histo-blood group antigen (HBGAs) or its derivative A-2 HBGAs (Coddens et al., 2017). ETEC infections are characterized by the production of two classes of toxins: the heat labile (LT) and heat stable (STs) toxins, that can be divided in two types, STa and STb. The ETEC F4 produces the LT, STa and STb toxins, while the ETEC F18 produce STa, STb and the rarer Shiga toxin (Stx2E). STb is more virulent in post-weaning pigs, STa instead is more relevant in diarrhea induction in humans, newborn piglets, and calves. The porcine LT variant is composed by 5 B-subunits and one A subunit that, after the bacteria binds to the receptor on the erythrocyte, is responsible for the activation of adenylate cyclase which increased the production of cyclic AMP (cAMP) and thus stimulating the cAMP-dependent protein kinase A. This mechanism increases the secretion of chloride ions and reduces the absorption of Na+ causing an imbalanced trans epithelial osmotic gradient that leads to diarrhoea (Barrett and Keely, 2000). STs enterotoxins connect to target receptors present on the brush border membrane of the small intestine epithelial cells, which trigger intracellular signaling cascades, disrupting electrolyte homeostasis and eventually lead to fluid secretion. In particular, STa and STb activate the cGMP-dependent protein kinase and calmodulin-dependent protein kinase II, respectively. The latter affects the transepithelial osmotic gradient by blocking the NaCl transport mechanism, while the other one influences the calcium regulation and activates the synthesis of prostaglandin E2 (PGE2) and 5-hydroxytryptamine (Peterson and Whipp, 1995). The structure of the Stx2E toxin is composed of an A subunit and five B subunits that bind to a specific glycolipid receptor on host cells. The A subunit has an N-glycosidase activity that inhibits protein synthesis in target cell by cleaving an adenosine base in the 28 S rRNA of the 60 S ribosomal subunits. The B subunits, instead, bind to the glycolipid receptor, globotriaosylceramide (Gb3) or to globotetraosylceramide (Gb4) receptors on the host small intestine where it’s internalized into target cells.

4.1 Genetic susceptibility to ETEC infection

PWD remains one of the main causes of mortality in the pre- and post-weaning period, ETEC F4ac and F18 infections are the principal cause of PWD. The ETEC adhesion to the intestinal epithelial is genetically programmed. The causative mutation responsible for genetic variation in susceptibility to ETEC F4 remains unknown so far (Schroyen et al., 2012). For ETEC F4 several positional candidate genes for resistance or susceptibility infection have been identified, comprising of *MUC* genes (*MUC4*, *MUC13* and *MUC20*) transferrin receptor gene (*TFRC*), *HEG1* and *ITGB5*.

- Mucine 4 (*MUC4*) is one of the most studied gene in relation to ETEC-F4ab and F4ac susceptibility. A single nucleotide polymorphism at position 8227 in intron 7 of MUC4 with the C recessive allele is associated to susceptibility, instead the G dominant allele is associated with resistance to ETEC F4ab and F4ac (Jørgensen et al., 2003).

- Another well studied polymorphism associated to ETEC-F4ab and F4ac susceptibility is that of the *MUC13* gene, that encodes for a transmembrane mucin expressed predominantly in the epithelial surface of the jejunum in piglets. *MUC13* gene encodes two transcripts: *MUC13A* and *MUC13B*. Both transcripts have the characteristic PTS domains of mucins consisting in distinct tandem repeats of proline, threonine and serine. *MUC13B* has more tandem repetitions, resulting in a heavy O-glycosylation and formation of the bacterium's binding site, making *MUC13B* responsible for susceptibility. *MUC13A*, on the other hand, lacks an O-glycosylation binding site due to a lack of *PTS* repeat domains (Zhang et al., 2008). As a result, ETEC F4ac–resistant pigs are homozygous for *MUC13A*, while susceptible animals have at least one *MUC13B* allele (Zhang et al., 2008).

- *MUC20* gene is mapped in the positional candidate region on SSC13, two 2 SNPs (MUC20-g.191CT and MUC20-c.1600CT) were identified to be associated with *in vitro* ETEC F4ab/ac adhesion and g.191CT was significantly associated with the ETEC F4ac adhesion phenotype (Ji et al., 2010).

- the transferrin receptor (*TFRC*) is involved in the delivery of iron into the cell from the transferrin protein. Iron availability is essential for *E. coli* survival and reproduction. A potential gene polymorphism related too ETEC susceptibility has been suggested based on the role of *TFRC* in the

host immune system and the gene's position in the target genomic region (SSC13q41). In the *TFRC* gene, five polymorphisms were discovered, one of which was completely linked with the ETEC F4ab/ac phenotype. None of them, however, was discovered to be the causal mutation (Jacobsen et al., 2011).

For ETEC F18 susceptibility two candidate genes have been identified so far: α(1,2)-fucosyltransferase (*FUT1*) and Bactericidal permeability-increasing protein (*BPI*):

- *FUT1* is the first identified candidate gene that can controls the adhesion of ETEC F18 to the intestinal epithelium (Vogeli, 1997). A SNP located at nucleotide 307 has been correlated with resistance to ETEC F18 infection (Meijerink et al., 1997). This mutation produces three genetic variants AA, AG and GG, and GG or AG genotypes are considered susceptible to infection while AA genotype is considered resistant. The G to A substitution causes a missense mutation (Ala to Thr) leading to a modification of the properties or quantity of the mature FUT1 enzyme. *FUT1* is involved in the formation of blood group antigens of the porcine AO blood group system by the addition of a terminal α(1,2) fucose residues (Vogeli, 1997). AA genotype show a different fucosylation pattern of carbohydrate structures expressed on cell surfaces, compared to AG and GG genotypes, preventing the adhesion of ETEC F18 to the epithelium (Hesselager et al., 2016)

- The *BPI* gene is located on chromosome 7q21-23 and encodes lipopolysaccharide binding protein. This protein is produced by neutrophils cells, and it involved in several immunological process because it can bind to LPS and other endotoxins and kill gram-negative bacteria. A mutation on the exon 10 has been suggested as a potential indicator for Salmonella infection in pigs (Levy et al., 2000), this same mutation have also been linked to ETEC F18 infection resistance. The gene produced AA, AG, and GG genotypes after HpaII digestion, with the AA genotype being linked with ETEC F18 resistant mice. Resistant genotypes have a higher expression of BPI in the duodenum and jejunum, compared to susceptible genotypes.

4.2 ETEC Vaccination

Based on available markers it is possible selecting for resistant piglets, but this can affect other selection parameters and the overall selective response. Another possible strategy to reduce PWD incidence, besides selecting for resistant piglets, involves vaccination. Different types of vaccines have been developed during the last years, but the most effective scheme in developing a proper mucosal immunity is represented by oral vaccination with inactivated *E. coli* (Melkebeek et al., 2013). Coliprotec, for example, is an oral live vaccine made from avirulent *E. coli* bacteria that express F4 and F18 fimbriae but do not produce enterotoxins. This vaccine has shown to be particularly effective in preventing the colonization of pig intestines following challenge with a virulent F4+ ETEC strain and F18+ ETEC strain, and therefore reducing the length and intensity of diarrhea (Fairbrother et al., 2017). The vaccine can be delivered via drinking water or administrated individually and is recommended for the vaccination of weaned pigs at the age of 17 days or more. In addition, the use of bivalent F4/F18 vaccine administered to pigs in drinking water has shown positive results in protecting piglets challenged with F4-ETEC and F18-ETEC within 7 days of vaccination (Nadeau et al., 2017).

Nevertheless, since PWD caused by F4+ ETEC generally starts within 3–10 days after weaning, pigs will remain unprotected for a period. To avoid this, immunization or, at the very least, immune system first stimulation, should take place during the suckling phase. However, it is likely that the presence of maternal antibodies in the gastro-intestinal tract during this time may interfere with the efficient colonization by the vaccine strain and, perhaps, successful immunization. Nevertheless, positive results have been observed when using a live F18ac-positive *E. coli* vaccine in suckling pigs born to sows with F18-specific colostrum IgA antibodies (Bertschinger et al., 2000). The possible explanation could be that maternal IgA antibodies favor the binding of the vaccine antigens to M cells. Indeed, results obtained in mice suggest that luminal antigens and microorganisms complexed with slgA could bind to M cell surfaces, and selectively transported to underlying macrophages and lymphoid cells (Weltzin et al., 1989).

Vaccine effects on immune response are well reported but nothing is known about the interaction between oral *E. coli* vaccine and the intestinal microbiota. In addition, it is not known if the effect of E.

coli vaccination on gut homeostasis can vary according to the swine genetic variants associated to the susceptibility to ETEC F4 and F18.

5 Feeding Strategies in the pre-weaning

The selection of iper-prolific sows led to an increase of frequency in low-birth-weight piglets and to a higher prevalence of piglets that have been subjected to intra-uterine growth retardation (IUGR), consequentially, there's been an increasing body weight variability of the litters. In this phase, therefore, the nutritional strategies are oriented to improve the survival rate, increasing weaning weight and lowering variability in body weight within the litter.

In addition, as discussed in the previous chapters, first colonization of the intestinal microbiota is critical for the proper development of the intestinal innate immune system and barrier function. In fact, during the early-life period, the composition and diversity of microbiota is unstable and highly influenced by several factors, including nutrition, exposure to stress, the use of antibiotics, and that can have a long-lasting effect on the gut microbiota and immune system (Schokker et al., 2015). Thus, programming the gut during early life could be beneficial throughout the entire production life of piglets.

5.1 Supplemental Milk

Milk contains bioactive compounds that are essential for the proper growth and development of the piglet GI tract. However, in some cases, with larger litters or when there are impairments in the milking ability of the sow, the use of supplemental milk can be useful in order to increase the weaning weight and reduce the weight variability within litters. Supplemental milk is defined as formula milk given to piglets that are reared by the sow. Studies where supplemental milk formulations have been used to manage large litters (13-14 piglets), have shown the improvement of small intestinal development and cell proliferation, as well as increased colon volatile fatty acids (AGVs) concentrations, and improving weaning weight (Greeff et al., 2016). In addition, milk replacer can also effectively reduce the incidence of pre- and post-weaning diarrhea, by modulating the colonization of the intestinal microbiota and altering the expression of mRNA encoding for jejunal inflammatory cytokines and

proteins that make up the gastrointestinal barrier (Jin et al., 2020). Another important aspect to consider is how milk replacer is formulated, for example, prebiotics are usually added to the formula to mimic the sow milk composition. These carbohydrate-in nature compounds can modulate gut microbiota, have a direct antimicrobial action, and be involved in the immune development. Taking another example, piglets receiving a formula milk supplemented with polydextrose and short-chain fructo-oligosaccharides (scFOS) had a similar peptidase activity, compared to piglets reared by the sows (Radlowski, 2012). In addition, the use of galacto-oligosaccharide (GOS) in milk replacer led to morphological changes in the duodenum (e.g., enlarged villus height, villus area and villus-to-crypt ratio) of piglets, suggesting an increase in nutrient utilization. Longer GOS exposure also seemed to improve gut functioning (e.g., nutrient digestion, barrier function) (Alizadeh et al., 2015). However, in these studies formula milk was provided by artificial rearing, where piglets were completely separated from the mother, and reared with only with artificial milk. These strategies already proved to be feasible, because piglets are fed *ad libitum* and without competing with other littermates for the milk, but they may be very expensive and are not considered by the EU legislation, because of the absence of behavior link with the mother. So, the effect seen in these studies may be less pronounced in sow-reared piglets. Nevertheless, several studies have shown a positive effect on live weight (Azain et al., 1996; Dunshea et al., 1999, 1998; Miller et al., 2012; Novotni-Dankó et al., 2015; Wolter et al., 2002) and reported in Figure 4.

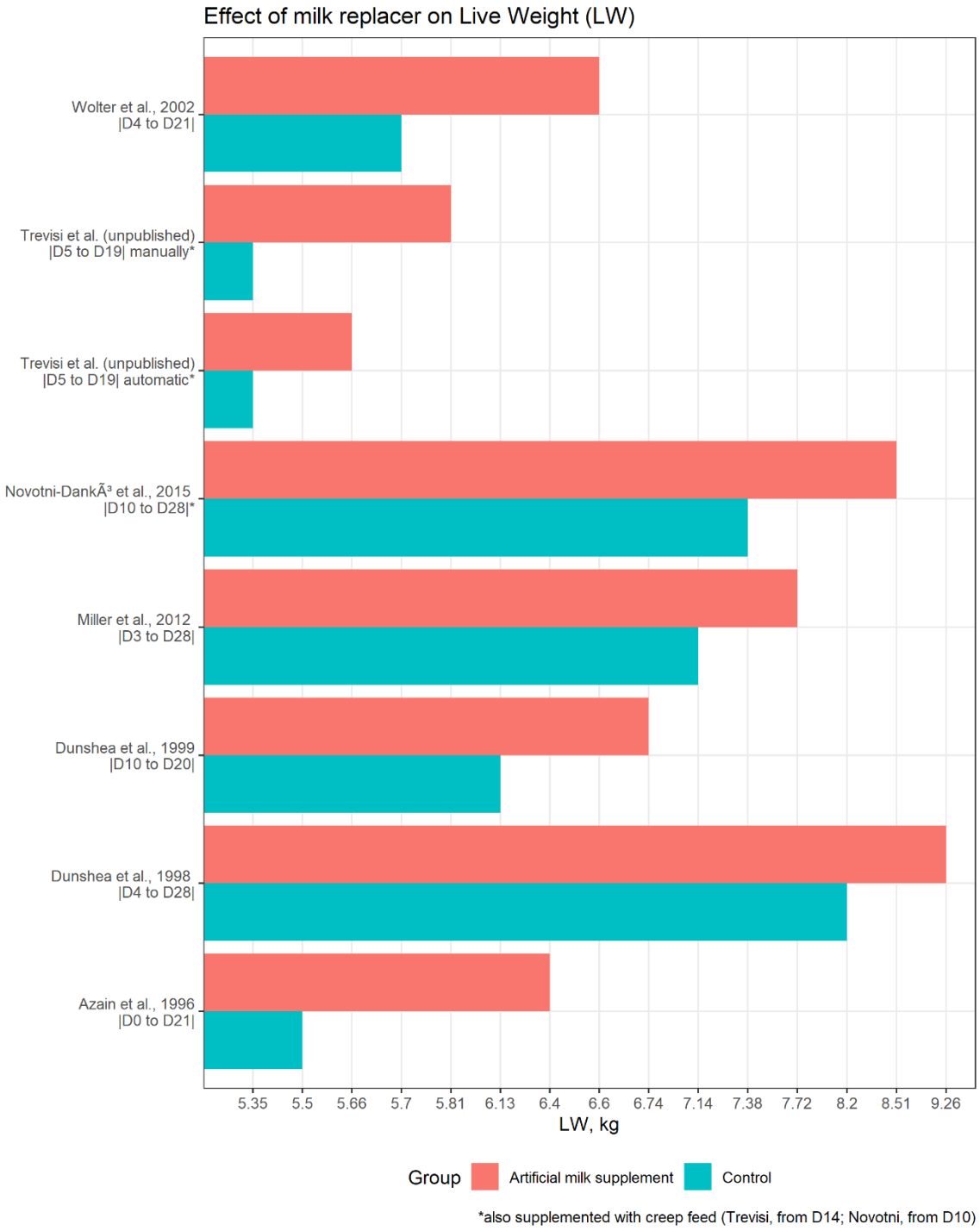

Figure 4. Studies showing the effect of milk replacer on live weight (LW), all the differences P-value <0.05.

5.2 Creep feed

The feeding shift from a diet mostly sow's milk to entirely solid feed is one of the major stressors that occurs during weaning, leading to the post-weaning fall in piglet health and performance. Creep feed is mainly used to increase weaning weights and improve post-weaning piglets' performance by stimulating the production of digestive enzymes associated with carbohydrate and protein digestion, provide tolerance to antigens present in the post-weaning diet and habituate the piglets to solid diet. Creep feed ingestion have also been linked to positive effects on gut morphology, cell proliferation and net absorption in the small intestine (Muns and Magowan, 2018), it can also affect the intestinal microbiota and its metabolism (SCFs production), as well as supporting digestive system development (Choudhury et al., 2021). The efficacy of the creep feed is tightly linked to multiple factors including: age of the animals, the amount of feed they ingest (intake level) and dietary and nutrient composition.

Among all factors, the intake level of the complementary feed is the most important factors for increasing growth performance and to influence the microbiota composition and is mostly related to the age of the animals and the amount of milk they drink. For example, piglets suckling the middle and posterior teats have been observed to consume more creep feed than piglets suckling the anterior teats. In fact, posterior teats produce lower quantities and quality of milk compared to the others, and central teats are more subject to dispute (Scheel et al., 1977).

To increase the creep feed intake several strategies can be adopted, for example mixing the solid feed with water or supplemental milk in the first days. In fact, during the neonatal period, piglets have the ability to suckle but the muscles involved in the mastication must develop in order to ingest solid food (Tucker and Widowski, 2009). Other strategy that can be adopted to increase the creep feed intake involves the use of softer pellets; this can be accomplished by increasing the size of the pellets, in fact several studies shown a positive effect of larger pellet on creep-feed intake in the pre-weaning (Table 1).

Table 1 Effects of the size of creep feed pellets on suckling pigs growth performance and health.

Authors	Pellet, mm	Start	End (weaning)	Mortality	ADG	Creeep feed disappearence	Postweaning performance (ADG)	Postweaning Health
(Craig et al., 2021)	4 x 4 vs 9 x12	D3	D26-26.5	=	=	From gilts, D3-D10, ↑ All, later, ↑	W-D21, ↑	Died=, Removed and medicated ~ ↓
(Clark et al., 2015)	~~Ø~~ 3.2 vs 12.7	D 10	D 21	↓	=	% eaters ↑	=	NA
(van den Brand et al., 2014)	~~Ø~~ 2 vs 12	D4	D25-28	NA	NA	Preference: ↑ (D4-D18)	NA	NA
	~~Ø~~ 2 vs 12	D3	D25-D30	=	=	Week 1, ↑	W-D10, ↑	Diarrhea score =
	~~Ø~~ 2 vs 10	D3	D25-D28	NA	=	Week 1&2, ↑	NA	NA
(Edge et al., 2005)	~~Ø~~ 1.8 vs 5	D10	D28	NA	=	Time on trough ↑	NA	NA

In addition to the strategies that lead to an increase in creep-feed intake, a number of dietary methods (e.g., highly digestible protein sources, feedstuffs with low fermentability) , and the addition of functional amino acids (e.g. glutamine), prebiotics (e.g., oligofructose), probiotics, symbiotics (Shim et al., 2005), medium-chain fatty acids (MCFAs), and different fiber sources (e.g., insoluble and soluble fibers) to creep feed, can be useful to modulate the gut health and improve the growth performance. Moreover, the use of a more fibrous diet or the increase of fermentable or non-fermentable fiber in the creep feed, can increment the fermentation activity in the large intestine (Hees et al., 2019). Another factor to consider is the nutritional and compositional similarity between creep feed and the post-weaning diet because piglets may be unable to get used to the post-weaning diet if there are significant differences between the two feeds, even if they consumed a significant amount of creep feed during the pre-weaning stage. For example, in a study by Heo et al. (2018), piglets were fed creep feed, a weaner diet, or a sow diet before weaning, and all of the groups were given the identical weaning diet after weaning: the piglets given creep feed had the highest feed intake before weaning. However, piglets given the weaner diet in both the pre-weaning and post-weaning phases had the highest feed intake and

body weight gain compared to the creep feed group in the first week post-weaning. These findings highlight the importance of feeding pigs the same or comparable pre-weaning diet in order for them to identify the post-weaning diet in terms of behavior (e.g., reduced feed neophobia) and physiology.

5.3 Oral Supplementation

Because of the limited feed intake in the first days of life, oral supplementation can be an effective strategy to properly administrate various additives, in order to improve BW and/or promote the maturation of GI tract and microbiota composition. Some studies, for instance, investigated the oral supplementation with probiotic *L. rhamnosus* GG to newborn piglets, showing positive results on the intestinal health by improving biological, physical, and immunologic barriers of intestinal mucosa (Wang et al., 2019b). Moreover, the oral supplementation with prebiotics like inulin, showed positives results on gut morphology and increased SCFAs production in the large intestine (Li et al., 2018). Other prebiotics like GOS can effectively modulate the microbiota composition (Wu et al., 2020) and increase the barrier function in the small intestine (Tian et al., 2018; Wu et al., 2020). In addition, the supplementation with other bioactive compound, like polyamines (spermine) can promote gut maturation by increasing the intestinal absorptive area at weaning and promote piglet growth after weaning. However, most of these studies involved the administration of the products daily or for prolonged periods, which makes it difficult to apply these methods in routine farm practices.

6 Feeding Strategies in the post-weaning

As we highlighted in the previous chapters, weaning involves a short-term anorexia, that combined with all the other stressors, leads to a compromised gastric barrier and function, intestinal inflammation, histological alterations in the small intestine (increase in the villi:crypts ratio), and the proliferation of pathogenic bacteria. The feeding strategies in this phase are oriented to increase the feed intake of the piglets, by using for example high palatable diets (Dong and Pluske, 2007), to increase feed digestion and absorption, modulate the gut microbiota composition to favor more beneficial bacterial species and to sustain the immune system development to enhance disease

resistance. In the following chapters the more studied feed supplements used to achieve these goals will be introduced.

6.1 Supplementary enzymes

Corn, wheat and barley are the most utilized feedstuffs in pigs diets across the world, for they starch content. However, these feeds include amounts of non-starch polysaccharides (NSP) and phytate-bound phosphorus. NPS are an important part of the plant ingredients, and most of them are composed by arabinoxylans, cellulose and β-glucans (Choct, 2015). Monogastric animals do not have the enzymes capable of digesting NSP, and this can be amplified in weaned pigs has they do not have developed yet the enzymatic ability to digest the vegetable diet (Lindberg, 2014). In addition, in the GI tract NSP can raise digesta viscosity, change the epithelial architecture of the gut, and impair nutrient digestibility (Lindberg, 2014; Passos et al., 2015). This may interfere with the animals' growth performance (Jha and Berrocoso, 2015). The use of exogenous enzymes may help to increase feed efficiency and to increase feed digestion especially in critical phases such as weaning. Among all the enzymes, the most used are fhytases and carbohydrases, then there are also proteases and lipases (Ravindran, 2013).

Phytases are the bestseller exogenous enzymes in pigs and poultry, since the majority of P in the seed-based ingredients is present inside the phytic acid molecule (Hexaphosphoric ester of myo-inositol). Thus, phytase can be a viable tool to spare phosphorus supplementation and can reduce the excretion of undigested P, which is ecologically beneficial. Furthermore, phytase may enhance macro and trace mineral availabilities and the utilization of protein/amino acids. The major effects of phytase in pigs have been summarized in a major review by Selle and Ravindran (2008).

Among the exogenous enzymes that target specifically NSP, xylanases are the most used. The effect on feed efficiency and growth performance is related their positive action by reducing viscosity and by degrading soluble arabinoxylans (Axs), or by hydrolyzing insoluble Axs into lower molecular weight arabinoxylan oligosaccharides (AXOS) releasing nutrients that are available to the animal and improving average daily gain and feed converition ratio (Cho et al., 2016; Lan et al., 2017). Recently it has been suggested that NPS degrading enzyme supplementation might influence the intestinal

microbiota through two main mechanisms: reducing the undigested substrates and releasing AXOS from cell wall NSP with potential prebiotic effects (He et al., 2020; Kiarie et al., 2013).

6.2 Probiotics

Probiotics can be defined as "Live microorganisms that when administered in adequate amounts confer a health benefit on the host" (Hill et al., 2014). Probiotics must be tolerant to their environmental conditions, while being alive and active in GIT, they must be resistant to saliva, gastric juice (acid and digestive enzymes), bile salts, and competitive conditions of gut. The most used probiotics are bacteria belonging to the *Lactobacillus*, *Bifidobacterium*, and *Enterococcus*. There is a large amount of studies who have reported beneficial effect of probiotic on intestinal health and growth performance (Barba-Vidal et al., 2018), but the mechanism actions related to this positive effects are not completely understood. The main hypotheses involve:

- Inhibition of pathogen adhesion to the intestinal mucosa by competitive exclusion (Roselli et al., 2005). Indeed, (Jin et al., 2000) reported that a strain of *Enterococcus faecium* inhibited the adhesion of enterotoxigenic E. coli (ETEC) K88 to the small intestinal mucosa. – Production of antimicrobial substances like bacteriocins and organic acids, i.e., Bacteriocin production is well characterized for *Lactobacillus salivarum* and *Enterococcus faecium* strains. – Modulation of the immune response by stimulating or suppressing the innate immune responses via several mechanisms including modulation of pro-inflammatory cytokines. Wang et al. (2020) observed that oral administration of *L. reuteri* I5007 can promote T-cell differentiation and stimulate ileal cytokine production, indicating that this probiotic strain could influence immunological function in weaned piglets.

A major limitation of probiotics is that the effects are treatment specific, depending on the strain, dosage, and context (Bosi and Trevisi, 2010; Li et al., 2012), and host-related physiological factors (e.g., health condition and genetics) or environment (e.g., sanitary status and food) (Dinan and Cryan, 2016; Mulder et al., 2009). In addition, there may be potential risks when using certain probiotics in animals with impaired gut health or pathogen pressure. For instance, during weaning the increased para/trans-cellular permeability in the enterocyte determined by inflammatory stress, can favor

bacterial translocation (Lallès et al., 2004). Moreover, it has also been reported that some probiotics may have immune-suppressive effects in the host (Siepert et al., 2014). Taken separately, these factors do not represent a problem in healthy piglets but can be a risk factor in pathological situation.

6.3 Prebiotics

The first definition of prebiotics was given by Gibson and Roberfroid, 1995 and were defined as "a non-digestible food ingredient that beneficially affects the host by selectively stimulating the growth or activity of one or a limited number of bacteria in the colon, and thus improves host health". However, only few carbohydrate compounds fitted this definition. In the last years, the discovery of new substances that have a positive effect on the gut bacteria colonization, and the discovery that prebiotics may have an influence for extraintestinal sites directly or indirectly, the definition was updated to "a substrate that is selectively utilized by host microorganisms conferring a health benefit" (Gibson et al., 2017). The beneficial effects of prebiotics are related to the fact that they are not digested in the upper GIT and are fermented by specific residential bacteria once they reach the colon. The fermentation of carbohydrates in the colon leads to the production of SCFA, (mainly acetate, propionate, butyrate) (Janssen and Kersten, 2015; Slavin, 2013). SCFA, in particular butyrate, represent the major energy source for colonocytes, even when competing substrates (e.g., glucose and glutamine) are available (Zambell et al., 2003). In addition, SCFA can lower the luminal pH and inhibits the proliferation of pathogenic bacteria and the production of toxic compounds, such as ammonia, amines, and phenolic compounds (Jarrett and Ashworth, 2018; Slavin, 2013). Prebiotics can also bind to pathogenic bacteria and prevent their adhesion to the intestinal epithelium (Hickey, 2012). Fructo-oligosaccharides (FOS), galacto-oligosaccharides (GOS), inulin, arabinoxylo oligosaccharides (AXOS) xylo-oligosaccharides (XOS), chito-oligosaccharides (COS), and dietary carbohydrates are among the most studied prebiotics. **Table 2** highlights the most significant recent results of various studies on the influence of prebiotics on intestinal health of weaned piglets.

Moreover, prebiotics can be used in combination with probiotics, in a relationship called symbiotic, to increase their residence by bacterial adhesion properties. In a study by Trevisi et al. (2008), the

combination of FOS and *Bifidobacterium animalis* increased the expression of TLR2 in the ileocecal lymph nodes of weaned pigs and thus may play a role in enhancing the innate immune response.

Table 2. Effect of prebiotics on intestinal health of weaned piglets

Prebiotics	Dosage	Duration	Outcomes	References
FOS	0.60%	7 d	↑ Bifidobacteria, *Lactobacillus* in jejunum ↑ IFN-g ↓ Proteobacteria in jejunum and ileum ↓ IL-4, IL-10	(Chang et al., 2018)
COS	100 mg/kg	3 wk	↑ *Bifidobacterium* ↑ Villus height, tight junction protein, IL-6, TNF-a ↓ *Escherichia coli*	(Wan et al., 2017)
COS	30 mg/kg	2 wk	↑ Intraepithelial lymphocytes number, goblet cells, IL-10, secretory immunoglobulin, ZO-1 ↓ Villus height, villus height-to-crypt depth ratio	(Xiong et al., 2015)
COS	400 mg/kg	2 wk	↑ Villus height, villus height-to-crypt depth ratio ↓ Crypt depth	(Aluko et al., 2017)
XOS	200 mg/kg	4 wk	↑ Villus height-to-crypt ratio ↑ Lactobacilli ↓ *E. coli*	(Li et al., 2018)
XOS	0.01%	Weaned	↑ Streptococcus, Turicibacter, ZO-1	(Yin et al., 2019)
AOS	100 mg/kg	2 wk	↑ Villus height, villus height-to-crypt depth ratio, goblet cells	(Wan et al., 2018)
AOS	100 mg/kg	2 wk	↑ Intestinal occludin, intestinal catalase activity	(Wan et al., 2018)
Resistant potato starch	5%	12 d	↑ *Terriporobacter*, *Sarcina*, *Clostridium sensu stricto 1* ↑ butyrate and lactate	(Trachsel et al., 2019)

6.4 Nucleotides

Nucleotides are essential in several physiological processes like encoding genetic information, mediating energy metabolism, and serving as coenzymes (Henderson and Paterson, 2014). Due to the absence of a nucleotide transport system and the presence of a high negatively charged phosphate group they cannot be absorbed by the intestinal epithelium but need to be first hydrolyzed in nucleosides (Uauy et al., 1994). Nucleotides are considered not essential nutrients. Some cells, however, such as intestinal epithelial cells and immune system cells, have minimal capacity to produce nucleotides through biosynthetic pathways and must rely on external supplies (Grimble and Westwood, 2001). Furthermore, the nucleotide need could be higher under particular condition such as fast growth, restricted food intake, illnesses, and immune response activation (Grimble and Westwood, 2001; Sauer et al., 2011), all factor related with weaning, so it is expected that the requirement for nucleotides during this this period is higher. In addition, nucleotide concentration tends to decrease during the lactation period (Sauer et al., 2012b) and the vegetal feed stuff used in the post-weaning diet diets luck of nucleotides.

Indeed, nucleotide supplementation can increase the villus to crypt ratio, stimulating cell division and differentiation, reducing apoptosis and modulates the innate immune responses (Daneshmand et al., 2017). Additionally, the roles of nucleotides in inflammatory responses (Giancamillo et al., 2016), composition and activity of the intestinal microbiota (Sauer et al., 2012a) in weaned piglets have been demonstrated.

On the other hand, as reviewed by Sauer et al. (2011), studies on the biological effects show contradictory results. This could me mainly related to heterogeneity of the nucleotide source used, in fact the most used are yeast cultures that may contain varying amounts of nucleotides and viable cells, cell wall components, and components of the medium in which the yeast cells were grown. As a result, attributing the reported biological effects to particular nucleotides contained in these products is challenging as studies using pure culture a limited.

7 Experimental model design to study PWD

The major limitation of studies testing the use of nutritional strategies in the post-weaning is that they are often carried out in very clean and sanitized research facilities, indicating that any results achieved may be difficult to transfer into the commercial practice. To overcome these limitations several studies relied on the use of an ETEC oral challenge. Hoverer, when using this kind of model there are several factors that need to be carefully considered including live weight, dosing concentrations of ETEC, health status, timing of the inoculum, genetic susceptibility, serotypes of pathogen (e.g. single or multiple strain) and rearing condition (single or multiple boxes). For instance, animals can have an age-related resistance for ETEC infection. In particular, the phenotypical expression of F4 receptors in the intestinal epithelium starts to increase from 7-day-old piglets up to 35-day-old pigs (Conway et al., 1990), while the timing of the expression of F18R is still debated but the animals appears to be susceptible to the infection starting from weaning (Coddens et al., 2007). In addition, the timing of the supplementation represents a critical factor, because piglets can still have some passive immunity coming from the mother milk, especially when the animals have been taken from farm with a recent history of ETEC infection. This can reduce the practical impact of the ETEC challenge. All the that and other factors involved in designing of ETEC F4/F18 challenge model have been extensively reviewed by Luise et al. (2019).

Alongside with that, with the increased use of ETEC vaccines in commercial farms, the use of experimental models that include susceptible piglets and ETEC vaccination can be considered. This induces an active immune response in the responsive piglets (ETEC-susceptible) and could allow a more accurate study of the effect of all those nutritional additives designed to support the development of the immune system.

8 Slow growing piglets

In piglets, reduced growth rate is often associated with low birth weight and low feed efficiency. There are some cases, instead, when piglets with equal birth weight have substantial variation in their growth rates already starting from the suckling period. At this stage, growth rates are closely related to the

muscle development that is linked to muscle fibers development. Indeed, Dwyer et al. (1993) found a positive relationship between the total number of muscle fibers and the postnatal development potential, demonstrating that littermates with a large number of fibers developed quicker and more effectively than littermates with a low number of fibers. However, muscles fiber proliferation ends at birth so the variations in postnatal development of muscle fibers depends on the primary fiber number and on the ratio of secondary to primary fibers as a lower secondary-to-primary fiber ratio is associated with reduced growth rate (Handel and Stickland, 1987).

Most of the research on this topic have been focused on the genetic and epigenetic factors (Estellé et al., 2008; te Pas et al., 1999; Zhao et al., 2011), but few investigated the possible link between microbiota composition and muscles fiber development. The interaction between gut microbiota and host organ systems has been recently an important object of research. The main reason is that hosted microorganisms produce several metabolites that are absorbed and released in the blood stream. Whatever is their further processing by the host they generate a communication between the gut microbiota and the organ system and can also be used as nutrients stimulating differently the host metabolism. The presence of potential effects of gut microbiota on muscle deposition and on the muscles fibers characteristics could have high relevance for the production of foods of animal origin. It is well known that several bacterial toxins, like LPS, can induce inflammation, and thus reduce in general the protein deposition. However, inflammation-independent effects on skeletal muscle metabolism can be predicted or have also been observed. A typical example is the use of different volatile fatty acid produced in the intestine as precursor for gluconeogenesis or for fatty acid synthesis. Indol derivatives, typically originating from bacteria, can stimulate myoblast glycolysis and pentose phosphate pathway *in vitro*, with implication for muscular deposition in mice (Grosicki et al., 2017). Nevertheless, a few research data suggest that also effects on meat quality cannot be excluded. Variation of gut bacteria profile was associated to pork intramuscular fat content (Fang et al., 2017). The possibility that gut microbiota can affect also muscle fibers characteristics is suggested by the observation that the porcine gut microbiota transplanted to germ-free mice reproduced the myocellular phenotype of the donor (Yan et al., 2016). An indirect confirmation is the association of analytes and metabolites with muscle quality in young, healthy adult humans (Lustgarten et al., 2015). A variation in

the ratio between fast and slow twitch fiber types may have relevance to the meat industry because of the different susceptibility to post-mortem glycolysis and for the final pH, that is one of the determinant factors influencing drip loss.

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Aim of the thesis

The objective of the thesis was first to study the changes that occur in the mucosal immune system and the microbiota of the small intestine before and after weaning. Then, the effect of vaccination on ETEC susceptible subjects was investigated, in order to define an experimental model that includes subjects susceptible to ETEC and subsequent vaccination. This model was used to evaluate nutritional interventions, to prepare piglets for weaning and to improve intestinal health, growth performance and development of the intestinal microbiome and immune system.

In particular, the first study aimed to increase the knowledge on the connection between the microbial colonization of the small intestine and the development of the local immunity of piglets, comparing the transcriptome profiles of jejunal Peyer patches and the peripheral blood, with the contemporary variation of the microbial in jejunum in the pre and post-weaning. In the second study, was focused on the effect of the *E. coli* F4/F18 live oral vaccine on the growth performance and profile of the faecal microbiota of piglets around weaning and also to investigate the effect of the genetic ETEC susceptibility on piglet's performance and faecal microbiota, as well as their interaction with the vaccination. Based on the knowledge acquired from the previous study, the third study was designed to evaluate the effect of an oral administration of nucleotides before weaning and in the immediate post-weaning on growth performance, health, intestinal structure, immunity, microbiota, and transcriptomic profile of piglets preliminary vaccinated against ETEC. In the fourth study, the use of bacterial xylanase in weaned piglets genetically susceptible to ETEC infection was tested. The effects were evaluated on the growth performance, intestinal microbiota and on some indicators of digestive homeostasis. The last study was designed to investigate the possible connection between gut microbiota and the muscles development in slow and fast-growing piglets during the suckling period.

Experimental studies

Manuscript 1

Weaning differentially affects the maturation of piglet peripheral blood and jejunal Peyer's patches

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Abstract

The study aimed to assess how the post-weaning condition changes piglet peripheral blood (PB) and jejunal Peyer's patches (JPPs) as compared to the suckling period, and how these changes are associated with intestinal microbiota evolution. Sixteen pigs were slaughtered and sampled for PB, JPPs and jejunal content (JC) at weaning (26 days) or at 12 days fed on a pre-starter diet. The PB and JPP transcriptomes were analysed using mRNA-seq. The Gene Set Enrichment Analysis was used to demonstrate enriched gene clusters, depending on sampling time. Jejunal microbiota was profiled using 16S rRNA gene sequencing. Post-weaning JPPs were enriched for processes related to the activation of IFN-γ and major histocompatibility complex (MHC) class I antigen processing which clustered with the reduced abundance of the *Weisella* genus and *Faecalibacterium prausnitzii* in JC. The post-weaning microbiome differed from that seen in just-weaned pigs. For just-weaned PB, the enrichment of genes related to hemoglobin and the iron metabolism indicated the greater presence of reticulocytes and immature erythrocytes. The JPP genes involved in the of I MHC and IFN-γ activations were markers of the post-weaning phase. Several genes attributable to reticulocyte and erythrocyte maturation could be interesting for testing the iron nutrition of piglets.

1. Introduction

The weaning period is a turning point in the life of commercial swine; the latter must be prepared for the robustness achieved during the suckling phase in order to avoid an excessive decrease in feed intake, transient gut inflammation and intestinal dysbiosis. Weaning implies providing a solid diet based on feeds of vegetal origin and the mixing of pigs from different litters. Thus, the gut of the newly weaned pig faces large quantities of poorly known molecules of dietary origin and of microorganisms scarcely encountered in its previous life. Suckling pigs progressively educate their mucosal lymphoid tissue9 upon the multiplication of microorganisms favoured by the presence of milk and, later, of additional offered feed. This is achieved in interdependence with the maturating activity of bone marrow and the thymus, and seeding from the peripheral blood (PB) (Sinkora and Butler, 2009).

The local porcine immune system is based on: i) both a diffuse system, mainly in the lamina propria, and on intraepithelial lymphocytes, ii) organised compartments: Peyer's patches (PPs) which are bud-shaped in the jejunum and continuous single structures in the ileum, and mesenteric lymph nodes; PPs have an important role in the host-microbiota cross-talk. Based on studies regarding surgical removal in rats (Enders et al., 1988) and gene knockout mice (Spahn et al., 2001), PPs are necessary for the production of immunoglobulin A (IgA) and to avoid intestinal pathogen translocation (Bermudez et al., 2010). In the lumen-protruding epithelium associated with PP follicles, specialised cells collaborate to detect and deliver particles from the lumen to the underlying tissue. B cells proliferate in the germinal centre of PPs, have somatic hypermutation and undergo selection upon the induction of T cells which are primed in the PP interfollicular area. Some recent evidence has finally suggested that ileal PPs (IPPs) initiate their development prenatally and accelerate after birth (Furukawa et al., 2020), being more involved in primary, undiversified IgA production (Butler et al., 2016) while jejunal PPs (JPPs) develop postnatally, being more responsible for initiating the production of diversified IgA.

Recent reviews have addressed the evolution of the gut microbial profile in piglet early life (Everaert et al., 2017; Guevarra et al., 2019). However, the major part of the microbiota data were not obtained from samples of the small intestine and did not compare the variability of the microbiota in the suckling pig with the maturation of PPs located in the second half of the small intestine. The need still exists to assess how the variations of the microbiota in the small intestine after the weaning period

compare with those induced in PPs and with pre-weaning values. The availability of a modern sequencing method to detail the transcriptome and microbiota gives important opportunities to disclose the involvement of under-considered biological pathways and potential marker genes. The PB is also an interesting area of study due to the minor invasiveness of the sampling and the possibility of replicating on the same individual. Their availability could speed the assessment of the efficiency of the dietary or management strategies proposed for the piglet in the transition from late suckling to the weaning period, and thereafter.

The general aim was to increase knowledge regarding the connection between the colonisation of the small intestine, the development of local immunity in piglets and their general health. The specific aim was to focus on the transcriptome profiles of JPPs and PB in healthy pigs at the time following separation from their mothers and at 12 d post-weaning, and to connect these results with the simultaneous variation of the microbiota in the jejunum.

2. Results

For PB and JPPs, 21,424 and 22,953 transcribed genes were recovered, with a mapping rate of 79 ± 3% and 78 ± 3%, respectively. The genes with useful attribution were 15,509 and 16,197, respectively. The separation between tissues was evident using preliminary multidimensional scaling plots. The JPP samples did not seem to cluster based on the timepoint; instead, the PB samples tended to cluster better (Figure 1a). Smear plots (Figure 1,b-c) showed that the majority of genes were centred around a log fold change (FC) of zero indicating that any composition bias between libraries was successfully removed.

2.1 Effect of weaning on differential gene expression analysis in JPPs.

The JPPs sampled at weaning and at 12 d post-weaning presented 114 and 31 significantly enriched gene sets (False discovery rate [FDR]<0.05), respectively. The transcriptome of the JPPs of the just-weaned pigs demonstrated intensive proliferative activity as compared to that at 12 d post-weaning. In fact, several gene sets related to DNA replication, elongation and repair; chromosome segregation;

chromatin and histone changes; mRNA export, regulation, maturation and ribosome activity, and translation activation were more enriched in JPPs at weaning (Figure 2).

Conversely, in post-weaning JPPs, the gene sets were mainly related to immune organisation and local structure, and the functions were enriched (Figure 3).

Biological processes related to interferon gamma (IFN-γ) production and response were involved, with a particular impact on the activation of the processing of major histocompatibility complex (MHC) class I antigens and their presentation. This, in turn, influenced several types of immune cells, including CD8+ cells and natural killer cells. Other enriched gene groups potentially related to immune functions were those related to the response to interleukin (IL) 15 and multi-vesicular body transport in cells. The transcriptome for structural organisation of the JPPs was more differentially affected by the enrichment of genes associated with keratinisation and cornification of cells. Genes for the metabolic process of cysteine and tricarboxylic acids were also upregulated.

The JPPs sampled at weaning and at 12 d post-weaning presented 126 and 164 genes positively differentially expressed, respectively (Supplementary Table 1). Melanotransferrin was the most upregulated gene at weaning and subtilisin-like proprotein convertase (*PCSK9*) at 12 d post-weaning.

2.2 Effect of weaning on differential gene expression analysis in the PB

The PB sampled at weaning and at 12 d post-weaning presented 114 and 31 enriched gene sets (FDR<0.05). In PB of pigs at weaning, Gene Set Enrichment Analysis (GSEA) evidenced primarily the enrichment of gene sets related to haeme biosynthesis and the metabolic process as compared to post-weaning (Figure 4). This was also associated with other sets typical of the PB: erythrocyte development, iron haemostasis, and regulation of coagulation. Other enriched sets were those related to the protein translation and post-translational processes.

The PB at weaning and at 12 d post-weaning presented 1195 and 242 up-regulated genes (Supplementary Table 2). Of those more expressed at weaning, there was haemoglobin subunit beta-like (ENSSSCG00000014727), not included in the list of gene sets. Bisphosphoglycerate mutase was the most upregulated gene at weaning (Padj=2.9 x e^{-9}). Peptide YY (*PYY*) and arginase 1 were among the genes not directly associated with haemoglobin function which were most upregulated at weaning.

2.3 Effect of weaning on the microbiota of the jejunal content

The sequencing procedure of the jejunal content (JC) produced 46,109 sequences on average per sample and a total of 882 amplicon sequence variants (ASVs). The ASVs were aggregated into 21 phyla, 96 families and 179 genera. The most abundant phylum was Firmicutes. accounting for 92.8 ± 5.3% of the total (mean ± d.s.) at both time periods, followed by Actinobacteriota 6.6 ± 1.4%. Lactobacillaceae and Streptococcaceae were the two most abundant families, accounting for 70.8 ± 8.2% and 14.3 ± 4.6%, respectively. *Lactobacillus*, 67.9 ± 7.6%, and *Streptococcus*, 14.3 ± 4.7%, were the most abundant genera. At 12 d post-weaning, the bacterial richness (Chao1) increased (Figure 6-a); this effect was not observed for the other measurementss used (Shannon and InvSimpson). For beta diversity, weaning affected the jejunum bacteria composition (R^2=0.13, *P*=0.001), as is also shown by the principal coordinates analysis (PCoA) plot in Figure 6-b, in which samples are clustered based on timepoint.

Several genera were enriched in the JC at 12 d post-weaning (Supplementary Table 3). The increased abundance of *Streptococcus porcorum*, *Collinsella aerofaciens* and of an ASV belonging to the *Blautia* genus was relevant for 4.28; 4.33; 8.74 Log2 Fold Changes, respectively as compared to the JC at weaning.

2.4 Effect of the clustering of weaned pigs of MHC class I antigens in JPPs on the jejunal microbiota

Due to the relevance of activation of the response of MHC class I antigens and IFNγ in JPPs sampled in the post-weaning phase, the post-weaning pigs were clustered into two groups differing as to the expression of 44 genes relevant to this response. The normalised expression of genes for cluster group 1 was, in general, higher than that of cluster group 2 (Supplementary Table 4). No effect of the attribution of each pig to the specific cluster group on Alpha and Beta-diversity of the jejunal microbiota was observed (Supplementary Figures 1 and 2). However, the pigs classified using statistical analysis as belonging to the cluster group with the higher activation of response to MHC class I antigens and IFNγ presented a highly significant reduction in the presence of two AVSs, belonging to the *Weissella* genus (mean abundance, cluster 1 =0; cluster 2 = 398.99, Log2 Fold Change =-11.1; adjusted P=0.00019) and *Faecalibacterium prausnitzii* (mean abundance, cluster 1 = 0; cluster 2 = 42.47; Log2 Fold Change=-23.13; adjusted P=3.5 x e^{-15}).

3. Discussion

3.1 Effect of weaning on the differential activations of MHC class I antigens in JPPs

Early stimulation by diverse microbiota acquired in the environment is important for developing the ability of inducing the complex activation of MHC in pigs to expand the intestinal immune system upon the encounter of different non-self antigens (Mulder et al., 2009). Compared to that of the just-weaned, the post-weaning condition of piglets was marked by switching to the activation of JPPs, related to the adaptive response associated with MHC class I stimulation and also to cells less dependent on antigen activation, such as natural killer cells (Beaulieu, 2021). This observation fits with the delayed maturation of CD8+ T cells (Vega-López et al., 1995) which are also located in the PP inter-follicular areas (Barman et al., 1997) and stimulated by class I antigens in the porcine jejunal lamina propria up to five weeks of age. Post-weaning did not affect the response related to MHC class II which was associated with the observation that CD4+ positive cells were typically stimulated by the molecules of that class and developed earlier in the small intestine of pigs(Vega-López et al., 1995). Furthermore, comparing the transcriptome of the IPPs of pigs still suckling at 21 d of age with those obtained from pigs weaned at this age but then reared for another week, the pathway Immune response MHC class I antigen presentation was positively affected (Inoue et al., 2015) Conversely, Immune response MHC class II antigen presentation was negatively affected (Inoue et al., 2015).

A complex array of phagocyte cells from different lineages of myeloid origin, under the general name of dendritic cells (DCs)(Da Silva et al., 2017) were aligned in the PP area. Some of them were located more in the interfollicular area, in the proximity of the aggregation of CD8+ T cells. These DCs play an important role of presenting other types of antigens, mainly of endocellular origin, priming naïve CD8+ T cells to polarise into cytotoxic T lymphocytes, in a local environment which is enriched by the secretion of IFN-γ from already primed T cells (Sato and Iwasaki, 2005). The molecular mechanism for processing antigens into the MHC I system is a kit which is the heritage of all nucleated cells; however, in PPs, a specific context is found for this process. The diffuse enrichment of several groups of genes involved in MHC class I activation after weaning in JPPs merits more specific discussion (Figure 7).

Weaning up-regulated almost all the genes associated with antigen processing and the presentation of the endogenous peptide antigen (Figure 7), including those coding for three subunits of porcine MHC class I (Swine Leukocyte Antigens, *SLA-1*, *SLA-2*, *SLA-3*) (Hammer et al., 2020). Other genes were those promoting entrance into the ER (*TAP1* and *TAP2*), the invariant light chain stabilising MHC class I, β-2-microglobulin (*B2M*), and the *TAPBP*; the eventual preliminary trimming of some peptides is carried out by the ER aminopeptidases (*ERAP1* and *ERAP2*).

In any cell, the majority of the antigenic molecules to be processed require reduction inside the proteasome complex. However, upon promotion by IFN-γ, this complex is integrated by three catalytic subunits (β1i, β2i and β5i) to form a immuno-proteasome (Kammerl and Meiners, 2016), specialised in the trimming of antigens for MHC I presentation. In post-weaning, the genes regulating the IFN-γ response were upregulated (IFN-γ is diffuse particularly in the interfollicular PP environment), and the three genes coding for the immuno-proteasome subunits (*PSMB8*, *PSMB9* and *PSMB10*) were also upregulated (Figure 7). Another gene (*UBD*), coding for a protein-like ubiquitin, the main actor starting the proteasome process, was also over-expressed. These observations suggested that the immuno-proteasome in JPPs maturates more after weaning, to improve the antiviral adaptive immune responses against intracellular infections. It could be proposed that *PSMB8*, *PSMB9* and *PSMB10* are important markers for testing JPP maturation.

The enrichment of the gene set related to expansion by the cell division of the T cell populations bearing the αβ type receptor, together with the upregulation of CD8A and several genes forming the T-cell antigen receptor complex (CD3D/CD3E/CD3G), additionally supports the involvement of CD8+ T cells (Figure 7) as a target of MHC I activation in post-weaning pigs. Interestingly, interleukin 15 (*IL-15*) and its receptor (*IL15RA*) were also upregulated in the post-weaning period. The maturation and survival of CD8 cells is promoted by IL-15, by the link to IL-15 receptors on these T cells (Ma et al., 2009).

Antigens accessible to MHC class I can derive from proteins of the viruses or microbes infecting the cells or were phagocytised. However, DCs can form a junction with infected non-immune cells and obtain fragments to process from them (Nierkens et al., 2013), producing a cross-presentation and priming. It is possible that this process was more stimulated after weaning because the gene set related

to multivesicular endocytic recycling was one of the most enriched (First genes: *RILP, TMEM50B, LYST*). In the context of JPPs it is also possible that mucosal antigens are transported by IgA to activate local DCs and prime T cells (Gayet et al., 2020). Finally, the need to recycle relevant quantities of MHC I molecules could explain the 1st ranking of *PCSK9*, involved in their complexation and additional lysosomal degradation (Liu et al., 2020).

The position of JPPs is strategic for sampling and controlling the proximal lumen. Emerging microbes could be useful in supplementing pigs in order to specifically orient the immune response in this phase. There was scarce evidence that membership of the pigs in the group with more activation of these pathways differed in the microbiota profile. However, the presence of the *Weissella* genus and *Faecalibacterium prausnitzii* apparently prevented the maximal activation of these pathways. *Faecalibacterium prausnitzii* is a member of Clostridium cluster IV, abundant in the human intestine in which it contributes to maintaining gut health by fermenting fibre and producing volatile fatty acids (Miquel et al., 2013). It has been isolated in pigs since 2014(Foditsch et al., 2014) and its presence in the gut content characterised the effect of supplementation with bovine colostrum to the standard weaning diet (Lo Verso et al., 2020) .This microbe is more documented in the porcine hindgut than in the small intestine(Ran et al., 2019); thus, in the future, variations of the abundance of this microbe in the jejunums of piglets deserves more attention in order to understand the local activation of the immune system. The *Weissella* genus includes lactic acid bacteria, currently found in the gut of mammals, which are the object of attention as potential beneficial microorganisms due to their ability to produce bacteriocins and also opportunistic pathogens depending on the strain (Abriouel et al., 2015).

3.2 Effect of piglet age and weaning on other gene sets in JPPs

The germinal centres of PPs are important for B cell proliferation and somatic hypermutation, and are inductive sites for IgA (Hara et al., 2019).The T-follicular helper cells interact with IgM+ naive B cells, inducing the class switching into IgA+ B cells (Stebegg et al., 2018). Nevertheless, no effect of age was seen regarding the gene sets related to controlling this process, indicating that, notwithstanding the increase in the volume of the JPPs with age (Barman et al., 1997; Prims et al., 2018), this was not the

main inductive site. It is possible that post-weaning affects the B cell receptor-signalling pathway in IPPs, considering that this pathway was up-regulated there as compared to JPPs (Maroilley et al., 2018).

Peyer's patches are more evident at anatomic inspection as protruding buttons as the pigs age. This requires more structure as evidenced by the presence of enriched gene sets related to cornification and keratinisation. The domes of PPs are marked by the presence of M cells, local cells specialised in the active transepithelial vesicular transport of microorganisms and macromolecules from the lumen to the subepithelial lymphoid tissue. Cytokeratin (KRT) 18 has been proposed as an M-cell marker in porcine PPs (Gebert et al., 1994). In the present trial, inside these gene sets, the most significant KRTs were *KRT19* and *KRT20,* instead of *KRT18.* This could mean that other KRTs, rather than *KRT18,* are more relevant for the overall structural maturation of PP*s*.

The younger and not yet weaned pigs showed a marked unidirectional pressure towards cell replication, evidenced by a large array of enriched gene sets as compared to older and weaned pigs. Inoue et al. (2015) evidenced that the transcriptome of the IPPs of suckling piglets between 21 and 35 d of age changed very little. Conversely, simultaneously weaned pigs differed sharply from up- and down-regulated genes as compared to unweaned pigs (Inoue et al., 2015) while the IPP volume increased until 28 d of age in suckling pigs but it was lower in simultaneously weaned pigs (Inoue et al., 2015). This indicated that, if the suckling period continues, the environmental stimuli tend to be constant and PPs just increase in volume. Thus, it can be hypothesised that this was also the condition of the JPPs in the present study while, in weaned pigs, the antigenic pressure tends to drive the JPPs towards qualitative maturation, as discussed in the previous section.

3.3 Effect of weaning on differential gene expression analysis in the PB

The PB is a very complex tissue due to the presence of cells of different series (erythrocytes, leukocytes, and platelets). These cells have a different origin and time of maturation, and it is not surprising that the changes seen in the PB transcriptome did not resemble those seen in JPPs, notwithstanding the fact that both contain some cells of common origin (immune cells).

Numerically, the dominant cells are erythrocytes which, in pigs, have a life of approximately 72 days (Withrow and Bell, 1969); thus, it was not surprising that the genes most represented were those related to haemoglobin (*HBA*; *HBB*; *HBB-like* gene) and functions related to its synthesis and iron regulation. In mammals, mature erythrocytes are anucleated and, thus, do not produce new mRNA but retain mRNA, for the most part in the mature form (Chen et al., 2017). This is indicated by the elevated relative presence of exonic sequences on total RNA and by the selective retention of mostly significant genes vs. nucleated erythrocyte progenitors. The majority of the remaining protein-coding mRNA found in erythrocytes codes for proteins functional for their characteristics (Chen et al., 2017), indicating that these cells are not just "bags of haemoglobin". This argues in favour of a selective process of deletion of unnecessary mRNA during the maturation of reticulocytes and their differentiation into erythrocytes. The degree of retention of mature mRNA in porcine mature blood cells has not been documented. However, the gene for erythrocytic spectrin beta (*SPTB*), an important marker of young erythrocytes (Ciana et al., 2017), among the most upregulated genes (Supplementary Table 2) at weaning supports the idea of the presence of less mature erythrocytes at this phase as compared to the post-weaning phase. Nevertheless, there are also reticulocytes in the PB; reticulocytes are young, still immature erythrocytes and are 2-7% of the circulating red blood cells (Miller et al., 1961); however, they do not replicate in circulation. Just-weaned pigs showed enriched gene sets related to haeme biosynthesis, cellular iron ion homeostasis, and erythrocyte development as compared to post-weaning pigs. This is the hallmark of a greater presence of cells of erythroid origin still under maturation. Erythropoietic activity is extraordinarily stimulated in newborn pigs (Kim and Luthra, 1977; Miller et al., 1961), and the still intense expression of genes could be related to the elevated presence of reticulocytes and/or of cells still retaining part of the functional mRNA. The greater percentage of reticulocyte cells in pigs was seen at 1 week or at 5-7 weeks of age (Miller et al., 1961). The pigs in the present study were regularly given intramuscular iron at d3–d4 of age; however, successively the intense growth typical of late suckling and the insufficient iron content of the sow milk could have stimulated intense erythropoiesis. Iron deficiency and anaemia in young pigs at weaning has been seen in recent studies when a single dose of iron was given within 48 h from birth (Bhattarai and

Nielsen, 2015; Perri et al., 2017). It is also possible that the lifespan of the reticulocytes increased in this phase, as in case of more intense release from the bone marrow (Kundrapu and Noguez, 2018).

Gene sets related to protein translation, particularly to proteins targeting to membrane, and those related to the binding of extracellular ligands to an integrin on the cell surface, were also upregulated in the PB before weaning. The erythrocyte membrane has to be particularly resistant; however, it also has peculiar selective properties. This is obtained by a reorganisation during maturation from the reticulocyte condition (Liu et al., 2020). Thus, these pathways are markers of the greater presence of erythroid cells under maturation in piglets.

Platelets are cellular fragments formed from the detached cytoplasm of large cells originating from bone marrow, megakaryocytes, and are anucleated, but can derive mRNA from the megakaryocytes, and are able to splice and post-transcriptionally manipulate it (Londin et al., 2014). Platelets store a complex mix of molecules in granules which are necessary for coagulation, and the retraction and resolution of the coagulum. In the PB of just-weaned pigs, the gene set PLATELET_DEGRANULATION was enriched more than in post-weaned pigs. This could be due to the specific activation of this pathway or the enriched presence of the platelets. In effect, as reported in a companion paper submitted regarding the same set of pigs, the number of platelets was higher in the just-weaned pigs than in the post-weaning pigs, supporting the hypothesis that the enrichment of PLATELET_DEGRANULATION was due to their increased presence in the PB. Nevertheless, this set of genes was the most affected of all the others related to the platelets, indicating that it is the most sensible to variations. Data agree with the peaking of platelet cells observed at approximately 4 weeks of age of piglets (Pliszczak-Król et al., 2016).

An interesting observation was the elevated expression of *PYY* and its upregulation in the PB from piglets at weaning (Ranking 41). *Peptide YY* encodes for a pre-protein which, depending on the site, pancreas or intestine, is cleaved to two different secreted peptides, related to controlling the meal and feed intake, a pancreatic peptide, or peptide YY. Thus, detecting these peptides in the blood is normal. However, no evidence was found in the research reported in the literature regarding a particular expression of *PYY* in the PB. In databases regarding gene expression in tissues, the PB is scarcely considered, and no evidence was found for *PYY*. The inspection of databases of trials reporting the

porcine PB transcriptome supports the Authors' observation (Maroilley et al., 2018; Ye et al., 2017). It is difficult to explain that the increased expression obtained at weaning in the PB; this could indicate that *PYY* originates from the presence of reticulocytes or from young erythrocytes. Interestingly, PYY also has a vasoconstriction action; however, an association with the eventual production of PYY from the blood has never been proposed. It is thus tempting to hypothesise that the activity of this gene could be the result of a particular need of erythroid cells to control the vessel environment.

Overall, these aspects merit more studies owing to the increasing interest in the use of the PB transcriptome as a response parameter in trials on pigs, not limited to diagnostic purposes in pigs.

In piglets sampled 12 d after weaning, the degree of differential expression of single genes was more limited than in piglets at weaning. However, several enriched sets were related to DNA replication, chromatin remodelling, and chromosome segregation. This can be explained by a greater replicative pressure on the leukocytes as reticulocytes (and erythrocytes) have no replicative ability. As will be reported in a companion paper, the number of leukocytes was sharply increased post-weaning as compared to at weaning. The results of PB transcriptome evidence showed that the systemic pressure on immune system was still present after weaning. This is consistent with JPP data, at least for those concerned the MHC I stimulation.

3.4 Effect of piglet age and weaning on jejunal microbiota

The microbiota of the content of the jejunum of piglets was dominated by the Firmicutes phylum which represented almost 97% of the total, with *Lactobacillus* and *Streptococcus* being the most abundant genus in accordance with previous studies (Crespo-Piazuelo et al., 2018; Mu et al., 2017). Weaning significantly affected the bacterial population leading to an increase in bacterial richness; this has also been well reported in the literature (Motta et al., 2019). A more diverse microbiota is considered to be a marker of a mature microbial community and is associated with functional redundancy, which contributes to a greater stability of the microbial ecosystem when it faces stressful situations, such as weaning, which can lead to dysbiotic conditions. A clear effect of weaning was also observed regarding the beta diversity, a parameter for which the samples clustered based on timepoint. The samples taken from piglets 12 days post-weaning had higher inter-variability as compared to piglets at weaning. The

opposite pattern has been reported in other studies (Bian et al., 2016; Motta et al., 2019) in which lactating piglets tended to have a higher inter-variability in relation to their individual intestinal maturation; then after weaning, there was a convergence towards a more stable microbial community. However, these studies mainly regarded faecal microbiota. Faeces represent almost the entire hindgut bacterial community which could potentially be less stable in the late suckling period in which the creep feed intake is variable between individuals, determining a variable adaptation of the microbiota (Choudhury et al., 2020). Conversely, milk intake maintains a more stable microbiota in the small intestine (Choudhury et al., 2020). On the contrary, in post-weaning pigs, it is also the small intestine which is impacted more by the relevant amount of solid feed, and the continuous influx of new bacteria from feed and from newly encountered piglets in the pen. In addition, the shift from a milk to a plant-based diet was accomplished by higher relative quantities of fibrolytic and/or short chain fatty acid producer bacterial groups, such as *Ruminococcus*, *Methanobrevibacter*, *Blautia* and *Subdoligranulum*, as also evidenced in several other studies (Choudhury et al., 2020; Guevarra et al., 2019). These microorganisms are generally associated with the hindgut; however, fermentative activity also takes place in the distal small intestine, as indicated by classical studies regarding the quantification of the entity of the short chain fatty acids produced in the different digestive tracts (Just et al., 1983). The acute increase in *Collinsella aerofaciens* abundance is inside this shift as this microbe is also a producer of the acetate and lactate abundant in colon of humans (Flint et al., 2015).

The dominant increased presence of *Streptococcus porcorum* was quite surprising as the primary isolation of this species in pigs (Vela et al., 2010) was not from the intestine. It is facultatively anaerobic, and the recovery of this microbe in porcines isolates from lesions of pneumonia and arthritis (Vela et al., 2010), and mesenteric lymph nodes and brain swab samples(Meekhanon et al., 2019) can render suspect its involvement under pathogenic conditions. However, no definitive conclusion regarding that has been reported, and the pigs in the present study were generally healthy.

In conclusion, JPPs sampled after weaning presented a general upregulation of the genes involved in the activation of class I MHC response and IFN-γ response as compared to just-weaned pigs. The intensity of this upregulation was only marginally associated with the variation found in the corresponding gut microbiota which, conversely, differs from that seen in just-weaned pigs. The

transcriptome of these pigs presented an interesting upregulation of the genes attributable to reticulocytes and erythrocytes under maturation. Several of these genes could be interesting for testing iron deficiency in piglets.

4. Material and Methods

On the day of weaning (26 days of age), 32 piglets ((Large White x Landrace x Duroc crossbred) from 8 litters (average live weight 7.18 kg, s.d. 0.77) were obtained from a commercial farm, and were assigned to 2 different times of slaughtering (the groups were balanced for the litter of provenience and live weight): weaning time or 12 d after weaning. The piglets were still with their sows when they were collected and were then immediately transported all together for one hour and half in a van to the experimental facility of the University of Bologna. There, the first group (9 females, 7 males) was immediately slaughtered while the second group (8 females, 8 males) was raised for twelve days with the same pre-starter feed used during the suckling period (Supplementary Table 5) and were finally slaughtered at 12 d (average live weight 9.76 kg, s.d. 0.57). After weaning, the pigs were reared individually and were penned inside a weaning room at pre-controlled temperatures and ventilation. Feed and water were freely available. On the morning of the day of sacrifice, they received half the dose of feed which had been given in the second meal of the previous day and access to the feed was closed an hour and half before the sacrifice.

After sedation by anaesthesia with tiletamine (15 mg/kg), the pigs were sacrificed with an intracardiac injection of a solution mixture of embutramide / mebezonium iodide / tetracaine hydrochloride (Tanax©, MSD Animal Health srl, Segrate, Italy; 0.5 mL/kg). Per each pig, a sample of PB was obtained by venipuncture on the vena cava before anaesthesia for mRNA sequencing, and a sample of JPPs was later collected from the jejunum, at approximately the 66% point of the small intestine length. The JC was also obtained at the same position, from a section of about 20 cm. The PB was collected using Tempus™ blood RNA tubes (Thermo Scientific, Waltham, MA, USA) while the JPPs and JC were collected in sterile tubes and were immediately frozen in liquid nitrogen. All the samples were then stored at −80°C until processed.

4.1 Pig mRNA extraction and sequencing

Total PB RNA was extracted from the Tempus™ tubes using the Tempus™ Spin RNA Isolation Kit (Thermo Scientific, Waltham, MA, USA) and from the JPP samples using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA), following the manufacturer's instructions. Contaminating DNA was removed by DNase treatment using the TURBO DNA-free™ DNA Removal Kit (Thermo Scientific, Waltham, MA, USA) following the recommended protocol. The RNA quantity and quality were evaluated using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis, respectively. An Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA) was used for testing RNA integrity. Libraries were prepared using the TruSeq Stranded mRNA Sample Preparation kit and were sequenced using the Illumina MiSeq system 2x100bp with 2x20 million sequencing depth.

4.2 Differential expression analyses of RNA-Seq data

The reads were quality controlled using the FastQC tool (v.0.11.9), filtered with Trimmomatic (v.0.36) (Bolger et al., 2014) by trimming leading and trailing bases with a Phred score less than 2 and dropping reads shorter than 15 bases long. Those with an average Phred score per base of less than 15. salmon (v.0.14.1) (Patro et al., 2015) were used to align sequences to the National Center for Biotechnology information (NCBI) *Sus scrofa* v11.1 reference transcriptome and are publicly available at the NCBI Sequence Read Archive under accession number SUB8684880.

An initial exploratory analysis of the expression profile was conducted using an MDS plot, based on count data normalised using variance stabilised transformation which was based on the count data of all the genes. In this plot, the samples were positioned according to the statistical distance of their expression profiles. The differential effect of the two different time points on single gene expression was carried out in R (3.6.2) using a DESeq2 package (v.1.26) (Love et al., 2014). The genes were considered to be differentially expressed when the FDR was <0.05.

4.3 Functional Enrichment Analysis

An exploratory analysis was conducted using GSEA software to evidence the enriched gene sets having a common biological function, depending on the time of sampling. The GSEA analysis was based on the C5 Biological Process collection of Gene Ontology (Liberzon et al., 2015) (MSigDB, Broad Institute, and UC San Diego). Gene sets with an FDR (q value) ≤ 0.05 were considered to be significantly enriched. The overlap and connections between the resulting different gene sets for the different times were produced by the EnrichmentMap Plugin (http://baderlab.org/Software/EnrichmentMap) for Cytoscape 3.8(Shannon et al., 2003), considering a q value of FDR <0.05 or < 0.01, depending on how it was later specified. The nodes were joined if the overlap coefficient was ≥0.375.

4.4 Microbiota

The bacterial DNA extraction from the JC was carried out using a HostZERO Microbial DNA Kit (Zymo Research, California, USA) following the manufacturer's instructions. The DNA concentration and purity (absorbance ratio 260/280 and 260/230, respectively) of the DNA isolated were checked using spectrophotometry on NanoDrop (Fisher Scientific, 13 Schwerte, Germany). The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified, amplicons were produced using the universal primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3' and Pro805R: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3'(Takahashi et al., 2014) using Platinum™ Taq DNA Polymerase High Fidelity (Termo Fisher Scientific, Italy) and sequenced using the Illumina MisSeq platform 300x2bp. The libraries were prepared using the standard protocol for MiSeq Reagent Kit V3 and were sequenced on the MiSeq platform (Illumina Inc., San Diego, Ca, USA). For the bioinformatics analysis, the DADA2 pipeline was utilised, (Callahan et al., 2016) using the Silva database (version 138)(Quast et al., 2013) as reference for the taxonomic assignment. The raw reads obtained from the 16s sequencing are publicly available at the NCBI Sequence Read Archive under accession number SUB9499525.

The statistical analyses regarding Alpha diversity, Beta diversity and taxonomic composition were carried out with R v3.6. The data were normalised using the variance stabilising transformation function provided by the DESeq2 package.(Love et al., 2014) A simple linear model assessed the effect of time on Alpha diversity. For the Beta diversity, a dissimilarity matrix using Euclidian distance was constructed; the results were plotted using an MDS plot, and a PERMANOVA was subsequently carried out to test for any correlation between community composition and age, with 10,000 permutations. The differential abundance analysis was carried out using the DESeq2 package, and the data were aggregated at the Genus level.

4.4 Clustering of weaned pigs for the MHC class I in JPPs and the effect on jejunal microbiota

After examination of the JPP transcriptome data (cfr. Results section), the pigs sampled at 12 d post-weaning were clustered into two groups based on the gene expression of a total of 44 genes related to the MHC class I antigen and to the response to IFN-γ production (Supplementary Table 6). In particular, they were those in the enriched core of the gene sets ANTIGEN_PROCESSING_AND_PRESENTATION_OF_ENDOGENOUS_ANTIGEN; INTERFERON_GAMMA_RESPONSE and of the MULTIVESICULAR_BODY_SORTING_PATHWAY. The transcriptome values were standardised using Z-score scaling so that, within each gene, mean = 0 and standard deviation = 1. The standardised sets of gene values for each pig were transformed using principal component analysis (PCA), with Proc PRINCOMP in SAS, to reduce the number of variables for additional testing. The distribution of samples was visualised on the score plot using Proc GPLOT of SAS. The components from the PCA which explained 85% of the total gene transcript variability and had eigenvalues for components >0.80 (Supplementary Fig.3) were used to create similarity clusters based on partitional clustering methods (Proc FASTCLUS in SAS). The resulting attribution cluster of each pig was used as a dependent variable to test the effect on the microbiota of the JC of the same pig. Alpha and beta diversity were analysed using a linear or PERMANOVA model, respectively, and using the Cluster attribution of each subject as a covariate. Taxonomic differences were tested using DESeq2, aggregating the data at the genus level.

Authors' contributions

P.B. and P.T. conceptualized and designed the study, F.C., P.B. and D.L. organized and performed the samplings, D.L. and F.C. performed the analysis, P.B., D.L. and F.C. analyzed and interpreted the data, P.B. and F.C. drafted the article. All authors critically reviewed the manuscript for intellectual content and gave final approval to the manuscript.

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None

Availability of data and material

Transcriptome and microbiome data are publicly available at the NCBI Sequence Read Archive under accession numbers SUB8684880 and SUB9499525, respectively.

Ethics approval

The procedures complied with Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna, Italy and by the Italian Ministry of Health (Prot. N. 675 − 3/96/2018).

The study was carried out in compliance with ARRIVE guidelines.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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Figures

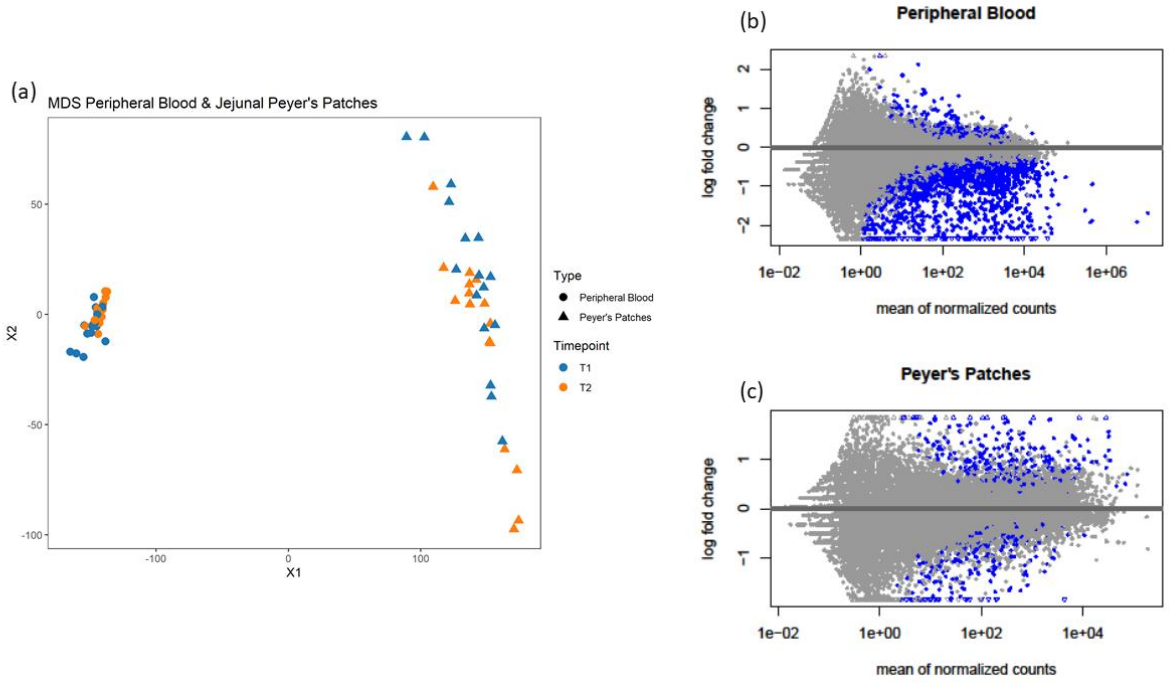

Figure 1. Summary plots of the gene expression profile of JPPs and PB at different times. The plots were multidimensional scaling (MDS) plots for the normalised gene expression levels of JPPs and PB at weaning (T1) and at 12 d post-weaning (T2) (a), and smear plots of the differential expression of the genes between T1 and T2 for JPPs (b) and PB (c), respectively. The smear plots show the relationship between the log fold change and the mean normalised count. The grey points represent non-significant differentially expressed genes whereas the blue points show genes which are significantly differentially expressed.

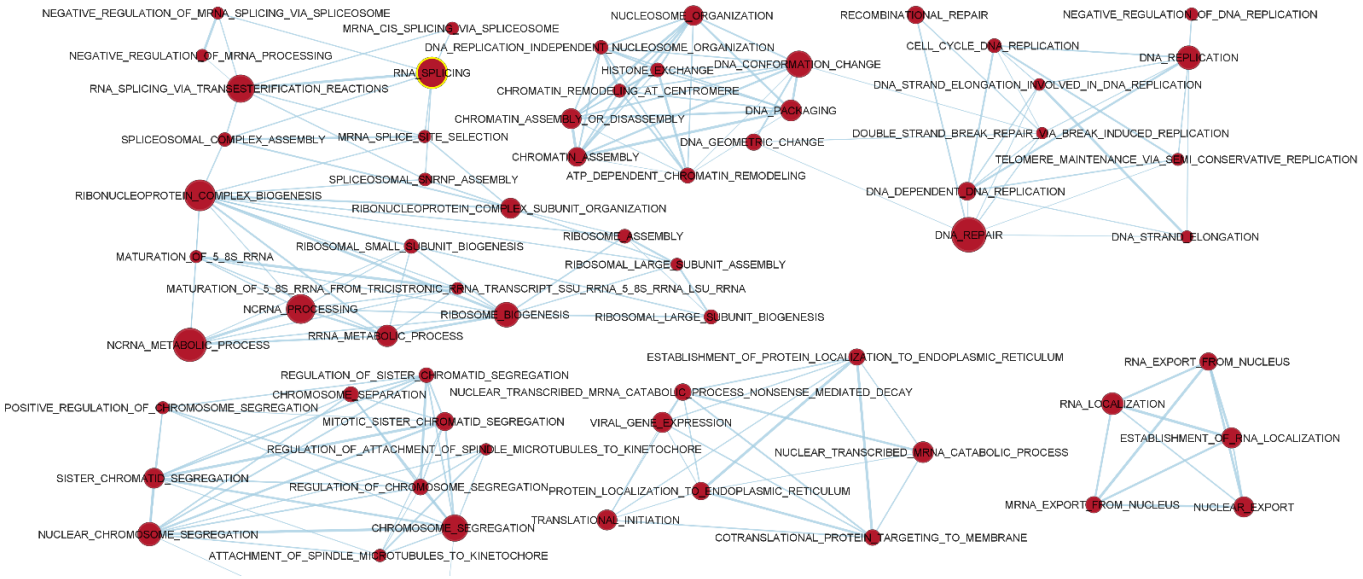

Figure 2. Gene sets upregulated in jejunal Peyer's patches of pigs on the day of weaning as compared to those sampled in pigs at 12 d post-weaning. The sets are enriched with FDR < 0.01. The edges represent the link of two or more gene sets sharing the same core group of genes, explaining the enrichment of each of the gene sets. Node colour intensity conveys enrichment significance (P-value) while their dimension increases with the number of their genes.

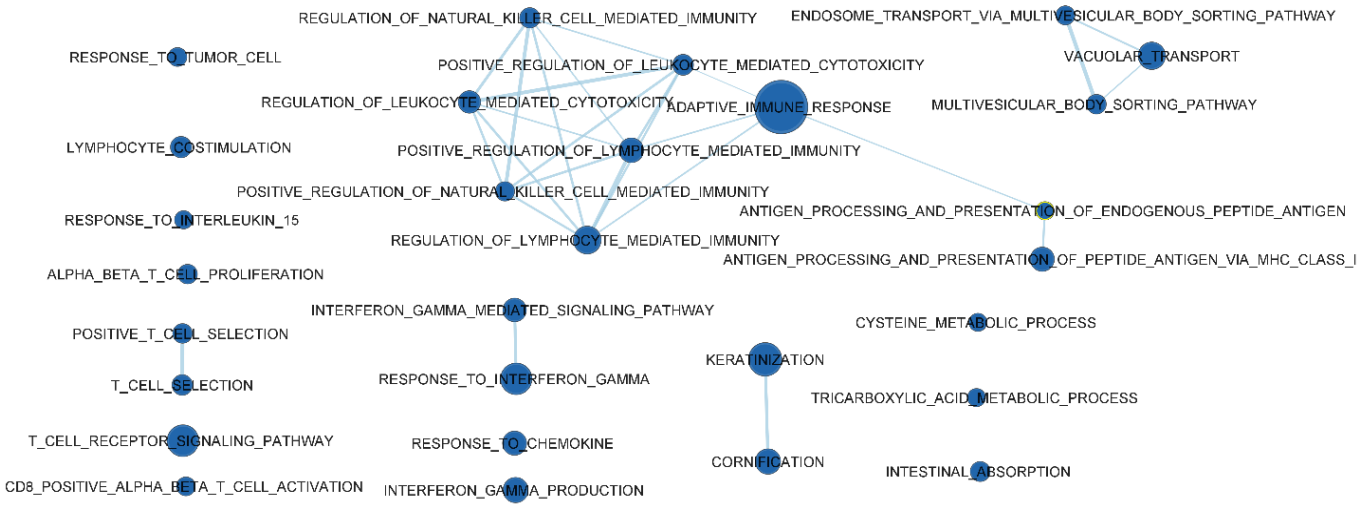

Figure 3. Gene sets upregulated in jejunal Peyer's patches of pigs sampled at 12 d post-weaningas compared to those on the day of weaning. The sets are enriched with FDR < 0.05. The edges represent the link of two or more gene sets sharing the same core group of genes, explaining the enrichment of each of the gene sets. Node colour intensity conveys enrichment significance (P-value) while their dimension increases with the number of their genes**.**

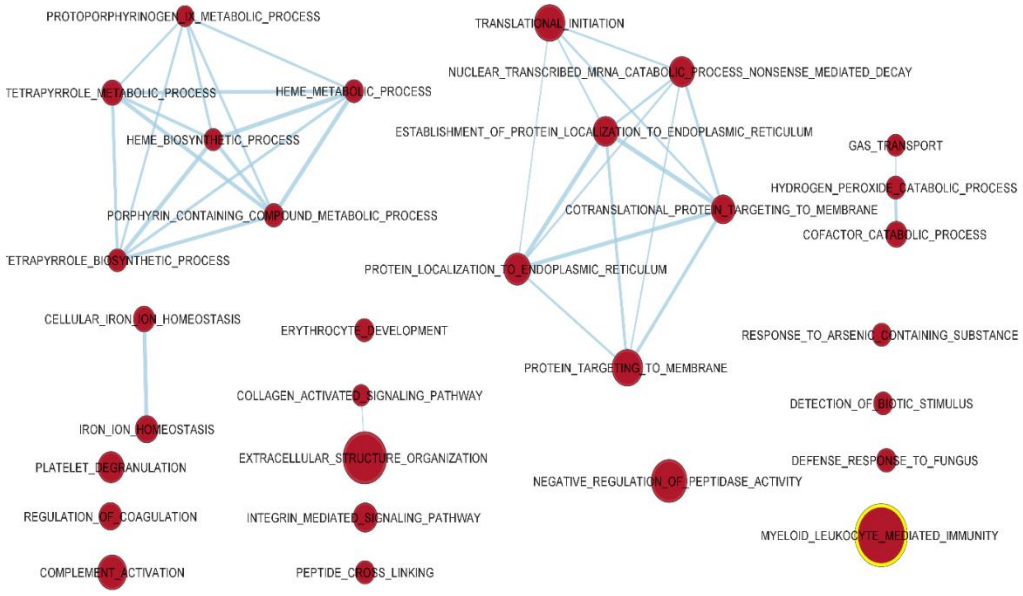

Figure 4. Gene sets upregulated in the peripheral blood of pigs on the day of weaning as compared to those sampled in pigs at 12 d post-weaning. The sets are enriched with FDR < 0.01. The edges represent the link of two or more gene sets sharing the same core group of genes, explaining the enrichment of each of the gene sets. Node colour intensity conveys enrichment significance (P-value) while their dimension increases with the number of their genes.

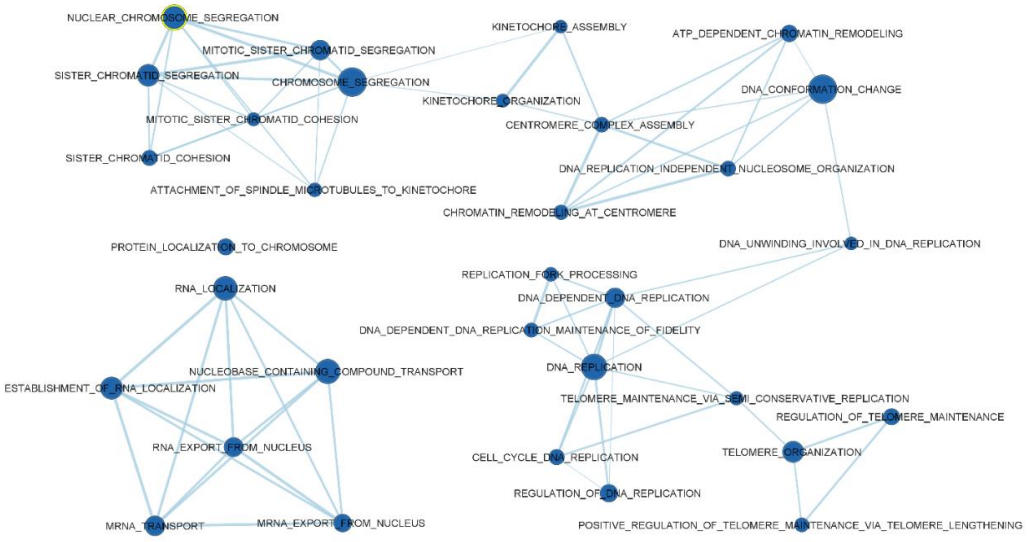

Figure 5. Gene sets upregulated in the peripheral blood of pigs sampled at 12 d post-weaning as compared to those on the day of weaning. The sets are enriched with FDR < 0.01. The edges represent the link of two or more gene sets sharing the same core group of genes, explaining the enrichment of each of the gene sets. Node colour intensity conveys enrichment significance (P-value) while their dimension increases with the number of their genes.

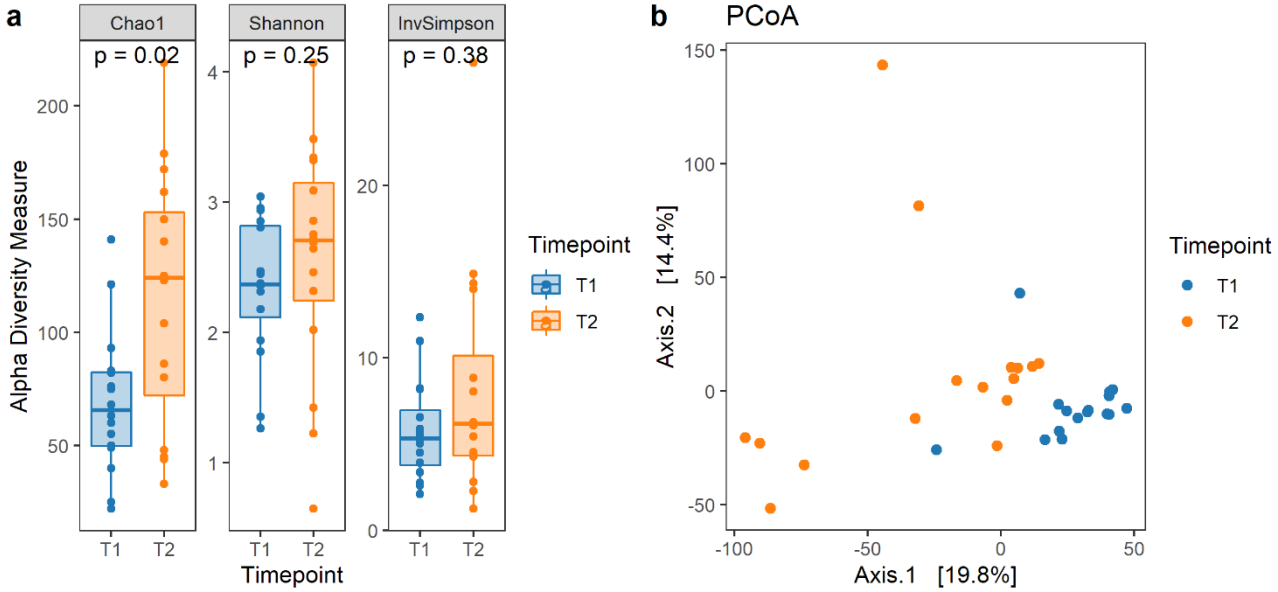

Figure 6. Descriptive plots for the bacterial diversity. (a) Boxplots of the Chao1, Shannon and InvSImpson α-diversity for each Timepoint. (b) NMDS plot using an Euclidian distance matrix for the 32 samples colored by timepoint.

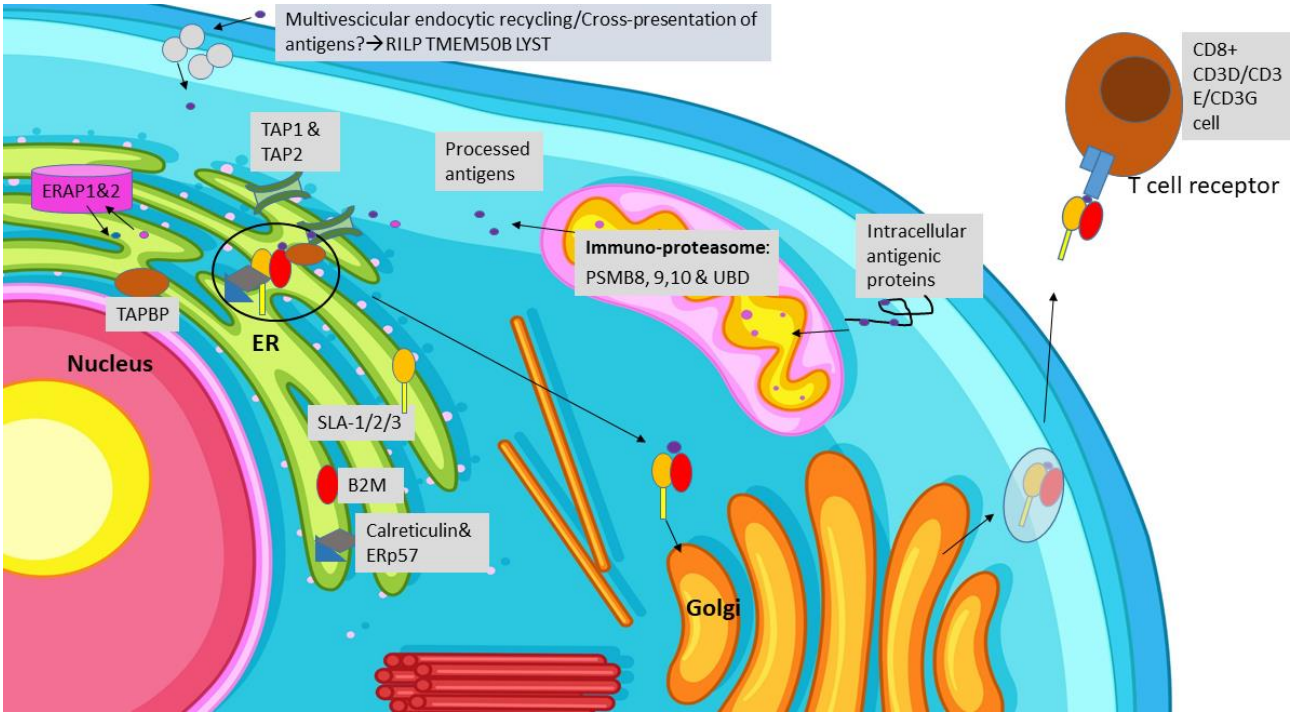

Figure 7. Scheme of the process related to MHC I activation upregulated in post-weaning pigs. Upregulated genes, alone or inside enriched groups, are in the figure in capital letters. In activated cells, such as DCs, a special proteasome complex is formed, thanks to 20S Subunit Beta 8, 9 and 10 (PSMB8/9/10) and ubiquitin D (UBD), to process intracellular antigenic proteins. Cross-presentation of external antigens (from neighbouring cells, cell fragments) can be processed using multivescicular body sorting (Rab Interacting Lysosomal Protein – RILP-, Transmembrane Protein 50B – TMEM50B -, lysosomal Trafficking Regulator –LYST). Entering into the endoplasmic reticulum (ER) by the channel made by Transporter 1 and Transporter 2, ATP Binding Cassette Subfamily B Members (TAP1 and TAP2), accepted antigens are integrated to MHC I (SLA-1, SLA-2, SLA-3) inside the complex, also formed by the TAP Binding Protein (TAPBP), β-2-microglobulin (B2M) calreticulin and ERp57. Some peptides must be preliminarily trimmed by endoplasmic reticulum aminopeptidase 1 and 2 (ERAP1 and ERAP2). The antigen-charged complex B2M – MHC I is then exported to the Golgi apparatus where it is packed in vesicle form to be exported from the cell. Design created in Microsoft PowerPoint 2016, and a part of the personal design freely obtained from http://www.freepik.com.

Supplementary Materials:

The following are available online at https://doi.org/10.1038/s41598-022-05707-9. **Supplementary Table 1:** List of genes with statistically significant differential expression in jejunal Payer's patches collected from pigs at weaning or 12 d post-weaning. **Supplementary Table 2**: List of gene with statistically significant differential expression in peripheral blood sampled from pigs at weaning or 12 d post-weaning. **Supplementary Table 3**: Amplicon sequence variants that were significantly more abundant in jejunal content collected from pigs 12 d post-weaning vs samples at weaning. **Supplementary Table 4**: Initial seeds of normalized expression of genes selected for activation of MHC class I and IFNγ in jejunal Payer's patches collected from pigs 12 d post-weaning, in the different cluster groups. **Supplementary Table 5:** Ingredients and calculated composition of the pre-starter feed expressed on as fed. **Supplementary Table 6:** List of genes of which it was used the expression, for the clustering of samples collected in the post-weaning period

Manuscript 2

Effects of *E. coli* bivalent vaccine and of host genetic susceptibility to *E. coli* on the growth performance and faecal microbial profile of weaned pigs

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Abstract

F4- and F18-positive enterotoxigenic *Escherichia coli* (ETEC) are well-known pathogens able to cause a severe swine disease with a worldwide economically importance in pig production. Vaccination of piglets with an oral live bivalent F4/F18 *Escherichia coli* vaccine has shown some promise and efficacy results in preventing ETEC post weaning diarrhoea (PWD); however, currently there is a lack of knowledge on its effect on the intestinal microbial community. Furthermore, is not known if the effect of vaccination on gut eubiosis can vary according to pig genetic susceptibility to ETEC F4 and F18. The aim of this study is to evaluate the effect of an *E. coli* bivalent vaccine and the potential influence of the host genetic susceptibility to ETEC growth performance and faecal microbial profile of healthy piglets around weaning. A total of 288 healthy piglets at 26 days of age (d0; weaning) were divided into two different groups balanced for body weight: i) vaccine (VAX: 6.33 kg) and control (CO: 6.45 kg). At d0 VAX group was orally inoculated with 2 mL of live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) vaccine (Coliprotec F4/F18; Prevtec Microbia an Elanco Company), while CO was orally inoculated with 2 mL of sterile water. At d0 and 18 days later (d18) piglets were individually weighted, and a faecal sample was collected for microbial profile of V3-V4 regions of the 16S rRNA gene using MiSeq Illumina platform.Bristles were collected to determine the genetic ETEC susceptibility by genotyping analysis of *MUC4* and *FUT1* polymorphisms. No effect of vaccination and genetic susceptibility was observed on growth performance. The vaccination modulated the faecal microbial composition, reducing the alpha diversity indices ($P<0.05$) and affecting the beta diversity ($R^2=0.02$; $P = 0.05$). VAX was discriminated from CO by beneficial bacteria genera including *Dialister, Prevotella, Blautia, Ruminiclostridium, Parabacteroides* and *Faecalibacterium.* No interaction between vaccination and animal genetic susceptibility to ETEC was observed. F4 resistant genotype showed a higher alpha diversity index than the susceptible ones pre- and post-weaning ($P< 0.05$). These findings contributed to the body of knowledge regarding the beneficial effect of oral live bivalent F4/F18 *E. coli* vaccine on piglets' gut homeostasis resulting in improved gut eubiosis.

Keywords: ETEC; Vaccination; Microbiota; Pigs

1. Introduction

The post weaning diarrhoea (PWD) is widely diffused in pigs resulting in an increase of animals' mortality and morbidity, slow growth and an increase of antibiotic uses and treatment costs. The enterotoxigenic *Escherichia coli* (ETEC) carrying the adhesive fimbriae F4 and F18 are recognized as the main pathogens associated with PWD in pigs (Luppi, 2017; Rippinger et al., 1995; Xia et al., 2015). These fimbriae allow the ETEC colonization of the intestinal mucosa and mediate the production of enterotoxins that induce an intestinal dysbiosis and a secretory diarrhoea (Luppi, 2017; Rippinger et al., 1995; Xia et al., 2015).

Non-pathogenic *Escherichia coli* (*E. coli)* strains bearing F4 and F18 fimbriae may competitively exclude ETEC F4 and F18 by occupying their intestinal receptors and can induce specific anti-F4 and anti-F18 IgM and IgA immune responses (Nadeau et al., 2017). Thus, the vaccination of piglets against ETEC with these non-pathogenic *E. coli* might represent a potential but not fully investigated strategy to contrast the PWD caused by these pathogens. Recently, it has been demonstrated that oral vaccination of piglets with a monovalent *E. coli* F4 vaccine (Fairbrother et al., 2017) and with a bivalent F4 and F18 vaccine (Nadeau et al., 2017) can exert protection against ETEC and can induce ETEC-specific protective immunity. However, scarce information is reported about the use of these vaccines in farm-scale and their effect on the piglet's growth and on piglet's intestinal microbiome.

For instance, although it is well accepted that intestinal microbiota can be influenced by administration of specific bacteria (Kiros et al., 2019; O'Toole and Cooney, 2008), diet (David et al., 2014), age (Motta et al., 2018), and genetics (Luise et al., 2019; Poulsen et al., 2018), nothing is known about the interplay between oral *E. coli* vaccine and commensal bacteria. Furthermore, it is not known if the effect of *E. coli* vaccination on gut homeostasis can vary according to the swine genetic variants associated to the susceptibility to ETEC F4 and F18, which influence the presence of the intestinal receptors for the bacterial adhesion in the mucosa (Jørgensen et al., 2003; Meijerink et al., 2000).

The aim of the present study was to assess the effect of a bivalent *E. coli* F4/F18 live oral vaccine on the growth performance and profile of the faecal microbiota of piglets around weaning. A secondary aim of the study was to investigate the effect of the genetic ETEC susceptibility on piglet's performance and faecal microbiota, as well as their interaction with the vaccination.

2. Material and methods

The procedures complied with the Italian law pertaining to experimental animals and were approved by the Ethic-Scientific Committee for Experiments on Animals of the University of Bologna (Protocol number 13855).

2.1 Animal and study design

A total of 288 healthy piglets from 48 litters (6 piglets per litter) were included in the study. The experiment was performed in a farrowing to growing farm with 400 sows and an all-in, all-out per barn swine production system, located in the municipality of Brescia, in Italy. The veterinarian responsible for this farm described a high occurrence of PWD in piglets and frequent isolation of ETEC F4 and F18 in the farm in the previous year. At weaning (26 ± 2 days of age (d0)), piglets were divided in two groups of 144 piglets balanced for body weight ((BW): 6.45 kg for the CO group and 6.33 kg for the VAX group. The control (CO) group was orally inoculated with 2 mL of sterilized water while the vaccine (VAX) group was orally inoculated with 2 mL of the bivalent vaccine Coliprotec® F4/F18 (Prevtec Microbia an Elanco Company, Montreal, Canada), a single-dose lyophilized vaccine consisting of live non-pathogenic *E. coli* O8:K87 (F4ac-positive, $1.3x10^8$ to $9.0x10^8$ CFU) and O141:K94 (F18ac positive, 2.8×10^8 to 3.0×10^9 CFU). After being rehydrated in water, the vaccine was orally administrated using a dosing device.

The day of the weaning, the piglets were weaned and located in the same room keeping the two groups in different not contiguous pens. Piglets were kept at a controlled temperature regulated at 28°C and relative humidity maintained at 60 %, with water and feed ad libitum. Piglets were fed a standard weaning diet (Table 1). The trial was carried out on two batches of 60 and 85 piglets, respectively.

Piglet mortality was monitored along the whole study. Piglets were individually weighted at d0 and 18 days later (d18; post-weaning). Bristles were collected from pigs in the second batch (85 piglets per group) for the genotyping analysis of *MUC4* and *FUT1* polymorphisms. Those polymorphisms were chosen as related to ETEC F4 and F18 susceptibility. From the same batch, from a subgroup of 30 piglets per group, a faecal sample was collected into a sterile tube at d0 (before vaccination) and at d18. The faecal sample was snap-frozen in liquid nitrogen and stored at −20°C for microbial analysis.

2.2 Animal genotyping

Genomic DNA of each pig was extracted from bristles following the procedure described by Luise et al. (2019). Briefly, the bristle bulbs were incubated in Proteinase K solution (10 mg/ml of proteinase K in buffer [20 mM Tris–HCl (pH 8.4), 50 mM KCl]) for 2 hr at 50°C; then, the proteinase was inactivated at 95°C and samples were stored at −20°C. Genotyping of the *MUC4* g.8227C>G, *FUT1* g.307 G>A, was carried out by Restriction Fragment Length Polymorphism PCR (PCR-RFLP) using specific primers, annealing temperature, condition enzymes reported by (Jørgensen et al., 2003) and (Meijerink et al., 2000), respectively. Pigs with the genotypes $MUC4^{GG}$ and $MUC4^{CG}$ were considered genetically susceptible to ETEC F4 (F4_S); pigs with the genotype $MUC4^{cc}$ were considered genetically resistant to ETEC F4 (F4_R). Pigs with the genotypes $FUT1^{CC}$ and $FUT1^{AC}$ were considered genetically susceptible to ETEC F18 (F18_S); pigs with the genotype $FUT1^{AA}$ were considered genetically resistant to ETEC F18 (F18_R).

2.3 Microbiota analysis

Total bacterial DNA was extracted from faeces using FastDNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, Ca, USA) following the manufacturer's instructions. The isolated DNA was controlled in its quantity and purity by spectrophotometry on the NanoDrop (Fisher Scientific, 13 Schwerte, Germany). In addition, a PCR for lactic acid bacteria (LAB) was performed following the protocol reported by Walter *et al.* (2001), to further confirm that bacterial DNA was extracted. The V3-V4 hypervariable regions of the 16S rRNA gene amplicons were generated using the primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3' and Pro805R: 5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3'.

The libraries were prepared using the standard protocol for MiSeq Reagent Kit v3 and sequenced on MiSeq platform (Illumina Inc., San Diego, Ca, USA). The raw reads obtained are publicly available at the European Nucleotide Archive (ENA) under the accession number PRJEB38181.

Generated sequences (approximately ~460 bp) were analysed using the DADA2 package version 1.5.0 and workflow (Callahan et al., 2016) in R version 3.6 (http://www.R-project.org). Briefly, the primers and reads with low quality were trimmed. Forward reads were trimmed at 280 pb, while reverse reads were trimmed at 210 pb. The first base pairs and instances of a quality score ≥ 2 were truncated. Reads

containing ambiguous bases and errors rate ≥ 2 were filtered out. Reads with identical sequences were collapsed to reduce computational time (dereplication). The amplicon errors were calculated and corrected using the DADA2 algorithm with default parameters. The denoised output of forward and reverse reads were merged and reads with mismatches were removed. Amplicon sequence variants (ASVs) shorter than 245 where removed. Chimeras were identified using the removeBimeraDenovo function and removed. Taxonomy was assigned using the Silva Database (release 132) (Quast et al., 2013).

2.4 Statistical and bioinformatical analysis

Data on BW and average daily gain (ADG) were analysed using an ANOVA model which included treatment (CO vs VAX), batches (1 and 2) and litter of origin as factors. Data obtained during the second batch were analysed using an ANOVA model including treatment, litter and the genetic susceptibility to ETEC F4 (F4_S, F4_R) and to ETEC F18 (F18_S, F18_R) as factors. Parity number of the sow a well as sex of piglets were initially included in the model and then removed because not significant.

Biostatistics analysis on ASVs was performed using vegan (Dixon, 2003), phyloseq (McMurdie et al., 2013) and mixOmix (Rohart et al., 2017) packages in R software (v.3.6.0) (Team, 2013).

For alpha diversity, Chao, Shannon and InvSimpson indices were calculated and statistical analysis was performed using an ANOVA model which included treatment (CO vs VAX), time (d0 and d18), susceptibility to ETEC F4 (F4_S, F4_R) and to ETEC F18 (F18_S, F18_S) and their interactions with the treatment and the repetition on each piglet per time. Then in order to investigate deeper the effect of vaccination and genetic susceptibility, the indices were analysed separately for the two time points using an ANOVA model that included treatment, genetic susceptibility and their interaction as factors.

Bray-Curtis Dissimilarity was calculated and used to compare the treatment differences in beta diversity using a permutational MANOVA (Adonis and pairwise.adonis procedure) including treatment, time and genetic susceptibility as factors. Then in order to investigate deeper the effect of vaccination and genetic susceptibility, a second permutational MANOVA model was computed separately for the two times including treatment, genetic susceptibility and their interaction as factors. The homogeneity of dispersion between the time-points (d0 *vs* d18) and treatments (CO *vs* VAX) was

tested using PERMDISP (with 999 permutations) function. Then Non-Metric Multidimensional Scaling Plots (NMDS) based of Bray-Curtis Dissimilarity distances were visualized in R software using ggplot2. The differences in taxonomic composition were tested by Welch's t test using the data after normalization through Cumulative Sum Scaling normalization (CSS).

Furthermore, in order to identify the discriminant taxa belonging to vaccination treatment the multivariate supervised approach sparse Partial Least Squares Discriminant Analysis (sPLS-DA) was performed on the data d18 after the CSS normalization. Data were aggregated at genera level. To validate the sPLS-DA results, the stability frequency scores of the selected taxa were calculated ("perf" function) with 10-fold cross-validation and 100 repetitions. The plotVar function was then used to identify the correlation between taxa matrix and the factor. Taxa showing a correlation >0.5 and a stability ≥80% were considered discriminative.

3. Results

A total of 16 piglets out of 288 (5.56%: 7 and 9 in the first and second batch, respectively) were excluded from the trial due to a severe impairment of the health conditions, 9 into CO group and 7 into VAX group. Overall, the other animals did not suffer of severe PWD.

3.1 Animal susceptibility to ETEC

The genotyping for the mutation localized on the *FUT1* gene was carried out on 163 out of 170 samples, since it was not possible to obtain DNA of sufficiently adequate quality for 7 subjects. A total of 154 F18_S pigs (94%) and 9 F18_R pigs (6%) were detected. The genotyping for the mutation localized on the *MUC4* gene was carried out on 162 out of 170 samples, the quantity and quality of DNA for 8 subjects being inadequate. A total of 108 F4_R pigs (67%) and 54 F4_S pigs (33%) were detected.

3.2 Piglets performance

No differences in the BW at d0 and d18 and in the ADG from d0 to d 18 were observed between CO and VAX groups (Table 2). No effect of genetic susceptibility to ETEC on piglet's performance was observed.

3.3 Faecal microbiota profile

Twenty samples (eighteen at d0 and two at d18) were excluded from the microbiota analysis due to problems in the sampling phase, and in the DNA extraction phase. A total of 4,686,603 reads were attributed to a total of 12578 ASVs distributed among samples as shown in S1 Table. The relative rarefaction curves are reported in Figure S1; it shows a plateau for all samples suggesting that the sequencing depth was sufficient to describe the variability within the analysed microbial communities. The taxonomic assignment allows obtaining 21 phyla, 28 classes, 67 families and 215 genera (Figure 1A e 1B).

3.3.1 Weaning transition

Time resulted the most significant factor influencing both alpha and beta diversity indices. No interactions were observed between time and treatment (vaccination) and between time and the genotypes for both alpha and beta indices. Thus, investigations for treatment and genotypes effects were performed separately in microbial data of d0 and d18.

Supplementary Figure S2 and S3 show the effect of weaning (age) on the faecal microbial profile of piglets. All the alpha diversity indices were significantly higher at d18 than at d0 ($P< 0.05$) (Figure S2). The homogeneity of dispersion between d0 and d18 was not significant ($P=0.86$). The Adonis test showed that weaning was significant in driving diversity assessed by Bray-Curtis Dissimilarity ($R^2= 0.05$; $P=0.001$). Figure S3 shows the effect of weaning on the faecal microbial structure (NMDS plot). The microbial structure of pigs at d 18 was less dispersed than the one of pigs at d0. A significant decrease of Proteobacteria, Fusobacteria, Synergistetes and Euryarchaeota phyla were found in post-weaning piglets, where there has been a significant increase of Firmicutes (Table S2).

3.3.2 *Vaccination*

No interaction between vaccine and genetic susceptibility of piglets to ETEC was observed for alpha and beta diversity indices. Before the vaccination (d0), the groups did not differ for alpha and beta diversity indices. Figure 2 details the microbial diversity indices among treatments at d18. Shannon, Chao and InvSimpsion richness estimate indicated that the microbial community in the faeces of CO

piglets at d18 were richer in terms of bacterial species than the community in the VAX piglets ($P < 0.05$). The homogeneity of dispersion between CO and VAX groups was not significant (P=0.78). Vaccination significantly influenced the beta diversity (R^2=0.02; P = 0.05). Figure S4 shows the effect of vaccination on the faecal microbial structure at d18 (NMDS plot), where the two clusters are partially overlapping. No significant differences at phylum, family and genus levels were observed between treatments using the Welch test (data not shown). Results of sPLS-DA analysis on faecal microbial profile at d18 are reported in Figure 3. The Score plot clearly differentiate the two clusters related to CO and VAX groups. The VAX group was discriminated by bacteria belonging to the genera *Dialister*, *Prevotella*_7, *Prevotella*_2, *Blautia*, *Ruminiclostridium*_6, *Parabacteroides* and *Faecalibacterium,* while CO group by those belonging to the genera *Methanobrevibacter* and *Bacteroides.*

3.3.3 Animal susceptibility to ETEC

No effect of susceptibility to ETEC F18 on the microbial profile has been reported both at d0 and d18, whereas the alpha diversity was significantly influenced by ETEC F4 susceptibility. At d0, piglets with the resistant genotype had a higher Chao1 index than susceptible ones (F4_R: 209 vs F4_S: 172; P= 0.03) while no differences were reported in the Shannon and InvSimpson indices. At d18, piglets with the resistant genotype (F4R) had a higher Shannon (F4_R: 4.99 vs F4_S: 4.80; P = 0.02) and InvSimpson (F4_R: 76.4 vs F4_S: 59.4; P=0.02) indices than susceptible ones (Figure 5). Beta diversity was not influenced by genetic susceptibility to ETEC F4.

4. Discussion

In line with previous studies (Pajarillo et al., 2014; Mach et al., 2015; Niu et al., 2015; Chen et al., 2017; Motta et al., 2019), our analysis of the alpha diversity indices shows a stark contrast between nursing (d0) and weaned (d18) animals with a remarkable increase in richness and alpha diversity of the gut microbiota at d18. This result confirms the notion that alpha diversity is affected by time (weaning transition) emphasizing that the increasing values in alpha diversity across age are linked to maturation of microbial community from birth to adulthood (Niu et al., 2015) and after the introduction of solid feed (Inoue et al., 2005; Konstantinov et al., 2006). In addition, the beta diversity (inter-

individual variation) was affected by weaning transition. After weaning (d18) a reduction of distance among samples and an increase of uniformity is observed compared with pre-weaning (d0). The increased uniformity of the faecal microbial profile is typical of the post-weaning period and can be attributed to the microbiota maturation (establishment of climax community), the stabilization of feed intake and the standardization of the environmental condition to which animals are exposed (Frese et al., 2015; Motta et al., 2019; Chen 2017; Isaacson and Kim 2012). In fact, it is well known that, after a first period of transient acute changes induced by post-weaning fasting, the voluntary feed intake resumption leads to a phase of intestinal maturation and stabilization (Lallès et al., 2007).

Besides the expected effect of weaning transition, this study evidenced the effect of an oral live bacterial vaccine on the faecal microbiota. The live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) supplied at one single dose just before weaning decreased richness and influenced the beta diversity of bacteria species. To the best of our knowledge, no previous data have been reported about the influence of bivalent *E. coli* vaccination on the microbial profile. However, the reduced richness of the bacterial community in the vaccinated group compared to the control may be related to the colonisation of the intestine by the non-pathogenic *E. coli.* Indeed, similar results are well recognised after probiotic supply (Kiros *et al.* 2019; Wang 2012) and after the one-time early oral inoculation with a live *Salmonella* strain in growing chicks that affects the microbial richness as much as a common probiotic administration (Ballou et al., 2016). Overall, these results would suggest the oral vaccination of young animals may favour a stabilization of the intestinal microbiota. Furthermore, in agreement with the study of Ballou et al. (2016), the oral vaccination influenced also the taxonomic composition, indeed, in our study the PLS-DA analysis showed that the *Dialister, Prevotella, Blautia, Ruminiclostridium, Parabacteroides, Faecalibacterium* genera dominated in the vaccine group. Those genera are generally recognised as short-chain fatty acid (SCFA)–producing bacteria (Liu et al., 2008; Haenen et al., 2013). We can assume that the microbial profile shifts to (SCFA)–producing bacteria might have induced a favourable colonic environment in the vaccine group. It is well documented that SCFAs have beneficial effect on gut health and, with immune and non-immune consequences, they could play a role in the inhibition of the expression of adhesin factors or the invasion genes of potentially harmful pathogens such as *Salmonella* Typhimurium and EHEC (Wong et al., 2004;

Wilhelm et al., 2012; Lebel et al., 2016; Tran et al., 2018). Although the high abundance of some genera, such as *Prevotella*, has been previously associated with an improvement of piglet's growth performance (Kiros et al., 2019; Mach et al., 2015), no significant difference was observed in our study in the growth performance between the experimental groups despite the vaccinated group was discriminated by *Prevotella*. However, the beneficial effects ascribed to *Prevotella* may depend on the species and some of these may instead cause a reduction of SCFAs and an alteration of the gut homeostasis (Iljazovic et al., 2020). An additional hypothesis for the lack of difference in the pig performance might be ascribed to a possible shift of the energy expenditure from growth to the immune response, as a known consequence of the vaccination is that the immune activity increases the energy expenditure (Martin et al., 2002; Trevisi et al., 2010). Although in our study it was not possible to monitor the immune response between groups, Nadeau et al. (2017) demonstrated that the bivalent *E. coli* vaccine used in our study induces immune responses and increases serum IgM and IgA, supporting our hypothesis. Finally, it is worth noting that the ADG was higher for the vaccinated pigs than the control group during periods from d18 to d72 and from d0 to d72 (gain measured per pen; data not shown), hence a potential long-term effect of vaccination related with an improvement of gut eubiosis and of immune development is not excluded. Further studies are needed to confirm or refute this hypothesis. In addition, further studies aimed at investigating the effect of the vaccination on the health parameters and the development of PWD in piglets would be desirable.

Regarding the role of genetic susceptibility in influencing the growth performance of piglets, as expected, no effect on the piglet BW and the ADG was observed. On the other hand, we found significant differences in the microbiota profile associated with the genetic susceptibility to ETEC F4 in the alpha indices both in the pre- and post-weaning periods. On the contrary, beta diversity was not influenced by F4 susceptibility. A higher microbial variability in animals resistant to F4 (F4_R) than susceptible (F4_S), confirms also that in addition to their resistance to the pathogenic species (ETEC F4), in these pigs a higher homoeostasis of the commensal gut bacteria is favoured (Luise et al., 2019; Massacci et al., 2019). In addition, no interaction between genetic susceptibility and vaccination was evidenced for the growth performance and microbial data. Further studies based on larger dataset would be useful to validate our results.

5. Conclusions

The ability of live bacteria orally administered to prevent the intestinal colonization by pathogenic species is well documented; while the effect of non-pathogenic *E. coli* supplied to healthy piglets is poorly investigated. The results reached in this study on the effect of an oral live bivalent F4/F18 *E. coli* vaccine on gut microbial composition highlighted modifications on faecal microbial profile, favouring a stabilization of the microbiota and the proliferation of SCFA producing bacteria, without affecting the piglet's performance. This study also reinforces the fact that piglet's genetic variance might have an effect of on their faecal microbiota, highlighting significant differences in the microbiota profile associated with *MUC4* genotypes.

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Tables

Table 1. Ingredients and calculated composition[1] of the piglet diet expressed (on as fed).

Item	Units	Content
Ingredients		
Maize	%	37.79
Wheat, soft	%	15.00
Wheat middlings	%	11.8
Soybean meal, 50% crude protein	%	11.00
Dehydrated skimmed whey, sweet,	%	10.00
Potato protein concentrate	%	4.00
Beet pulp, dehydrated	%	3.00
Lard	%	3.00
Dicalcium phosphate	%	1.42
Calcium carbonate	%	0.65
L-Lysine HCl	%	0.64
Sodium chloride	%	0.30
DL-Methionine	%	0.28
L-Threonine	%	0.28
L-Valine	%	0.17
L-Tryptophan	%	0.12
Choline chlorydrate (at 75%)	%	0.05
Vitamin-mineral premix[2]	%	0.50

Calculated chemical composition		
Metabolizable energy	MJl/kg	13.74
Crude protein	%	17.5
Crude fat	%	5.48
Ash	%	5.44
NDF	%	11.92
Lysine	%	1.39
Threonine	%	0.96
Methionine	%	0.56
Cysteine	%	0.29
Tryptophan	%	0.31
Isoleucine	%	0.72
Valine	%	1.01
Leucine	%	1.41
Phenylanine	%	0.80
Tyrosine	%	0.60
Histidine	%	0.41
Calcium	%	0.854
Phosphorus	%	0.703

[1]The values were estimated by the EvaPig® software Version 1.4.0.1 (Noblet et al., 2008) using information from the INRA-AFZ tables of feedstuff composition.

[2]The premixture supplied the following per kg complete diet: vitamin A, U.I. 15,000; vitamin D3, U.I. 2,000; vitamin E, 100 mg; vitamin K3 (MNB) 1.625; vitamin B1, 1.65 mg; vitamin B2, 5.375 mg; vitamin B6 (pyridoxine hydrochloride), 3.125 mg; vitamin B12, 0,05 mg; biotin, mg 0.50; betaine hydrochloride, 290 mg; folic acid, 2.50 mg; niacine, 30 mg; calcium D-panthotenate, 18 mg; iron (iron (II) sulphate monohydrate), 120 mg; iodine (anhydrous calcium iodide), 1 mg; copper (copper (II) sodium pentahydrate, 50 mg; copper (copper (II) chelate of glycine hydrate), 23.7 mg; manganese (manganese (II) oxide), 103 mg; zinc (zinc oxide); 105 mg; selenium (sodium selenite), 0.35 mg; phytase (EC.3.1.3.26), 1000 U

Table 2. Effect of bivalent E. coli vaccination on piglet's growth performance.

Items	Treatment, mean		SE	P-value		
	CO	VAX		Treatment	Batch	Body weight at d0
Body weight d0 (kg)	6.45	6.33	0.11	0.430	0.760	-
Body weight d18 (kg)	8.96	8.79	0.11	0.240	0.280	<0.0001
Difference in body weight d18-d0 (kg)	2.56	2.39	0.11	0.240	0.280	0.110
ADG (d0-d18) (kg/day)	0.15	0.14	0.005	0.250	0.049	0.120

CO=Control, not vaccinated pigs; VAX=Vaccinated, pigs vaccinated with live non-pathogenic E. coli O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) at d0 (26±2 age of life).

Figures

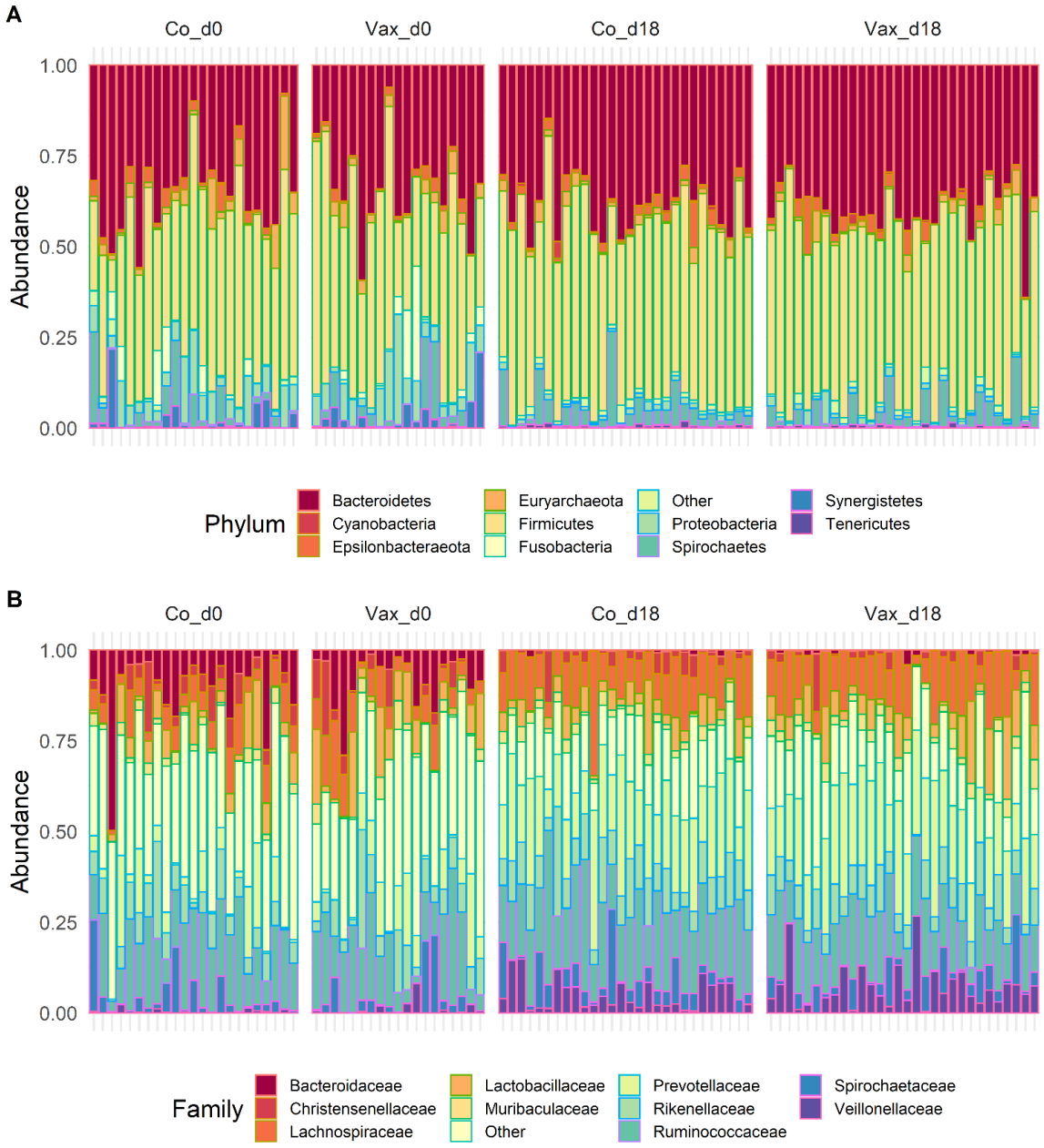

Figure 1. Taxonomy assignment at phylum (A) and family (B) level on fecal samples of piglets according to treatment and time. Co= control, not vaccinated pigs; Vax=vaccine, pigs vaccinated with live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) at d0 (26±2 age of life); d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning.

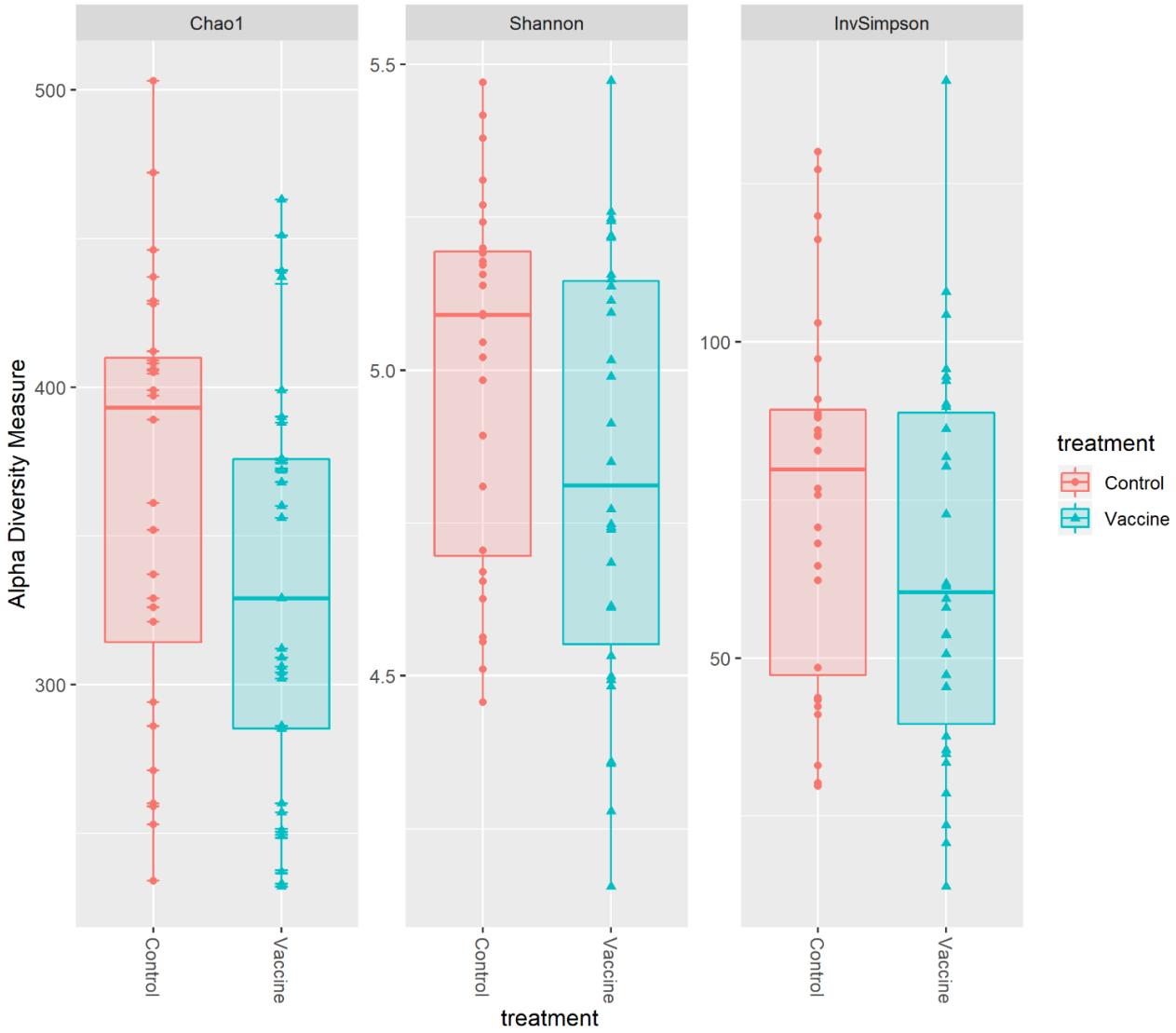

Figure 2. Effect of treatment on alpha indices of faecal microbiota of piglets at eighteen days post weaning. Boxplot of Alpha diversity indices (Chao1, Shannon and InvSimpson) values. Control= not vaccinated pigs; Vaccine= pigs vaccinated with live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) at d0 (26±2 age of life).

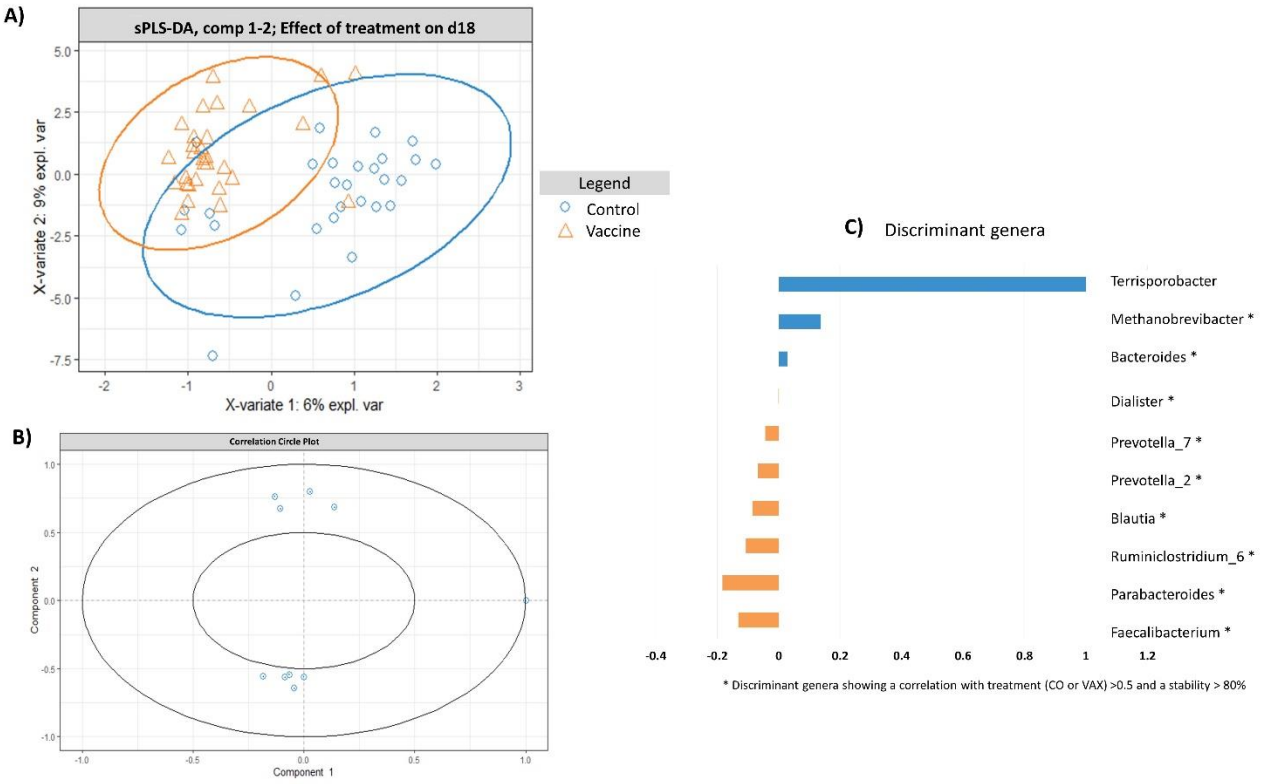

Figure 3. Results of sPLS-DA analysis on fecal microbial composition of piglets at eighteen days post weaning. A) score plot resulting from the sPLS-DA analysis; Control= not vaccinated pigs; Vaccine= pigs vaccinated with live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) at d0 (26±2 age of life). B) Correlation plot for PC1 and PC2 identified the significant taxa discriminating the CO and VAX groups. C) Significant discriminant taxa for Control (blu colour) and vaccine (orange colour) groups.

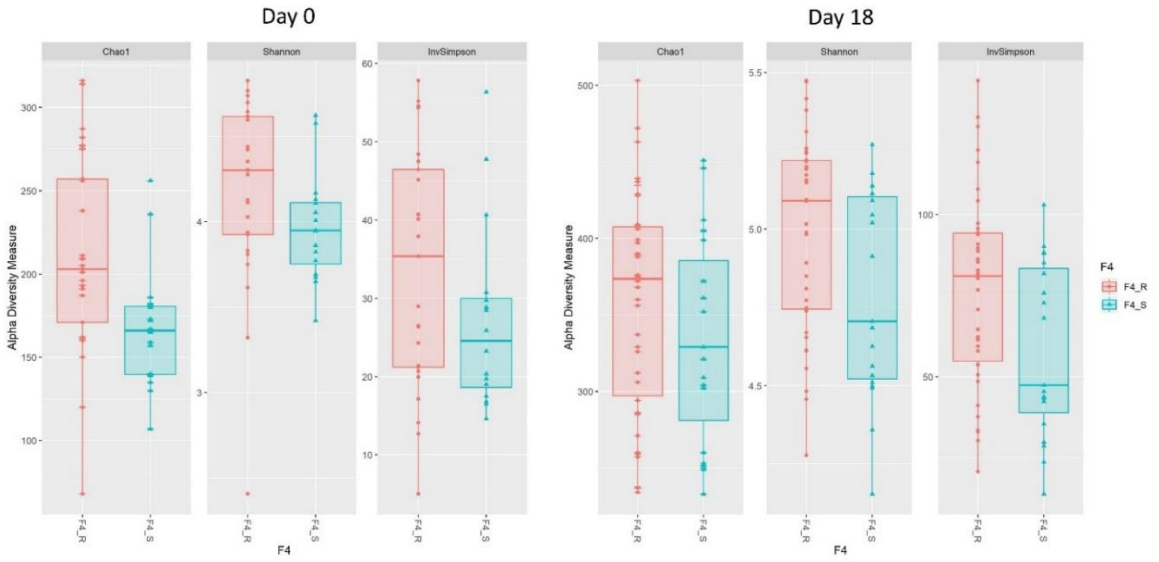

Figure 4. Effect of genetic susceptibility to ETEC F4 on the alpha indices of faecal microbiota of piglets. Boxplot of Alpha diversity indices (Chao1, Shannon and InvSimpson) values. F4_S= pigs genetically susceptible to ETEC F4; F4_R= pigs genetically resistant to ETEC F4; d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning.

Supplementary Materials:

The following are available online at https://doi.org/10.1016/j.livsci.2020.104247, **Supplementary Figure 1.** Rarefaction curve of fecal samples resulted by sequencing of V3–V4 regions with MiSeq platform (Illumina Inc., San Diego, Ca, USA). **Supplementary Figure 2.** Effect of age on alpha indices of faecal microbiota. Boxplot of Alpha diversity indices (Chao1, Shannon and InvSimpson) values. d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning. **Supplementary Figure 3.** Effect of age on faecal microbial structure of piglets. NMDS plot on Bray Curtis distance matrix. d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning. **Supplementary Figure 4.** Effect of bivalent *E. coli* vaccination on faecal microbial profile of piglets at 18 days post vaccination (d18). Control= not vaccinated pigs; Vaccine= pigs vaccinated with live non-pathogenic *E. coli* O8:K87 (F4ac-positive) and O141:K94 (F18ac positive) at d0 (26±2 age of life). **Supplementary Table 1**: Per sample information about sequencing depth (reads abundances),ASVs abundances and alpha diversity indices (Chao, Shannon and InvSimpson).**Supplementary Table 2**: Significant different Phyla in the fecal samples of piglets before and after weaning. d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning. **Supplementary Table 3**: Significant different families in the fecal samples of piglets before and after weaning. d0= the day of weaning (26±2 age of life); d18= eighteen days post weaning.

Manuscript 3

Investigation of Early Supplementation of Nucleotides on the Intestinal Maturation of Weaned Piglets

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Simple Summary: Nucleotides represent a group of bioactive compounds essential for the developmentof the gastrointestinal tract and immune function. This study aimed to evaluate the short-term effect of oral administration of nucleotides before and after weaning on growth performance, health, development of the intestinal immunity and microbiome of piglet. A nucleotide-based product (NU) was orally given four times before weaning and once after to one group of piglets, while a second group was used as a control (CO). The NU pigs did not grow more than the control until 12 days post-weaning but had increased hemoglobin and hematocrit values. At weaning, feces of NU pigletshad a microbial profile more typical of growing pigs, while those of CO were more representative of suckling pigs. The upregulation of genes in the blood of control pigs at weaning was indicative of more activation towards an inflammatory response, while genes of erythropoiesis were more active in NU pigs post-weaning. NU supplementation stimulated genes for proliferative activity in the intestinal immune system, a sign of possible anticipated maturation. NU supplementation did not influence the growth performance of piglets but may have expressed a positive effect on pig microbiota anticipating its maturation at weaning, with possible immunostimulant activity on the intestinal immune system.

Abstract: Nucleotides are essential for the development of the gastrointestinal tract and immune function, but their intake with milk by piglets could be insufficient. The effect of nucleotides on growth and health was tested on 98 piglets divided into two groups: NU, orally administrated with 4 mL of a nucleotide-based product (SwineMOD®) at 10, 15, 18, 21, 27 days, or not (CO). Blood and feces were sampled at weaning (26 d, T1), and at 38 d (T2). Per each group and time-point, eight piglets were slaughtered and jejunal Peyer's patches (JPPs) were collected. NU increased hemoglobin content and hematocrit, but not growth. At weaning, the NU fecal microbiota was characterized by the abundance of Campylobacteraceae, more typical of the growing phase, compared to CO, with a greater abundance of Streptococcaceae. For the blood transcriptome, an initial greater inflammatory activation was seen in CO, while at T2, NU enriched gene sets related to erythropoiesis. The activation of gene groups ranging from epigenetic response to transcriptional regulation evidenced an intense proliferative activity in NU JPPs. NU supplementation did not influence the growth performance of piglets but could

have expressed a positive effect on pig microbiota anticipating its maturation at weaning. This immunostimulant activity in the JPPs could moderate the inflammation in the immediate pre-weaning.

Keywords: immunity; intestinal maturation; microbiota; nucleotides; piglet; transcriptome

1. Introduction

Nucleotides are a group of bioactive compounds representing building units of nucleic acids (DNA and RNA) and are involved in various biochemical processes. They are composed of a nitrogenous base (pyrimidine or purine) bound to a pentose (ribose or deoxyribose) sugar to which one, two or three phosphate groups are connected. They are considered essential for tissue with high cellular turnover (Van Buren and Rudolph, 1997), such as the intestinal epithelium and lymphoid tissues, that lack de novo synthesis of nucleotides (Uauy et al., 1994) and rely mostly on the salvage pathway that requires an external supply of nitrogenous bases. In some physiological conditions such as weaning, there is a limited nutrient intake and a higher cellular proliferative activity of the gut and immune system that are not fully developed. In these cases, nucleotide supplementation as growth promoters and immune stimulators could be essential in piglets (Grimble and Westwood, 2001; Sauer et al., 2011).

In addition, suckling and weaning are, for the piglet, the two most critical phases when it undergoes enteric pathologies that can have detrimental effects on its health, requiring antibiotic and therapeutic interventions. Furthermore, pre-weaning piglets are frequently vaccinated to improve their resistance to the main causes of colibacillosis (Enteropathogenic strains of *Escherichia coli* = ETEC), and this requires an active and mature response of the immune system. Nucleotides have shown potential value to mitigate the effect of weaning on piglet growth and/or health (Grimble and Westwood, 2001; Mateo, 2005; Sauer et al., 2011). There are also some pieces of evidence that nucleotides could favor the development of a healthier microbiota. In pigs, this was by increasing lactobacilli and bifidobacteria and reducing *Clostridum perfringens* counts (Mateo et al., 2004). In pathogen-free chickens, it was by increasing intestinal bacterial diversity and the abundance of *Lactobacillus* (Wu et al., 2018).

During the suckling phase, the main source of nucleotides is represented by the maternal milk, but their concentration in sow milk tends to decrease with the proceeding of lactation (Mateo et al., 2004).

Therefore, it is possible that the contribution to the specific nutritional requirement of the digestive and immune system by the milk nucleotides tends to decrease progressively, thus also delaying the maturation of the digestive system and immunity. This factor can influence the stabilization of the intestinal microbiota and the growth performance in the post-weaning phase. Curiously, most of the studies conducted on nucleotides are focused on the post-weaning phase, not considering the possible supplementation during suckling.

The aim of this study was to evaluate the effect of oral administration of nucleotides before weaning and in the immediate post-weaning on growth performance, health, intestinal structure, immunity, microbiota, and transcriptomic profile of weaned piglet preliminary vaccinated against ETEC.

2. Materials and Methods

2.1. Experimental Design and Sampling

The trial was carried out in a 300-sow farm that uses a vaccine prophylaxis for diarrhea caused by ETEC expressing F4 and F18 fimbriae. A total of 8 sows from homozygotes susceptible to *E. coli* F4 infection were selected. This was done to obtain all susceptible piglets (homozygotes or heterozygotes, determined by the sire) from these sows, that for this reason were potentially immunologically reactive to the vaccination (Fairbrother et al., 2017). For the genotyping, DNA was extracted from the bulbs of bristles obtained from each sow and genotyped for MUC4 g.8227C>G, by restriction fragment length polymorphism PCR (PCR-RFLP) using specific primers (Jørgensen et al., 2003). The sows belonged to two temporarily consecutive batches of farrowing. At 10 days of life, a total of 96 piglets (3.5 ± 0.6 kg), balanced by live weight and litter of origin, were randomly assigned into the two experimental groups. In the treatment group (NU = 48; 3.48 ± 0.72 kg body weight), piglets were orally supplemented with 4 mL of a solution containing a total 100 mg of a product standardized in nucleotides (swineMOD®, Prosol, Madone -BG-, Italy), and in the control group (CO = 48; 3.52 ± 0.63 kg body weight), pigs were orally supplemented with 4 mL of pure water. The supplements were orally administered using a dosing device at 10, 15, 18, 21 days of life, and 1-day post-weaning. The use of an oral solution was preferred to the integration of the creep feed to ensure an equal intake for all subjects.

Creep-feed was available from day 15. The creep-feed ingredients and estimated analytic composition are reported in Table 1.

At weaning (26 days of age, T1), piglets were transferred to the experimental facility of the University of Bologna. There, a total of 16 piglets (8 piglets per group) balanced by body weight and litter were slaughtered. The rest of the piglets were raised for twelve days with the same pre-starter feed used during the suckling period, and at the end of the experimental trial (12 d post-weaning, T2), another 16 piglets (8 piglets per group) derived from the same litters were slaughtered. After weaning, pigs were reared individually, penned inside a weaning room at pre-controlled temperatures and ventilation. The pens (100 cm × 33 cm each) were side-by-side, allowing contacts of the muzzles of neighbor pigs. Feed and water were freely available.

2.2. Growth Performance and Samplings

Piglets were weighed individually at 10, 21, and 26 days of age and then at the end of the experimental trial (12 d post-weaning). After weaning, the amount of feed supplied to the piglets was recorded daily and corrected for any residual feed to calculate the average feed intake.

The incidence of diarrhea was assessed as the number of days of diarrhea per pig, considering those with diarrhea as having a fecal score value above 3. The fecal score was defined using a five-point scale from 1 = hard feces to 5 = liquid feces.

For the analysis of the microbial profile, fecal samples at T1 (16 pigs selected for the slaughtering) and at T2 (80 pigs, including 16 pigs destined to the second slaughtering) were collected. At the same time points, the same piglets were sampled for peripheral blood (PB). Samples were collected from the jugular vein, using BD Vacutainer with EDTA K3 and BD Vacutainer with clot activators for serum collection. Blood was centrifuged at room temperature after 2 h of incubation to obtain serum. On the samples, a hemogram was determined. The hemochromocytometric analysis was performed using the automatic analyzer CELL-DYN 3700R® (Abbott Laboratories; Abbott Park, IL, USA).

The pigs to be slaughtered were chosen based on the average body weight (T1, 7.02 ± 0.81 kg; T2, 9.76 ± 0.57 kg), sedated by anesthesia with Zoletil 100 (15 mg/kg) and slaughtered with an intracardiac

injection of Tanax ® (0.5 mL/kg). From the same pigs, an additional PB sample was taken before anesthesia for mRNA sequencing. The PB was collected using Tempus™ blood RNA tubes (Thermo Scientific, Waltham, MA, USA) and stored at −80. In addition, a sample of jejunal Peyer's patches (JPPs) was collected from the distal third part of jejunum, and also for mRNA sequencing. JPPs were selected for testing post-natal immune activation because more involved in the diversification of immunoglobulin production and less in their primary undiversified production than ileal PPs in swine (Butler et al., 2016). JPPs were collected in sterile tubes and immediately frozen in liquid nitrogen and stored at −80 °C until processed.

2.2.1. Statistical Analysis of Growth Performance and Blood Parameters

Data on body weight (BW), average daily gain (ADG), feed intake (FI), feed to gain ratio (F:G), and hematocrit were analyzed with an ANOVA model considering diet, litter of origin and sex as factors, using GLM procedure of SAS (SAS Inst. Inc., version 9.4, Cary, NC, USA).

2.3. Microbiota Profiling

The bacterial DNA extraction was carried out using QIAamp Fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany). The V3-V4 region of the 16S rRNA gene was amplified using Pro341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3′ and Pro805R:5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3′ (Takahashi et al., 2014), using PlatinumTM Taq DNA Polymerase High Fidelity (Thermo Fisher Scientific, Italy). The PCR reaction conditions for amplification of DNA were as follows: initial denaturation at 94 °C for 1′, followed by 25 cycles of denaturation at 94 °C for 30″, annealing at 55 °C for 30″ and extension 65 °C for 45″, ending with 1 cycle at 68 °C for 7′. The libraries were prepared using the standard protocol for MiSeq Reagent Kit v3 and were sequenced on the MiSeq platform (Illumina Inc., San Diego, CA, USA). For the bioinformatics analysis, the DADA2 pipeline was used (Callahan et al., 2016) using the Silva database (version 132) as reference for the taxonomic assignment.

2.3.1. Statistical Analysis of Microbiota

The statistical analysis on alpha diversity, beta diversity, and taxonomic composition was carried out with R v3.6, using the PhyloSeq (McMurdie and Holmes, 2013), Vegan (Dixon, 2003) and lme4 (Bates et al., 2015) packages. An ANOVA and a PERMANOVA ("adonis" procedure) models were used to test the effect of age and treatment, on alpha and beta diversity, respectively. These models were applied on the datasets divided according to the two time points, while the effect of time was tested on the entire data set. The differences in taxonomic composition were tested using the DESeq2 package based on negative binomial generalized linear models (Love et al., 2014).

2.4. Transcriptome

2.4.1. Pig mRNA Extraction and Sequencing

For blood samples collected in Tempus™ tubes, total RNA was isolated using the Tempus™ Spin RNA Isolation Kit (Thermo Scientific, Waltham, MA, USA) following the manufacturer's instructions. For tissue samples, total RNA was extracted using the GeneJET RNA Purification Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's instructions. DNase treatment was performed to remove contaminating DNA using TURBO DNA-free™ DNA Removal Kit (Thermo Scientific, Waltham, MA, USA) following the recommended protocol. RNA quantity and quality were evaluated using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies Inc., Wilmington, DE, USA) and agarose gel electrophoresis, respectively. RNA integrity was evaluated through Agilent Bioanalyzer 2100 (Agilent Technologies, Santa Clara, CA, USA). Libraries were prepared using the TruSeq Stranded mRNA Sample Preparation kit and sequenced using the Illumina MiSeq system 2 × 100 bp with 2 × 20 million sequencing depth.

2.4.2. Differential Expression Analyses of RNA-Seq Data

After quality control using the FastQC tool (v.0.11.9), reads were filtered with Trimmomatic (v.0.36) (Bolger et al., 2014) by trimming leading and trailing bases with a Phred score less than 2 and dropping reads shorter than 15 bases long and those with average Phred score per base less than 15. Sequences were aligned to the NCBI *Sus scrofa* v11.1 reference transcriptome using salmon (v.0.14.1) (Patro et

al., 2015). Differential expression analysis was carried out in R (3.6.2) using the DESeq2 package (v.1.26) (Love et al., 2014), testing for the effect of the diet in the two different time-points separately. Genes were considered differentially expressed (DE) when a p-value adjusted (p adj) for a false discovery rate (FDR) < 0.05 and a Log2FC > 2.

2.4.3. Functional Enrichment Analysis

For the functional enrichment analysis, an exploratory analysis was conducted using the Gene Set Enrichment Analysis (GSEA) software (Bild and Febbo, 2005), which performs an analysis of gene sets, defined as groups of genes with common biological function, chromosomal position, or regulation. The GSEA analysis was based on the C5 sub-collections of Gene Ontology and on the Hallmark collection (Liberzon et al., 2015) (MSigDB, Broad Institute, and UC San Diego), and the gene sets were considered significantly enriched with a q value of FDR ≤ 0.05. Finally, to evaluate the differences between diets by combining either times or tissues, the EnrichmentMap Plugin (Merico et al., 2010) for Cytoscape 3.8 (Shannon et al., 2003) was used, which displays the overlap and connections between different gene set, considering a q value of FDR <0.05 or <0.001, depending on what is specified later, per each presented enrichment map. The nodes were joined if the overlap coefficient was ≥0.375.

3. Results

3.1. Growth Performance and Blood Parameters

Table 2 shows the growth performance data. In general, no statistically significant differences were observed for all the parameters analyzed.

For the blood parameters (Table 3), there was no statistically significant interaction between the diet and sampling time-point; consequently, the effect of the two factors was assessed separately and the value for the diet is inclusive of the two sampling times. Pigs treated with nucleotides (NU) had a higher hemoglobin content (12.0 vs. 11.8, $p < 0.05$) and hematocrit percentage (36.9 vs. 35.4, $p < 0.05$) than pigs in the CO group.

3.2. Microbiota Profile

The sequencing process produced a total of 3,459,059 reads (69,348 on average). Two samples did not produce enough reads, and thus were removed from the analysis. In total, the number of subjects in the analysis were 93 (46 NU, 47 CO, 16 T1, 77 T2). A total of 8591 amplicons sequences variants (ASVs) were identified, which resulted in 23 different phyla (Firmicutes 42.48%, Bacteroidetes 41.71%), 66 Families (Prevotellaceae 21.55%, Ruminococcaceae 16.91%) and 204 genera (Prevotellaceae_NK3B31_group 7.68%, Rikenellaceae_RC9_gut_group 6.45%).

The alpha diversity values (variability within communities), measured with Chao1, Shannon, and Simpson indexes (Figure 1), did not differ between the two diets both at weaning (T1) and at the end of the trial (T2). However, at T1, there was a strong trend for the Simpson index and the CO group had a higher value than NU group (CO: 0.965, NU: 0.937, $p = 0.06$).

For the beta diversity (compositional similarity between microbial communities), the results were plotted using a nonmetric multidimensional scaling plot (NMDS; Figure 2). The effect of age was evident and visible in two distinct clusters and it was confirmed by the Adonis test ($R^2 = 0.06$; $p = 0.01$). There were no differences between the two diets at both time-points (T1 and T2).

For the taxonomic composition, at weaning, the phylum Epsilonbacteraeota was significantly more abundant in the NU group compared with the CO group (log2FC: 2.30, p adj < 0.01); within this phylum, the most represented family in NU was that of Campylobacteraceae (log2FC: 3.14, p adj: 0.03), while Streptococcaceae were more abundant in the CO group (log2FC: —5.36, p adj < 0.01). There were no statistically significant differences regarding the taxonomic composition between the two diets at T2.

3.3. Transcriptome Profile

Transcriptomic analysis was carried out on a total of 32 samples per tissue (PB or JPPs), divided into the two timepoints (weaning or 12 d post-weaning). However, for a single blood sample (NU, T2 group), it was not possible to obtain an adequate quantity and quality of RNA, so sequencing was not performed. For PB and JPPs, a total of 21,424 and 22,953 transcribed genes were obtained, with a

mapping rate of 79 ± 3% and 78 ± 3%, respectively. Of these transcribed genes, an important part still has no nominal attribution, according to the referenced NCBI and Ensembl porcine genome databases. Therefore, the number of genes with useful attribution was, respectively, 15,509 and 16,197. The expression profile was explored with a multi-dimensional scaling plot (MDS plot) for each tissue, based on count data normalized via variance stabilized transformation. In this plot, samples are positioned according to the statistical distance of their expression profiles, showing no clear separation based on dietary group (Figure 3).

Differential expression analysis revealed that in PB samples at T1 two genes, pancreatic trypsin inhibitor (*PTI*) (Log2FC = 5.0, lgfSE = 1.1, *p* adj < 0.01) and PIGY Upstream Reading Frame (*PYURF*) were DE in favor of the NU group; the latter was also DE in JPPs, in favor of NU (Log2FC = 22.7, lgfSE = 2.9, *p* adj < 0.01). At T2, no DE genes were found for PB samples, whereas in the JPPs of NU pigs, there was a higher expression of *REG3G* compared to CO (Log2FC = 5.4, lgfSE = 1.1, *p* adj < 0.01).

The enrichment analysis conducted using the Gene Set Enrichment revealed the upregulation of 24 gene sets for CO, and 1 for NU at weaning (T1), in the PB samples (Table 4). Conversely, at the end of the trial (T2), 10 gene sets were enriched in the NU group against 4 in CO (Table 5).

Figure 4 presents the effect of the diet on peripheral blood gene sets enriched in the two sampling times, using the Hallmark collection. In general, at weaning, many typical blood sets (haeme metabolism, coagulation, angiogenesis, complement, anoxia) related to cell differentiation and inflammatory response (response to interferon-alpha and gamma, signal through TNF, IL2, etc.) were dominant in the CO group, and the response was reversed 12 days after weaning. Some gene sets such as those related to the response to external organisms and to the reactive oxygen species were enriched with over-regulated genes in the CO group.

For the JPPs, a more articulated gene set collection was used, which considers the biological processes detailed by Gene Ontology and includes 7530 sets. With this collection, 62 enriched gene sets were observed in T1 in the CO group and 92 in the NU group, respectively. At weaning, 445 gene sets were enriched for CO and 341 for NU. The effect of the diet on the enriched gene sets is shown in Figure 5.

In this figure, the names of the gene sets are abbreviated, while Supplementary Figure S1 shows the full names. The combined representation shows that in general, the response is consistent in the two time-points. NU supplementation enriched a rather large set of genes involved in cell replication, epigenetic regulation, DNA, messenger RNA, mitochondrial RNA, and protein synthesis. On the other hand, in the CO group, there was an activation of genes related to the structuring of JPPs (junctions and cell-matrix), the organization of local smooth muscles, and neuronal control through synaptic vesicles.

Figure 6 shows the effect of the diet on the gene set enrichment in the two types of tissue at the time of weaning. This showed that in the samples collected at weaning in the NU group, the gene sets involved in the processes of rRNA and mRNA (including its maturation) and chromatin were enriched, compared to CO, whatever the tissue. On the other hand, for the CO group, there was a constant activation of two types of gene set associated with the type 1 response to interferon.

In the case of the samples collected at the end of the trial, there was no equal response in the two tissues, although many gene sets had been differently involved. Consequently, Supplementary Figure S2 reports only the graphical visualization of the different responses in the two tissues at T2.

4. Discussion

This study investigated the effect of oral administration of nucleotides before and after weaning to piglets. Even though nucleotides did not affect the growth performances, they could explicate a positive effect on the microbiota and on the immunological maturation of the GI tract, by increasing the abundance of bacterial taxa associated with age and by increasing the proliferative activity of the JPPs.

Nucleotides represent essential compounds involved in several biological processes, as they are the building blocks of RNA, DNA, and ATP. Tissues with high cellular turnover, such as intestinal epithelium and lymphoid tissues, have a higher need for nucleotides. Additionally, in a stressful situation like weaning, a higher intake of nucleotides could be beneficial by enhancing the immune response and reducing the intestinal inflammation associated with it. The effects of nucleotide supplementation on growth performance are heterogeneous. Several studies reported no effect on growth performance (Domeneghini et al., 2004; Lee et al., 2007; Martinez-Puig et al., 2007), in agreement with our results. On the other hand, in a study by Jang and Kim (2019) (Jang and Kim,

2019), a supplementation of 50 and 150 mg/kg of nucleotides to newly weaned pigs increased ADG in the first week post-weaning. In another study, Perricone et al. (2020) (Perricone et al., 2020) found that a higher dose (0.8 g) of nucleotide in post-weaning increases ADG, BW, and FI. These studies were mostly focused on the post-weaning phase, none of these investigated the effect starting from the suckling phase. In this context, the effect of nucleotide supply on growth performance could be related to dose, time of administration, and supplementation method (in the feed or in oral solution).

The values of the blood parameters fall within the range commonly observed for weaned piglets (Perri et al., 2017). However, piglets in the NU group had a higher level of hematocrit and hemoglobin than piglets in the CO group. Revilla et al. (2019) (Revilla et al., 2019) found a favorable correlation between the hematocrit and hemoglobin values at weaning and the piglet robustness index, concerning the first 7 weeks after weaning. Robustness is defined as the ability of an animal to maintain a certain phenotype regardless of the characteristics of the external environment (Colditz and Hine, 2016). This is associated with resilience, defined as the ability to cope with environmental disturbances and to quickly return to the "pre-challenge" state (Colditz and Hine, 2016; Revilla et al., 2019). Furthermore, a positive relationship was observed between the hemoglobin and hematocrit values and daily weight gain in the first 3 weeks post-weaning (Bhattarai and Nielsen, 2015). In particular, a 1 g increase in hemoglobin per dL of blood would correspond to an increase in weight gain of 17 g per day (Bhattarai and Nielsen, 2015). In the present study, an absence of interaction between diet and age on hematocrit values indicates that the effect of supplementation on the hematocrit persists after twelve days of feeding with pre-started diets. The litters of origin were the same and the feed used was identical for the two groups and well supplemented for iron, thus dietary iron could not have been a negative factor affecting the iron state of pigs. Therefore, it can be assumed that the observed differences were associated with a direct early effect of the nucleotide supplementation and are attributable to other factors, such as the general state of health and body hydration. Conversely, no effect of nucleotides was seen on the hematocrit of piglets when given in weaning (Sauer et al., 2011), indicating that starting in the suckling period with more immature pigs is relevant.

Studies involving the effect of nucleotides on microbial profile are limited. There are no studies investigating the effect of nucleotides using 16S amplicon sequencing on pig microbiota, a study by

Wu et al. (2018) on poultry found that nucleotides increased the alpha diversity indices (Chao1 and Shannon) in intestinal contents. However, in the present study, no effect on alpha diversity was seen. In addition, in the present study, no effect of nucleotides was evidenced for the beta diversity and limited differences were found in the abundancy of taxa. Wu et al. (2018) found a higher abundance of *Lactobacillus* and a decrease in bacteria from *Blautia* and *Ruminiclostridium_5* genera with dietary yeast nucleotide supplementation in specific pathogen-free chickens. In the present study, a higher abundance of Campylobacteraceae and a decrease in Streptococcaceae was associated with the nucleotide supply starting from 10 days of age. The Campylobacteraceae family belongs to the Epsilonbacteraeota phylum that was consequently also more abundant in NU. In the gastrointestinal tract of the piglet, the Campylobacteraceae family is made up of both pathogenic (e.g., *Campylobacter jejuni*) and commensal bacteria (De Rodas et al., 2018). Furthermore, this taxon is generally associated with an increase in the maturation of piglet gut microbiota as its abundancy increases with age in the post-weaning phases (De Rodas et al., 2018); in addition, a greater abundance of the Campylobacteraceae family in the caecal mucosa has been associated with better feeding efficiency in growing pigs (Metzler-Zebeli et al., 2018). Streptococcaceae, on the other hand, are a predominant taxon in the gut microbiota of piglets in the suckling phase (Petri et al., 2010). Therefore, the lower abundance of Streptococcaceae and the greater abundance of Campylobacteraceae in the NU pigs at weaning may indicate that the intestinal microbiota of piglets receiving the nucleotide supply was already more mature than that of the control. Furthermore the ability of adhering to the intestinal mucosa, recognized in several strains belonging to Campylobacteraceae (Scanlon et al., 2013), could be a factor associated with the increased immune response observed with NU. Nevertheless, more studies are needed to better understand the role of commensal Campyloabaceraceae in young pigs.

The differences seen in the presence of some bacteria associated with the addition or not of the nucleotide-based product could be related to a different activation of the local immune system (Grimble and Westwood, 2001; Sauer et al., 2011). Furthermore, all the pigs in the trial were stimulated at 18 d of age with anti-ETEC vaccination, and for this purpose we selected ETEC-susceptible pigs. To investigate the effect of nucleotides supplementation on gene expression profile, we used a global transcriptomic approach to evidence mRNA changes in both PB and JPPs. At weaning, the

transcriptomic profile of the PB samples evidenced a higher expression of *PTI* and *PYURF* genes in the NU group, and the latter was also DE in JPPs samples. *PYURF* encodes the upstream open reading frame of the bicistronic transcript that encodes for the biosynthesis of a phosphatidylinositol glycan anchor protein (PIGY) (Bruford et al., 2020). In pigs, this gene is located in the chromosome 8, in a QTL that was associated with porcine hematocrit variation (Ponsuksili et al., 2016). Interestingly, hematocrit values were also positively associated with NU. However, generally, the *PYURF* expression was low and limited to some subjects, thus further studies are required to clarify this aspect.

PTI is a gene for which there is not a true homolog in humans, coding for a pancreatic trypsin inhibitor, long known for pigs (Bartelt et al., 1977). In the pancreas, it has a protective action, while in the blood it could have relevance as an antifibrinolytic factor on the homology of the molecule of bovine origin (Gregorczyk and Maślanka, 2019). Regulation of the activation of *PTI* has not been studied previously, even though there is increasing interest in medicine regarding this kind of protein. The upregulation of *PTI* in the present case may be associated with the antifibrinolytic effect and in general with the slightly different percentage of blood volume occupied by erythrocytes. However, using a rapid visualization of the plot of data, no association between *PTI* expression and hematocrit was seen.

At T2, no DE gene in the PB was found, and conversely in JPPs, the nucleotide supplementation increased the *REG3G* expression. The homologous protein transcribed from this gene, with known antimicrobial action, is typical of the intestinal epithelium and its activation can be stimulated by both beneficial microorganisms, such as *Lactobacillus plantarum* (Gross et al., 2008) or pathogens (like *E. coli* K88, Trevisi et al., 2018). In our case, in healthy subjects, it could be an indicator of increased activation of epithelial defense. In fact, it should be underlined that the pigs were vaccinated with two non-pathogenic *E. coli* strains bearing both F4 and F18 fimbria, and that ETEC-susceptible healthy pigs also previously showed higher expression of *REG3G* (Luise et al., 2019).

The enrichment analysis showed that nucleotide supplementation induced a greater activation of the replicative and productive functions of the JPPs that was consistent in the two sampling times. Lymphocytes are the main cellular component of Peyer's patches; thus, it can be assumed that the activation of these genes concerns mainly this class of cells. The intensification of lymphocytic

replication in the jejunum and ileal plaques is a typical indicator of their maturation with age (Pabst et al., 1988). It would be tempting to state that this greater activation was maintained by the early provision of the dietary addition of the nucleotide-based product, in agreement with the high mitotic index observed in ileal mucosa of weaned pigs (Domeneghini et al., 2004). In fact, lymphocytes have a limited ability to produce nucleotides to optimize their proliferation and maturation (Carver, 1999). Their higher multiplicative and productive activity in supplemented pigs may also explain the better ability of Peyer's patches to produce more inflammatory cytokines of post-weaning pigs reared in poor environmental condition (Waititu et al., 2017) and the increased IgA production (Sauer et al., 2011). Conversely, this mechanism was not more active in standard rearing conditions (Perricone et al., 2020), where Peyer's Patches activation of inflammatory cytokines was not seen with the addition of nucleotides, while this addition promoted growth response of piglets.

On the other hand, in the CO group, a higher activation of genes related to JPPs structuring (junctions and cell-matrix), organization of local smooth muscles, and neuronal control through synaptic vesicles, was seen. The effects were consistent in both the time-points considered. The presence of groups of genes typical of other tissues is not surprising because overall JPPs are an aggregate of complex cellular structures in which cells of natural endothelial and keratinocytic types also play a role, which also gives them a circulatory function and a structural consistency. In particular, the porous nature of the local lamina propria assures the migration of antigens from the surface epithelium and M cells to the dendritic cells in the sub-epithelial dome (Panneerselvam and Budh, 2020; Takeuchi and Gonda, 2004). Interestingly, cytokeratin (18) was proposed as a marker of M-cells in porcine Peyer's patches, presumably contributing to their specific shape (Gebert et al., 1994). In general, the structural cells supporting Peyer's patches received scarce research attention. It is possible that due to the increased activation of lymphocytes in the treated pig group, relatively more mRNA was present in the control related to these structural genes.

For PB samples, the time-dependent different presence of enriched gene sets in the two treatment groups apparently contrasts with the dietary effect in the absence of a significant interaction with time, observed for some of the blood cell counts. For instance, the enrichment of gene sets associated with heme metabolism, coagulation, angiogenesis, and anoxia in the immediate pre-weaning in the control

group may be explained by the need to attain a higher volume for the blood cells in PB like the one observed in piglets supplemented with the nucleotide-based product. Conversely, the enrichment of the same gene sets in samples obtained from supplemented pigs on day 12 post-weaning, in the presence of a relatively higher hematocrit, suggests that these pigs were further activating their erythropoiesis to improve their oxidative status. Taken together, these data may indicate that nucleotides delayed the need for oxygen support for the oxygen-demanding growth in the post-weaning.

No other direct evidence of that has been reported, but in neonatal rats, nucleotides increased the concentration of 2,3-diphosphoglycerate in erythrocytes (Scopesi et al., 1999), possibly improving the exchange of oxygen in tissues, because hemoglobin has more affinity for this molecule compared to oxygen. Moreover, the nucleotide supplementation improved the unsaturation index of red blood cell phospholipids in human neonate (DeLucchi et al., 1987) and weanling rat (Boza et al., 1992), with a possible impact on their regulation of the metabolic activity of these cells. This, however, was not seen in preterm babies (Axelsson et al., 1997). Taken together, these observations in other species, indicating a possible better degree of systemic oxidation and a reduced need of activation of hematopoiesis with nucleotide supplementation, can explain the reduced activation of genes related to heme metabolism in the immediate pre-weaning. However, it also contrasts with the constantly higher hemoglobin content and blood cell density with the supplementation of nucleotides that needs other explanations. Finally, concerning hematocrit variations, we calculated the simple correlations of this parameter with the expressions of gene in PB (data not shown), and it is interesting to report that the best correlation across time and feeding groups was seen for Junctional Cadherin 5 Associated (*JCAD*) ($r = +0.591$). The same was also with hemoglobin ($r = +0.647$). Scarce information is available on *JCAD*. However, the protein coded by *JCAD* was located in blood endothelial cell–cell junctions from human tissues (Shigeoka et al., 2020) and mutations of that gene were associated with atherosclerosis and hypertension (Williams and Stein, 2019). Thus, it is tempting to propose this gene as an indicator of blood erythrocyte density and to consider it for further studies.

A time-dependent effect of the diet on PB transcriptome was also seen on several gene sets related to inflammatory response (primarily INTERFERON_ALPHA_RESPONSE, INTERFERON_GAMMA_RESPONSE, TNFA_SIGNALING_VIA_NFKB). In the immediate pre-

weaning, in the NU group, a lower initial activation of these gene sets was seen compared to the CO group, and this agreed also with the reduced enrichment of the set of the other collection, RESPONSE_TO_ TYPE_I_INTERFERON, observed for both PB and JPP (Figure 5). For PB, no effect of the diet was seen on the number and relative partition of different classes of leucocytes, thus it can be assumed that the effect on transcriptome was related to the modulation of some of them. This could be associated with the increased activity of JPP and to the ability to control the translocation of microorganisms to the whole-body system in NU compared to CO, such as increased production of IgA (Sauer et al., 2011). On the contrary, the increased presence of enrichment sets on day 12 post-weaning in NU could depend on a shift in the diffuse immune system related to the continuing maturation progress. However, the overall regulation seems quite complex, as indicated conversely by the divergent response of transcriptome between PB and JPPs.

Overall, the transcriptomic profile showed an intense proliferative activity in the JPPs of piglets of the NU group, exhibited by the activation of a series of gene sets, ranging from epigenetic response to transcriptional regulation. This could be a sign of a more advanced state of maturation, which may have been favored by the supplementation of nucleotides.

5. Conclusions

Nucleotide supplementation did not influence the growth performances but could have favored an early maturation of the gastrointestinal microbiota by increasing the abundance of bacterial taxa associated with older pigs (Campylobacteraceae). For the transcriptomic profile, a complex, time- and tissue-dependent effect was seen. In fact, the nucleotide supplementation induced a higher proliferative activity in the JPPs, possibly reduced the inflammation in the immediate pre-weaning, and increased the erythropoietic activity in the post-weaning in the PB. Further studies are needed in order to investigate the effect of nucleotides on the pig microbiota structure and the immune maturation of the gastrointestinal tract.

Data Availability:

The raw reads obtained from the 16s and mRNA sequencing are publicly available at the NCBI Sequence Read Archive (SRA) under the accession numbers SUB8665787 and SUB8684880, respectively.

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Tables

Table 1. Ingredients and calculated composition of the feed expressed on as fed.

Ingredients	Units	Content
Bakery former food	%	20.00
Barley	%	15.00
Soybean Protein Concentrate	%	13.50
Wheat, soft	%	12.20
Maize	%	11.75
Whey, sweet, dehydrated, skimmed	%	9.00
Wheat middlings	%	5.00
Spray dried porcine plasma	%	3.00
Lard	%	2.00
Beet pulp, dehydrated	%	1.50
Dicalcium phosphate anhydrous	%	1.40
Dextrose	%	1.20
Medium chain free fatty acid mixture	%	1.00
Organic acid mixture	%	1.00
l-Lysine HCl	%	0.55
Calcium carbonate	%	0.53
Sodium chloride	%	0.30
Vitamin and trace mineral mixture [1]	%	0.30
dl-Methionine	%	0.28
l-Threonine	%	0.28
l-Valine	%	0.11
l-Tryptophan	%	0.10

Calculated values [2]		
Metabolizable energy	Kcal/kg	3340
Crude Protein	%	18.00
Crude Fat	%	6.44
Crude Fibre	%	2.75
Ash	%	5.71
Lysine	%	1.34
Cysteine	%	0.30
Methionine	%	0.51
Threonine	%	0.95
Tryptophan	%	0.29
Valine	%	0.99

[1] Provided per kg of diet: vitamin A (retinyl acetate): 7500 IU; vitamin D3 (cholecalciferol): 1000 IU; vitamin E (dl-α_tocopheryl acetate): 80 mg; vitamin K (menadione sodium bisulfite): 1 mg; riboflavin, 2.3 mg; calcium-d-pantothenate: 9.0 mg; niacin: 17.5 mg; pyridoxine hydrochloride: 1 mg; folic acid: 0.5 mg; biotin: 0.10 mg; thiamine: 1.45 mg; vitamin B12: 15 µg; ferrous sulphate monohydrate: 302.8 mg; zinc oxide: 118.2 mg; manganous sulphate monohydrate: 18.48; copper sulphate: 347.6; sodium selenite: 0.55 mg; potassium iodide: 1.31 mg. [2] The values were estimated using EvaPig® software (Noblet et al., 2008) with information from the INRA-AFZ tables of feedstuff composition. Noblet, J., Valancogne, A., Tran, G., Ajinomoto Eurolysine sas, 2008. EvaPig®.[1.0. 1.4]. Computer program.

Table 2. Performance data (weaning at d 26; CO = control group; NU = nucleotides group; BW = body weight, ADG = average daily gain, FI = feed intake, F:G = feed to gain ratio).

Item	CO	NU	SEM	*p*-Value
BW, d 10, kg	3.49	3.47	0.09	0.872
BW, d 21, kg	6.38	6.22	0.14	0.409
BW, d 26, kg	7.25	7.09	0.17	0.450
ADG, d 10-d21, g	263.2	250.1	7.8	0.231
ADG, d 10-d26, g	235.2	226.4	7.2	0.381
BW, d 38, kg	9.55	9.38	0.20	0.559
ADG, d 10-d38, g	216.5	211.0	5.2	0.448
ADG, d 28-d38, g	190.1	188.1	7.5	0.849
FI, d 26-d38, g	222.2	216.4	5.5	0.453
F:G, d 26-d39	1.33	1.20	0.08	0.225
Days with diarrea, n	0.53	0.71	0.17	0.433

Table 3. Blood data obtained on fresh samples, for subjects sacrificed at weaning and 12 days later.

Item [1]	Diet		SEM	Sampling Time		SEM	*p*-Value		
	CO	NU		Weaning	d 12 Post-Weaning		Diet [1]	Sex	Sampling Time [2]
RBC, M/µL	6.52	6.73	0.11	6.40	6.86	0.12	0.114	0.844	0.013
HGB, g/dL	11.6	12.0	0.2	11.7	11.9	0.2	0.032	0.256	0.517
HCT, %	35.4	36.9	0.6	35.2	37.1	0.6	0.026	0.354	0.037
MCV, fL	54.5	55.1	0.6	55.3	54.3	0.7	0.469	0.196	0.327
MCH, pg	17.9	18.0	0.2	18.4	17.4	0.2	0.736	0.182	0.003
MCHC, g/dL	32.9	32.7	0.2	33.5	32.1	0.2	0.403	0.776	0.0001
RDW, %	24.4	23.2	0.6	23.5	24.0	0.7	0.102	0.203	0.624
PLT, K/µL	507	537	33	580	464	35	0.434	0.955	0.028
WBC, K/µL	13.8	13.7	0.8	11.4	16.0	0.85	0.897	0.012	0.0004
Neutrophils, K/µL	6.87	7.09	0.605	6.94	7.03	0.64	0.757	0.432	0.926
Lymphocytes, K/µL	5.43	4.99	0.62	3.22	7.20	0.65	0.545	0.023	0.0001
Monocytes, K/µL	1.15	1.26	0.15	1.05	1.36	0.16	0.559	0.496	0.209
Eosinophils, K/µL	0.13	0.11	0.040	0	0.25	0.045	0.653	0.365	<0.0001
Basophils, K/µL	0.19	0.18	0.040	0.15	0.22	0.045	0.744	0.042	0.294
Neutrophils, %	51.9	51.6	2.4	59.7	43.8	2.56	0.901	0.543	<0.0001
Lymphocytes, %	36.7	37.1	3.0	29.5	44.3	3.14	0.929	0.486	0.002
Monocytes, %	8.95	9.29	0.995	9.33	8.91	1.06	0.774	0.568	0.793
Eosinophils, %	0.97	0.71	0.28	0	1.75	0.295	0.434	0.155	0.0001
Basophils, %	1.43	1.23	0.22	1.37	1.29	0.23	0.432	0.225	0.830

[1] WBC—white blood cells; RBC—red blood cells; HGB—hemoglobin; HCT—hematocrit; MCV—mean corpuscular volume; MCH—mean corpuscular hemoglobin; MCHC—mean corpuscular hemoglobin concentration; RDW—red blood cell distribution width; MPV—mean platelet volume. [2] The interaction between diet and sampling time was not statistically significant, and thus was excluded from the statistical model.

Table 4. Significantly enriched gene groups in PB, respectively for CO and NU groups, according to the Hallmark collection, at weaning (T1).

Gene Set Name	Total Number of Genes	Normalized Standard Error	*p*-Value	FDR q-Value
CO Group				
HEME_METABOLISM	185	2.922	0.000	0.000
INTERFERON_ALPHA_RESPONSE	87	2.820	0.000	0.000
INTERFERON_GAMMA_RESPONSE	177	2.672	0.000	0.000
IL6_JAK_STAT3_SIGNALING	80	2.106	0.000	0.000
TNFA_SIGNALING_VIA_NFKB	192	1.903	0.000	0.000
MYOGENESIS	188	1.863	0.000	0.001
P53_PATHWAY	187	1.772	0.000	0.002
KRAS_SIGNALING_UP	183	1.772	0.000	0.002
INFLAMMATORY_RESPONSE	188	1.767	0.000	0.001
MITOTIC_SPINDLE	198	1.759	0.000	0.001
ANGIOGENESIS	31	1.696	0.002	0.003
COMPLEMENT	185	1.678	0.000	0.004
ESTROGEN_RESPONSE_LATE	174	1.646	0.000	0.005
XENOBIOTIC_METABOLISM	169	1.585	0.000	0.009
APOPTOSIS	154	1.571	0.001	0.010
APICAL_JUNCTION	184	1.559	0.001	0.011
IL2_STAT5_SIGNALING	191	1.544	0.001	0.013
COAGULATION	116	1.530	0.006	0.014
WNT_BETA_CATENIN_SIGNALING	39	1.524	0.025	0.015
EPITHELIAL_MESENCHYMAL_TRANSITION	185	1.508	0.001	0.017
REACTIVE_OXYGEN_SPECIES_PATHWAY	49	1.460	0.034	0.026
HYPOXIA	186	1.429	0.006	0.033
HEDGEHOG_SIGNALING	32	1.422	0.055	0.033
UV_RESPONSE_UP	145	1.405	0.009	0.036
NU Group				
MYC_TARGETS_V1	193	−2.469	0	0

Table 5. Significantly enriched gene groups in PB, respectively, for CO and NU groups, according to Table 2.

Gene Set Name	Total Number of Genes	Normalized Standard Error	*p*-Value	FDR q-Value
CO group				
OXIDATIVE_ PHOSPHORYLATION	167	2.826	0.000	0.000
MYC_TARGETS_V1	193	2.408	0.000	0.000
DNA_REPAIR	143	2.159	0.000	0.000
FATTY_ACID_ METABOLISM	140	1.999	0.000	0.000
NU group				
EPITHELIAL_ MESENCHYMAL_ TRANSITION	185	−1.823	0.000	0.014
INTERFERON_ ALPHA_RESPONSE	87	−1.720	0.000	0.017
UV_RESPONSE_DN	136	−1.696	0.000	0.015
TNFA_SIGNALING_ VIA_NFKB	192	−1.665	0.000	0.016
HEME_METABOLISM	185	−1.664	0.002	0.013
INTERFERON_ GAMMA_RESPONSE	177	−1.550	0.002	0.031
ANGIOGENESIS	31	−1.526	0.039	0.032
HEDGEHOG_ SIGNALING	32	−1.516	0.029	0.032
INFLAMMATORY_ RESPONSE	188	−1.456	0.004	0.048

Figures

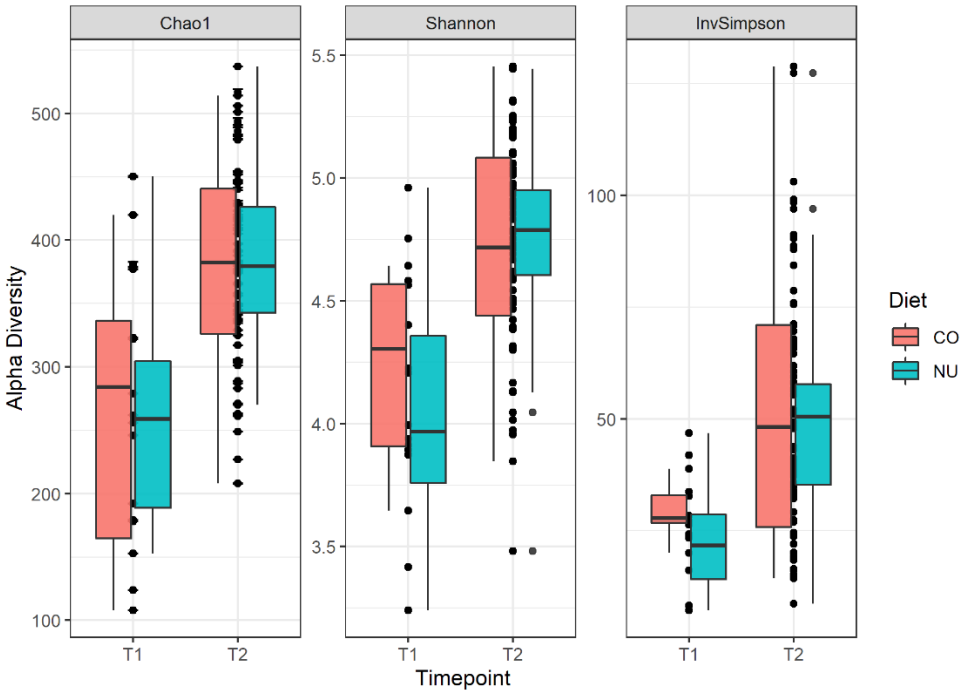

Figure 1. Alpha diversity boxplots with Chao1, Shannon and Simpson indices for the piglets divided into the 2 experimental groups (NU, CO) and the 2 sampling times (T1,T2). Simpson index, $p = 0.06$.

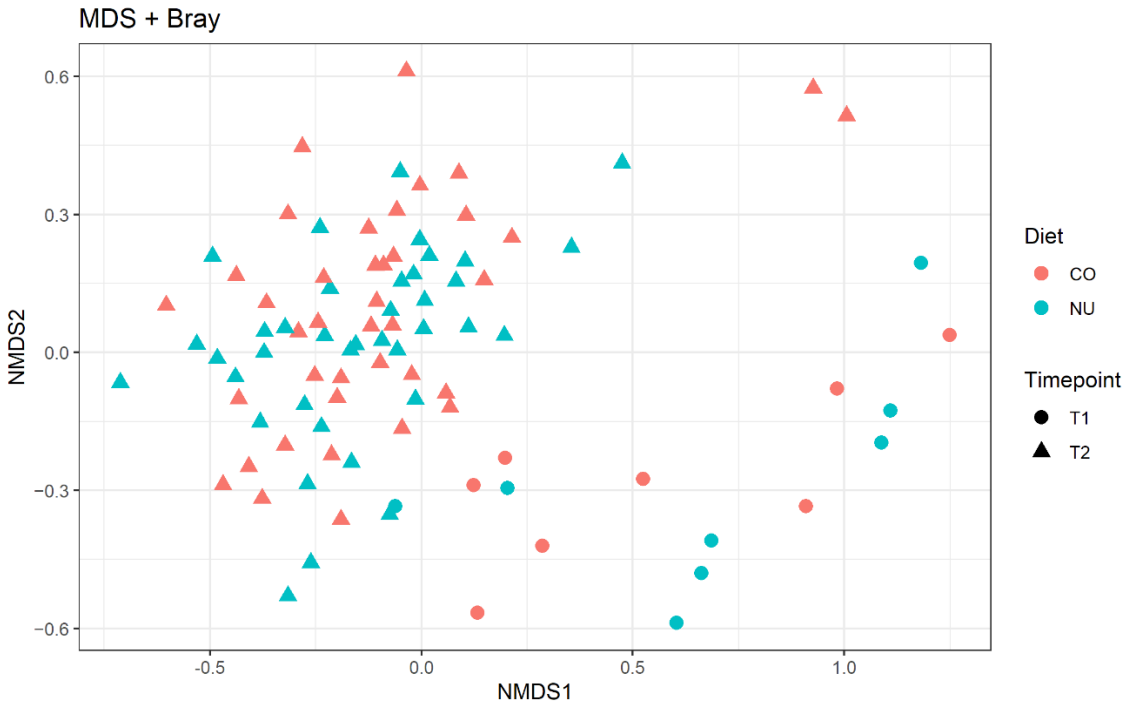

Figure 2. Non-metric multi-dimensional scaling (NMDS) plot on Bray–Curtis distances at ASVs level. CO: control group, NU: nucleotide supplementation. T1: weaning. T2: 12-d post weaning.

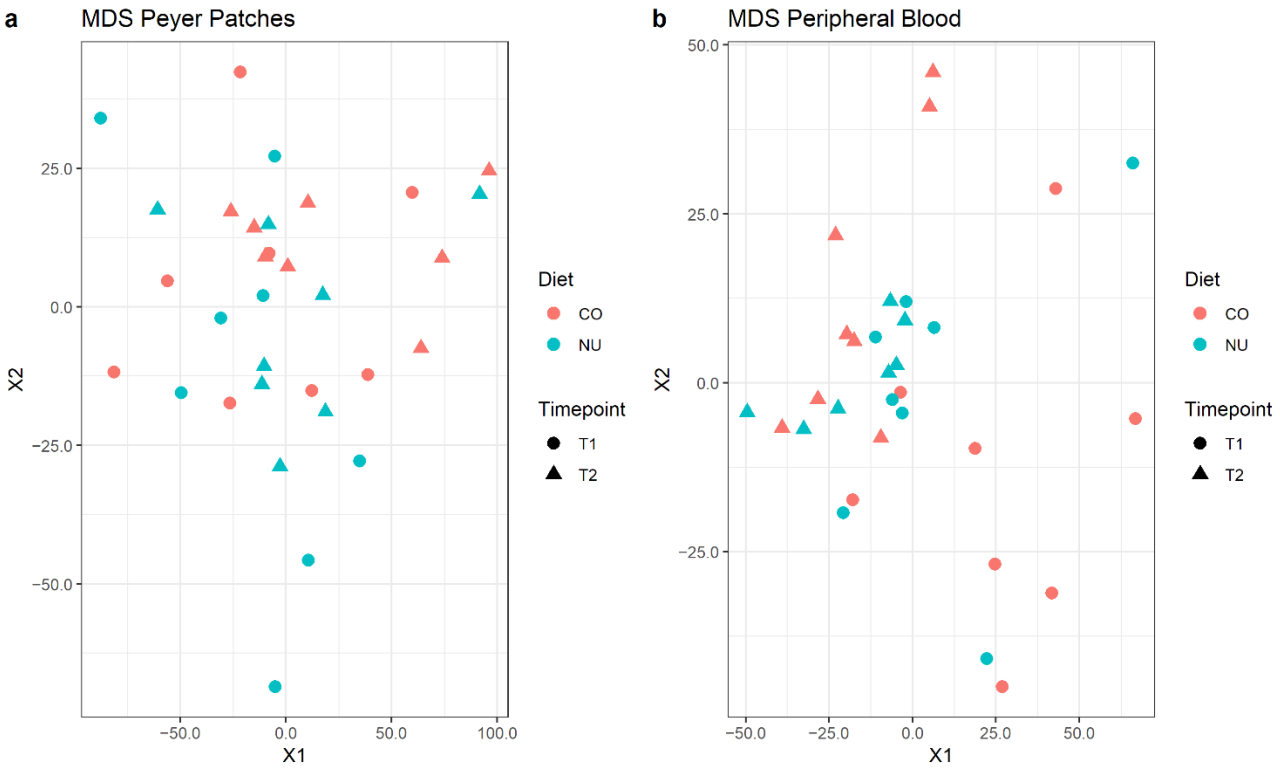

Figure 3. Multi-dimensional scaling (MDS) plots for normalized gene expression levels of JPP (**a**) and PB (**b**) at weaning (T1) and 12 d-post-weaning (T2), in control (CO) and nucleotide (NU) groups.

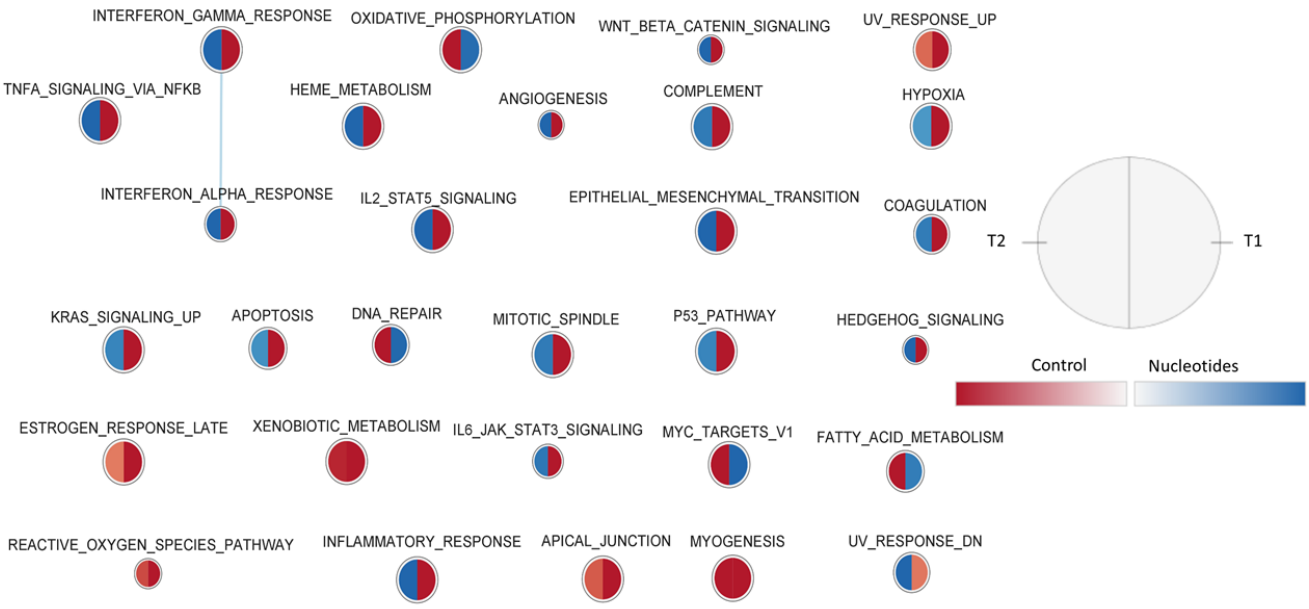

Figure 4. Effect of diet on peripheral blood gene sets enriched in the two sampling times, using the Hallmark collection. The nodes represent the enriched gene sets in the CO group (red) and NU group (blue). For each node, the right semicircle represents T1 and the left semicircle represents T2. The node size represents the number of genes in each gene set. The threshold for node insertion was FDR q-value < 0.05. The nodes were joined if the overlap coefficient was ≥0.375.

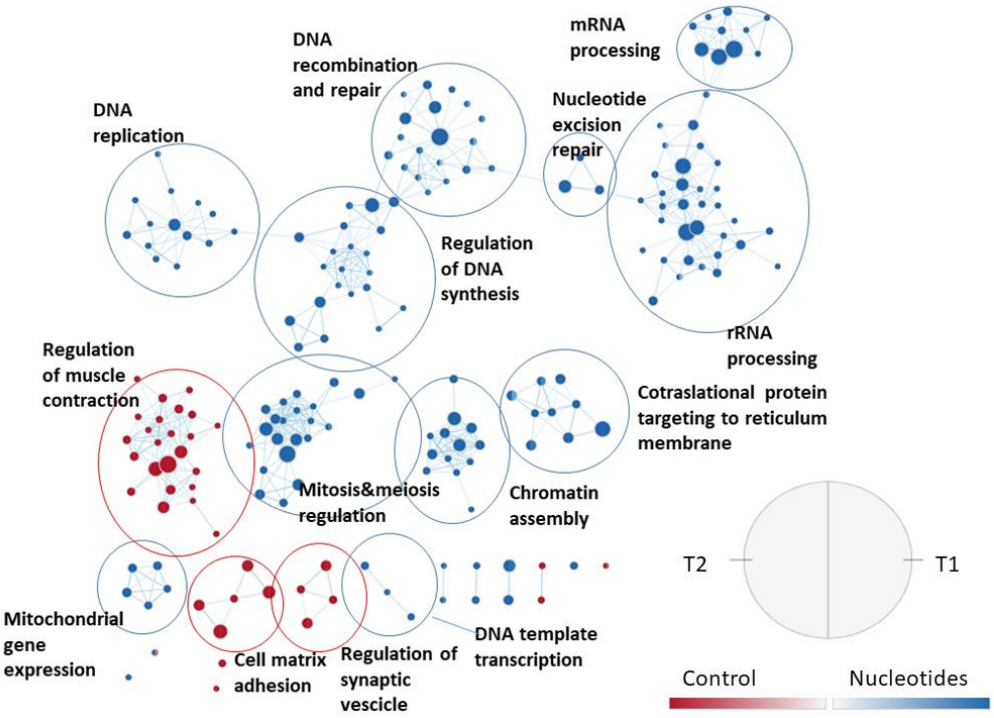

Figure 5. Effect of the diet on jejunal Peyer's patches gene sets enriched in the two sampling times, using the collection of biological processes according to Gene Ontology. The nodes represent the enriched gene sets in the CO group (red) and NU group (blue). For each node, the right semicircle represents T1 and the left semicircle represents T2. The node size represents the number of genes in each gene set. The threshold for node insertion was a FDR q-value < 0.001. The nodes were joined if the overlap coefficient was ≥0.5.

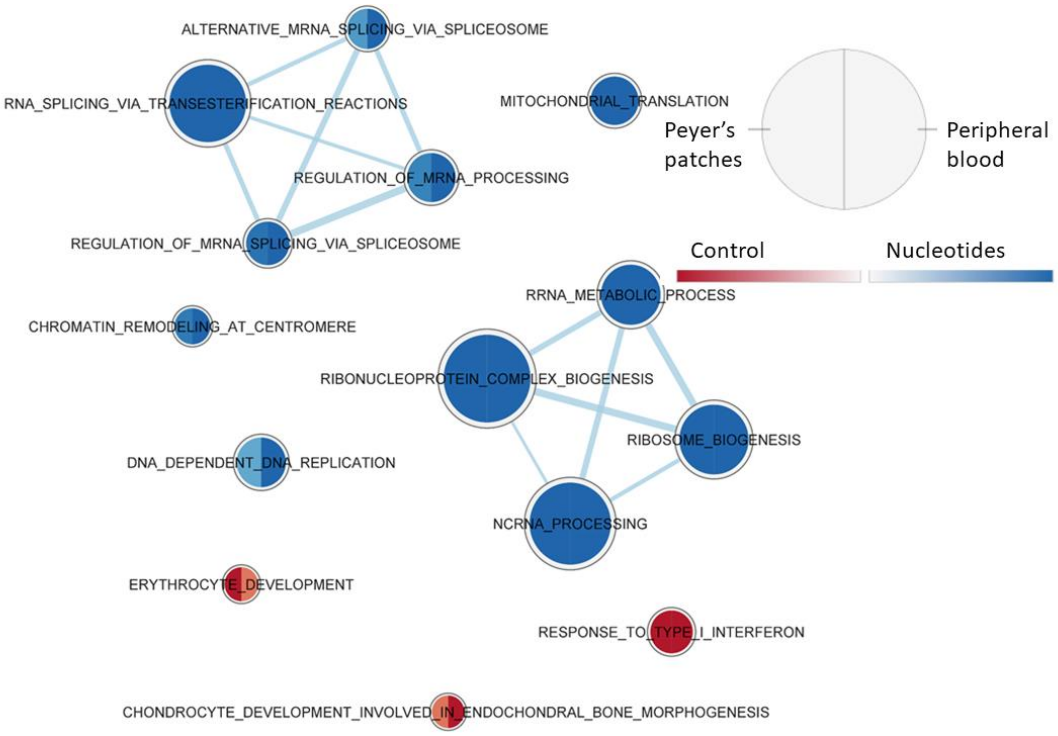

Figure 6. Effect of diet on PB and JPPs gene sets enriched at weaning (T1), using the collection of biological processes according to GO. The nodes represent the enriched gene sets in the CO group (red) and NU group (blue). For each node, the right semicircle represents the JPPs and the left semicircle represents PB. The node size represents the number of genes in each gene set. The threshold for node insertion was an FDR q-value < 0.001. The nodes were joined if the overlap coefficient was ≥0.5.

Supplementary Materials:

The following are available online at https://www.mdpi.com/article/10.3390/ani11061489/s1 , **Figure S1**: Effect of the diet on jejunal Peyer's patches gene sets enriched in the two sampling times, with the full name of each enriched gene set. The nodes represent the enriched gene sets in the CO group (red) and NU group (blue). For each node, the right semicircle represents T1 and the left semicircle represents T2. The node size represents the number of genes in each gene set. The threshold for node insertion was a FDR q-value < 0.001. The nodes were joined if the overlap coefficient was ≥0.5. **Figure S2**. Effect of diet on PB and JPPs gene sets enriched at 12 day post-weaning (T2), using the collection of biological processes according to GO. The nodes represent the enriched gene sets in the CO group (red) and NU group (blue). For each node, the right semicircle represents the JPPs and the left semicircle represents PB. The node size represents the number of genes in each gene set. The threshold for node insertion was an FDR q-value < 0.001. The nodes were joined if the overlap coefficient was ≥0.5.

Manuscript 4

The supplementation of a corn/barley-based diet with bacterial xylanase did not prevent diarrhoea of ETEC susceptible piglets, but favoured the persistence of *Lactobacillus reuteri* in the gut

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Abstract

Exogenous enzymes can favour the release of shorter polymers of the dietary fibre, favouring the development of a beneficial digestive microflora. The addition of bacterial xylanase to a weaner pig diet was tested for its impact on the intestinal microbiota and digestive homeostasis. Thirty-two pigs genetically susceptible to enterotoxigenic *Escherichia coli* (ETEC), equally divided into two experimental groups, were used to increase the risk of diarrhoea and test the response of xylanase under conditions representing those severe situations which are frequently present on farms. Pigs, weaned at 25 $\pm$ 1 days, were fed a corn/barley standard diet without (Group CO) or with (Group XY) 100 g/t xylanase from BELFEED NV, Belgium. Blood samples (for measuring the reactive oxygen metabolites) and faeces were taken 14 and 28 days from the beginning of the trial. On day 28, the pigs were euthanised and jejunal samples were collected. The faecal bacteria16S rRNA gene was sequenced using a MiSeq Reagent Kit V3-V4 on a MiSeq-Illumina platform. The pigs had diffuse diarrhoea starting from day 4. On the morning of day 8 and for the two following days, all the pigs were treated with Enrofloxacin intramuscularly. The efficacy of the Enrofloxacin was confirmed using the ETEC F18 growth inhibition test. Four animals in each treatment group died or were suppressed to reduce pain. The diet did not change growth, the faecal score, or the reactive oxygen metabolites in the blood. The XY treatment trended to increase villus length in the jejunum ($p=0.066$). The operational taxonomic unit (OTU) distribution was homogeneous, the microbial diversity indices were not changed by the treatment, and the per phylum abundances were homogenous among the diets and were dominated by Bacteroidetes and Firmicutes. The beneficial xylose-fermenting *Lactobacillus reuteri* persisted after weaning in the XY treatment group ($P< 0.05$). The Beta diversity was clusterised for the time of sampling ($P= 0.003$). The supplementation with xylanase did not improve growth or protection against ETEC, but the effect on some beneficial bacteria species is merits additional study.

Keywords: enterotoxigenic *Escherichia coli*; exogenous carbohydrases; intestinal microbiota; jejunum; weaning pig; xylanase

1. Introduction

Exogenous carbohydrases are included among dietary additives in order to release polymers of lower molecular weight from the complex carbohydrates of the dietary fibre and to improve digestion. Xylanases can deploy their favourable action by reducing viscosity, mainly due to their action on soluble arabinoxylans (AXs), or by hydrolysing insoluble AXs into lower molecular weight arabinoxylan oligosaccharides (AXOS) making entrapped nutrients available to the animal. In corn and barley, more than in wheat, insoluble AXs largely predominate over soluble AXs (Englyst, 1989; Choct, 2015). Thus, in corn and barley, xylanase is mainly expected to hydrolyse insoluble AXs and release AXOS.

In pig production, the use of antibiotics and zinc oxide for therapy is already or is going to be more strictly limited by law, in order to prevent the risk of antimicrobial resistance, and/or by the pressure of consumer requests, depending on the country. Especially in piglets, antibiotics are very frequently used for the therapy of intestinal disorders which are, in the majority of cases, generated by pathogenic bacterial overgrowth. The possibility of reducing or excluding the use of antibiotics in the diet of weaning pigs cannot be based only on the availability of other equally powerful antimicrobials, but should also be based on the synergistic action of different dietary and additive factors.

The fermentability of AXOS is inversely proportional to their degree of polymerisation and proportional to the arabinose to xylose ratio (Rivière et al., 2016). As dietary supplements, AXOS may have a prebiotic function in the gut of monogastrics, with an increase in lactic acid bacteria, Clostridium spp and the hindgut production of acetate (Chen et al., 2019). In a piglet diet, AXOS upregulated the gene of an important intestinal defence protein (REG3G, Niewwold et al., 2019). The use of endo-1,4-β-xylanases (xylanase) can favour the release of AXOS useful for the development of a beneficial digestive microbiota and a more effective intestinal barrier. In broiler chickens, the addition of xylanase to a wheat-based diet sharply reduced the intestinal presence of the causative agent of necrotic enteritis, Clostridium perfringens (Choct et al., 2006), and improved the caecal microbiota profile when added to a corn-based diet (Kadhem et al., 2016). In pigs, xylanase supplementation reduced the molecular weight of indigestible carbohydrates of wheat distiller by-products (Pedersen et

al., 2015). The addition of xylanase to barley- or corn-based diets was tested on growing-finishing pigs (Barley, Boudry et al., 2017; corn distiller's dried grains, Tsai et al., 2017). Conversely, there are scarce data regarding weaning pigs, and the impact on gut microbiota which is particularly relevant for this phase has, in general, been poorly addressed. Experimental models which reproduce the predisposing conditions to intestinal disorders or diarrhoeic syndromes were, in general, not considered when testing the addition of xylanase.

The genetic susceptibility to some enterotoxigenic *Escherichia coli* (ETEC) can affect the growth response to the diet (Trevisi et al., 2015) and is an important driver of antibiotic treatment. In fact, upon experimental infection with ETEC, non-susceptible pigs showed no improvement of feed efficiency when orally dosed with colistine plus amoxycycline while susceptible pigs needed the antibiotic supplementation to prevent death and maintain good feed efficiency (Bosi et al., 2004). Thus, the use of pigs genetically susceptible to ETEC has often been considered in experimental models aimed at testing dietary solutions in post-weaning pigs (Luise et al., 2019) in order to better reproduce the effect that diets can have on farms where this intestinal pathology is frequently present.

The aim of this study was then to test the use of bacterial xylanase in weaning diets based on corn and barley by evaluating its impact on the growth performance, intestinal microbiota and on some indicators of digestive homeostasis.

2. Materials and Methods

2.1. Ethical approval

All the procedures complied with EU Directive 2010/63/EU for animal experiments, were approved by the Ethical-Scientific Committee of the University of Bologna and were sent to the National Health Ministry (protocol number 91/2017-PR) by this Institution.

2.2. Animals and dietary treatments

Pigs genetically susceptible to the most common pig enteric pathogen, ETEC, were used in this study to increase the risk of diarrhoea and to thus have a greater chance of highlighting the eventual animal

response with the addition of xylanase to the diet of post-weaning pigs with diarrhoea during the trial. A total of 32 pigs were used for the trial, selected from 6 litters based on potentially susceptible parents, and confirmed for susceptibility to ETEC F4, based on the genetic marker Mucine 4 (Jørgensen et al., 2003). The pigs were weaned at 25 ± 1 day of age and were equally assigned to Control or Xylanase treatment, balanced for litter of origin (Supplementary table 1), sex (7 females and 9 males per group) and body weight.

The piglets were fed with the same basal diet (standard diet according to NRC (2012), Table 1) without antimicrobial, or any type of growth promoter, and a single batch lot was used for all pigs. The xylanase diet was prepared mixing"on top" the enzyme with the basal feed using a small mixer (25 kg per batch). The xylanase used in this trial was a bacterial endo-1,4-beta xylanase from Bacillus subtilis (BELFEED NV, Belgium) and was added to the feed at the recommended dose of 10 IU/g of feed which is the registered concentration based on safety and efficacy tests (European Food Safety Authority (EFSA) Panel on Additives and Products or Substances used in Animal Feed, 2016). The presence of the enzyme in the diet at the expected level was confirmed by analysis, following the procedure used for the approval of the additive (European Union Reference Laboratory for Feed Additives, 2014). Briefly, 10 g of the feed mixture are extracted in 100 ml of 0.1M acetate buffer (pH 4.7, minutes) and centrifuged (5 minutes). A commercially available cross-linked arabinoxylan substrates (Xylazyme tablets) is then added to an aliquot (0.5 ml) of the supernatant and then incubated (50° C, 30 minutes). The reaction is stopped with 5 ml of a Trizma base solution and the solution is vigorously mixed twice on a vortex mixer, and finally paper filtered and the absorbance measured against water as blank at 590 nm. Calibration is performed on standards prepared from identical blank feed samples fortified with a reference standard with a known enzyme activity. The observed concentration was close to the predicted values (9.17 IU/g in the supplemented mixture, not detected in the control).

From their arrival, the pigs were reared in individual cages inside the weaning rooms at pre-controlled temperatures and ventilation. Each pen measured 100 cm x 33 cm. The pens were side by side and the separation between them allowed contact of the piglets' muzzles. Feed and water were freely available.

diet: vitamin A (retinyl acetate): 7,500 IU; vitamin D3 (cholecalciferol): 1,000 IU; vitamin E (DL-α_tocopheryl acetate): 80 mg; vitamin K (menadione sodium bisulfite): 1 mg; riboflavin, 2.3 mg; calcium-D-pantothenate: 9.0 mg; niacin: 17.5 mg; pyridoxine hydrochloride: 1 mg; folic acid: 0.5 mg; biotin: 0.10 mg; thiamine: 1.45 mg; vitamin B12: 15 μg; ferrous sulphate monohydrate: 302.8 mg; zinc oxide: 118.2 mg; manganous sulphate monohydrate: 18.48; copper sulphate: 347.6; sodium selenite: 0.55 mg; potassium iodide: 1.31 mg.

2ME: Metabolizable energy. The values were estimated using EvaPig® software (Noblet et al., 2008) with information from the INRA-AFZ tables of feedstuff composition.

2.3. Experimental procedures

All the pigs were weighed at the start of the trial and then weekly for four weeks until the end of the experiment. The feed intake of each animal was recorded daily. At the second and the fourth week (end of test) 5 mL of blood were collected from the jugular vein of all pigs in order to evaluate the level of the oxidative stress marker. At the same time, the faeces were collected.

The severity of the diarrhoea was evaluated at the end of each week in each pig by a five-point faecal score (1 to 5: 1 = hard, 5 = watery faeces) by the same operator. Based on diarrhoeic evidence, detailed in the Results and Discussion sections, and to avoid discomfort for the piglets, on the morning of day 8, all the pigs were treated with Baytrill (5 mg / Kg live weight, Enrofloxacin, Pharmily, Arborea, Italy) intramuscularly, and this treatment was repeated for the next two days.

At the end of the test, the pigs were sedated by anaesthesia with Zoletil 100 (Virbac, Milano, Italy; 15mg / kg pv) and were sacrificed with an intracardiac injection of Tanax ® (embutramide 200 mg/m, mebenzonium iodide 50 mg/ml tetracaine hydrochloride 5 mg/ml) (0.5 mL/kg BW; Intervet Productions srl, Aprilia, Italy).

A segment of the distal jejunum (at 75% of the total jejunal length) was sampled at necropsy for histologic measurements of the morphometry of the villi, including the calculation of the absorbing surface (Mazzoni et al., 2016). The samples were tautly pinned to balsa wood and immersed in 100

ml/l buffered formalin (pH 7.4). This procedure allowed good distension of the wall and consequently of the villi.

2.4. Histology and morphometric analysis

Tissue samples of the jejunum were fixed overnight in 10% neutral buffered formalin and embedded in paraffin. Formalin-fixed, paraffin wax-embedded 4 μm thick sections were deparaffinised in xylene and stained with hematoxylin–eosin. At least 10 sections were obtained from each paraffin-embedded sample and mounted on poly-L-lysine-coated slides. For each sample, the height and the width of 10 villi, and the width and the depth of 10 crypts were measured; only villi and crypts perpendicular to the muscularis mucosae were considered suitable for morphometry. The sections were examined at low magnification with a conventional microscope interfaced with a digital camera and a personal computer equipped with Cytometry software (Byk Gulden, Milan, Italy). The morphometric evaluations were carried out in a blind fashion by two expert investigators (MM and DL). Villus height was measured as the distance from the crypt opening to the top of the villus whereas crypt depth was measured from the base of the crypt to the level of the crypt opening. The mucosal-to-serosal amplification ratio (M), representing the absorptive mucosal surface in the jejunum, was calculated as previously reported by Kisielinski et al. (2002) in the rat and successively applied to the pig by Trevisi et al. (2009) and Mazzoni et al. (2016). This ratio is based on the mean values of the villous surface (calculated using length and width of the villous), mucosal unit bottom (determined by villous and crypt width) and villous width: M = (villous surface + unit bottom − villous bottom)/unit bottom, where villous surface = π (villous length × villous width), unit bottom = π (villous width/2 + crypt width/2)2 and villous bottom = π (villous width/2)2.

2.5. Reactive oxygen metabolites

The serum was analysed for reactive oxygen metabolites (ROMs) colorimetrically using the d-ROMs test kit (Diacron International Sr1, Grosseto, Italy) as their presence is an indicator of damage to the intestinal mucosa. For the automatic analysis (Brambilla et al., 2002), the sera were diluted 1:20 in distilled water, incubated for 5 min at 37°C with mixture containing 0.01 M acetic acid/sodium acetate

buffer pH 4.8 and N,N-diethyl-p-phenylenediamine as chromogen. Absorbance was read at 520 nm. Duplicates of each sample were analysed.

2.6. Microbiota analysis

Total bacterial DNA was extracted from 48 faecal samples (12 per group at two timepoints) using the FastDNA SPIN Kit for Soil (DBA Italia, Segrate, Italy) following the manufacturer's instructions. The library formation and sequencing of the 16S rRNA gene were carried out using the MiSeq® Reagent Kit V3-V4 (Illumina Netherlands, Eindhoven the Netherlands) on a MiSeq-Illumina® platform. One sample from the Control Group at the first time point and one sample from the Control Group at second time point were excluded due to a poor sequencing yield (<1000 reads).

2.7. Bioinformatics and Statistical analysis

The results were analysed using ANOVA (GLM procedure, SAS, SAS Inst., inc., Cary, NC) taking into account diet and the litter origin of pigs. Sex was excluded from the analysis as it was never statistically significant after a preliminary inclusion in the model. The data of the ROMs in blood measured at the two time points were analysed using the MIXED procedure of SAS with the option of repeated measurements. The statistical analyses followed the preliminary check of homogeneity of variances between the two feeding treatments using Levene's test which was never statistically significant. Significance was declared if $P \leq 0.05$ and a trend was considered when $0.05<P< 0.10$.

QIIME version 1.9.1 (Caporaso et al., 2017) was used for the bioinformatic analysis of the microbiota. Briefly: the reads were demultiplexed and quality filtered with a cutoff of Q20. The open-reference operational taxonomic unit (OTU)-picking strategy (Rideout et al., 2017) was applied using uclust (Edgar, 2010) with a cutoff of 97% sequence similarity; the representative sequences were aligned and taxonomy was assigned using PyNast (Caporaso et al., 2010) against the Greengenes database V13_8 (De Santis, 2006) witha 90% confidence threshold. The data were low-abundance filtered by removal of the OTUs with a relative abundance ≤0.005% across all samples (Bokulich, 2013) and chimera checked using the Blast fragments approach (Altschul et al., 1990). An outlier sample (Control group, Time point 1) was excluded from additional analyses.

Biostatistics regarding Alpha Diversity, Beta Diversity and Taxonomic Composition were carried out in R v3.3.2 using the phyloSeq (McMurdie, 2013), Vegan (Dixon, 2003) and mixOmics (Lê Cao et al., 2016) packages. The effect of the timepoint (14th d or 28th d of sampling) and of the type of diet were tested using ANOVA for alpha diversity indices ("aov" procedure) and with PERMANOVA for beta diversity ("adonis" procedure). The differences in taxonomic composition abundances between the experimental groups were tested using STAMP software (Parks et al., 2014) with the non-parametric Kruskal-Wallis test, followed by Welch's post hoc test; the p-values were corrected using the Benjamini-Hochberg method.

For the sPLS-DA (sparse Partial Least Squares Discriminant Analysis), Cumulative Sum Scaling normalisation (CSS) (Paulson et al., 2013) was applied to the genus aggregated data.

3. Results and discussion

3.1. General consideration regarding health.

All the piglets were healthy when they arrived at the experimental facility immediately after weaning (day 0). From day 4, diarrhoea appeared in 10 control pigs and in 9 xylanase-supplemented pigs, initially as mild and then more watery. Three pigs (two control and one xylanase supplemented) were excluded from the trial on day 7, for severe health impairment presumably due to an ETEC infection. At the same time, 4 faecal samples were taken from the other pigs with diarrhoea (two control and two xylanase supplemented), delivered to the local health Regional control service (IZSLER), and the presence of ETEC F18 was confirmed by plate count and by polymerase chain reaction (PCR) for fimbriae and toxins (Supplementary fig.1). Two days later, on a sample of bristle bulbs, all pigs were also confirmed to be genetically susceptible to this enteric pathogen, based on the polymorphism of the fucosyltransferase 1 (FUT1) gene (Coddens et al., 2007) (Supplementary fig.2). The possibility of the development of diarrhoea related to colibacillosis was implicit in the programmed choice of pigs genetically susceptible to ETEC F4. This was hypothesised to test the supplementation of xylanase under conditions representing those severe situations which are frequently present on farms. In reality, the cause of the diarrhoea was ETEC F18 instead of ETEC F4, but these are the two enteric pathogens

most frequently isolated from weaned pigs and associated with post-weaning diarrhoea syndrome (PWDS) (Luppi, 2017); thus, what was observed was, in some way, among the possibilities associated with the experimental design.

The all the piglets were then treated with Enrofloxacin for three days, starting from day 8. Enrofloxacin efficacy against ETEC F18 isolates on the farm of origin of the pigs in the present study was confirmed by an antimicrobial susceptibility test carried out by IZSLER. Nevertheless, the effects of the infection lasted for additional days; several other pigs had a faecal score greater than 3, and 5 others were excluded from the trial and suppressed for severe health impairment during the following ten days to prevent suffering. Thus, a total of 4 pigs in each group were out of the trial.

3.2. Growth performance.

Growth performance is reported in Table 2. In general, growth was not affected either by the diet or faecal score. The trial was not specifically designed to test growth performance. The absence of the effect of xylanase supplementation had also been observed in recent trials on piglets where xylanase had been added to a diet mainly based on corn (Li et al., 2018) or corn plus corn distiller by-products (Tsai et al., 2017). One could hypothesise that the absence of an effect on growth from xylanase supplementation may have also been due to the presence of xylanase inhibitors as the cultivars of cereals (Elliott et al., 2003; Gebreurs et al., 2010) can counter the effect of xylanase addition, particularly for barley (Goesaert et al., 2001), and can thus affect diet digestibility. The degree of anti-xylanase activity of the barley used in this study was not checked; nevertheless, the inhibitor described in barley is similar to that described in wheat, which was also found to mainly inhibit fungal xylanases (Flatman et al., 2002). However, Goesaert et al. (2004) found that the xylanase from Bacillus subtilis, as was used here, was less inhibited than the fungal xylanases they used in their comparative trials. In growing pigs fed barley diets with either high and low xylanase inhibition, fungal xylanase increased the apparent ileal digestibility of the dry matter but not that of the organic matter of the diet (Nørgaard et al., 2019). Conversely, total tract digestibility was not improved by fungal xylanase regardless of the barley varieties (Parks et al., 2014). Overall, the absence of the effect of xylanase in the present trial was not consistently supported by the hypothesis of a specific inhibition of xylanase activity on barley

in the diet. In a corn plus corn distiller by-product diet, the addition of xylanase improved faecal digestibility (Cho et al., 2016; Tsai et al., 2017) and AX digestibility (Tiwari et al., 2018). Conversely, the results for faecal or ileal digestibility in a corn-based diet were inconsistent (Passos et al., 2015; Abelilla et al., 2019). An effect of the nutritive quality of the corn varieties can be excluded because, in a dedicated study (Petry et al., 2019), improved digestibility was observed when xylanase was added to diets based on corn of either higher or lower digestibility. In corn, the presence of xylanase inhibitors has not been proven (Elliott et al., 2003; Gebreurs et al., 2010); therefore, a more stable consistent efficacy of xylanase should thus be expected. However, when an improvement in digestibility was observed with the addition of xylanase, this was often not associated with improved growth. Overall, it seems that more knowledge is required regarding the effect of xylanase on the digestibility of mixed diets based on corn and barley.

Furthermore, in the present trial, the impaired health status could have affected the animal growth response. However, one can presume that it was affected in a similar way for both the control and the supplemented animals as indicated by the similar faecal scores, by the comparable need for antibiotic treatment and by the same mortality rate.

No effect of the diet was seen for the levels of reactive oxygen metabolites in blood at the two timepoints (Table 3). The absence of an effect can be associated with the overall similar health conditions of the pigs in the two groups. The addition of xylanase to the diet also did not change the values of malondialdehyde, an indicator of the degree of lipid peroxidation which occurs in vivo, when tested in the blood plasma and in the small intestinal mucosa of weaning pigs fed a corn-DDGS diet (Tiwari et al., 2018).

A marginal increase in villus length in the jejunal mucosa was observed with xylanase supplementation (+14.5%, P= 0.066). With xylanase treatment, there was a numerical increase in the absorptive mucosal surface calculated (+14.5%, P= 0.109). No effect was seen for the other morphometric measurements. In broilers fed wheat-based diets, xylanase supplementation increased villus height in the ileum when given alone (Wu et al., 2004b) or, when fed in combination with microbial phytase (Wu et al., 2004a), also in the duodenum, jejunum (but only at 1000 units/kg) and ileum (Luo et al., 2009). Conversely,

Yang et al. (2008) and Amerah et al. (2008) did not see any effect on the morphology of the intestinal villi. With a rye-based diet, broilers had higher villi when supplemented with xylanase coupled with β-glucanase (Mathlouthi et al., 2002). Apparently, when observed, the favourable effect of xylanase on wheat-based diets was associated with a reduction in viscosity (Wu et al., 2004a). The differences seen in broiler trials may have depended on their age, on the origin of the xylanase or on the cereal particle size (Amerah et al., 2008). In growing pigs fed a diet containing corn as a cereal base, no effect on the intestinal morphology was observed (Mathlouthi et al., 2002). This was explained by digesta viscosity rather than by wheat or rye due to the reduced presence of soluble AXs in the corn cultivar. In the present trial, it was possible that the improvement in the villus morphology was also induced by the mild variations seen in the gut microbiota. Variations in the intestinal bacteria profile in the small and large intestine were seen by the addition of one xylanase together with a second xylanase, blended with cellulase and β-glucanase, to the diet which were associated with the upregulation of occludin and claudin 3 genes, markers of improved villus barrier integrity, in colon mucosa (Li et al., 2019). However, this effect on the intestinal mucosa was not seen with the addition of the xylanase alone (Li et al., 2019). In broiler chickens fed a corn-based diet, the favourable effect of xylanase on the morphometry of the jejunum mucosa was also associated with an increase in Clostridium cluster IV microbes (Kadhem et al., 2016) which produce butyrate and promote enterocyte growth (Rivière et al., 2016). However, these bacteria are largely restricted to the large intestine (Rivière et al., 2016) and, in the present trial, no effect on the prevalence of these bacteria was seen.

3.3. Microbiota results

From the 45 samples analysed (Control Group T1=10; Control Group T2=11; Xylanase Group T1=12; Xylanase Group T2=12), a total of 1,687,216 quality checked reads were obtained which returned 1367 different OTUs in total, distributed among the samples as shown in Supplementary Table 2.

3.3.1 Alpha and Beta diversity

The OTUs distribution in the experimental groups was fairly homogeneous and the Shannon and InvSimpson index values (Figure 1a) did not show significant differences regarding treatment, time point or their interaction.

For the Beta diversity (Bray-Curtis distance) there was a significant effect of the time point (P= 0.003), and two clusters are visible in the Non-Metric Multidimensional Scaling (NMDS) plot of the Bray-Curtis distance matrix (Figure 1b).

Considering the effect of the treatments at the two different time points, no clear clustering and no significant differences were observed regarding the treatment at either time point (Figures 1c, 1d).

3.3.2. Taxonomic composition

The dominant Phyla were Bacteroidetes (52%) and Firmicutes (43%), while six other Phyla were identified, the majority of which were, on average, scarcely abundant (<1%). The distribution of phyla between the experimental groups was quite homogeneous, and no significant differences were found between the different timepoints and treatments (Figure 2). However, for the xylanase treated group at the timepoint I, the Firmicutes were more abundant (50%) than the Bacteroidetes (46%). These two Phyla are normally dominant in the large intestine and faecal content of growing pigs, both including several fibre-fermenting species. However, it has been frequently seen that the ratio between the abundances of these Phyla in the gut content can change according to the fibrous substrate (Mu et al., 2014; Ferrario et al., 2017; Maier et al., 2017). Thus, in the present trial, it could mean that the presence of the xylanase induced a mild change in the substrate availability for different bacterial strains.

At the genus level of taxonomic classification, 53 different genera were assigned and 25 were unassigned (Supplementary Table 3); the dominant genus was Prevotella (average abundance 39.5%) followed by an Unassigned genus belonging to the Ruminococcaceae family (average abundance 12.0%). Prevotella and Ruminococcus genera were identified as the dominant markers of two different bacterial clusters identified in the pig microbiota (Ramayo-Caldas et al., 2016), suggesting the presence of characteristic enterotypes in this species.

The sPLS-DA enabled the selection of the most discriminative features in the data which helped to classify the samples (Lê Cao et al., 2011). The Authors used this technique to reduce the background noise and to try to identify the bacterial genera which could be associated with the dietary supplementation (Figure 3). The sPLS-DA identified a greater distinction between treatments at time point 2, but the high error rate (~50%) of the model did not allow evaluating the results regarding the discriminating genera to be reliable. The genera abundance was therefore tested using the Kruskal-Wallis test, and none of the genera showed significant differences regarding treatment effect.

Normally, the genus level is the maximum taxonomic depth which can be reached by 16S profiling; however, it is possible to classify some reads up to the species level. Analysing the taxonomic level of species, it was seen that *Lactobacillus reuteri* remained longer after weaning in the experimental group treated with xylanase (Figure 4). The Authors therefore speculated that xylose released by the action of xylanase may have favoured the presence of *Lactobacillus reuteri* which is capable of using xylose (Tannock, 2004; Staudigl et al., 2012). This species is one of the most studied having the aim of providing humans and domestic animals with a probiotic effect. In pigs, several strains were tested with several positive effects on gut health as reviewed by Hou et al. (2015). A favourable effect on the growth of lactic acid bacteria by the dietary addition of AXOS has already been observed in broilers (Courtin et al., 2009). Furthermore, the growth of a strain of another *Lactobacillus* (*L. plantarum*) was increased when the diet was supplemented with xylanase (Vandeplas et al., 2009). Research data regarding the impact of the addition of xylanase to pig diets on the gut microbiota are scarce. Furthermore, the few studies carried out supplemented xylanase together with other carbohydrases, and the study of the gut microbiota was limited to the counts of a few genera (Kiarie et al., 2007; Zhang et al., 2014). Contrary to the present observation regarding the abundance of L. reuteri, the addition of xylanase to a corn-based porcine diet decreased the relative abundance of lactobacilli at the genus level, both in the ileum and caecum content, but not in the colon (Li et al., 2019). The different response may have been related to the presence of different AXs in the diet in the present study which also contained 25% barley, or to the fungal origin of the different xylanase used in the trial of Li et al. (Li et al., 2019). In broilers, xylanase supplementation to a barley-based diet did not affect the caecum content of the Lactobacilli (but decreased the relative amount of Enterobacteriaceae) (Jozefiak et al., 2010). The

infection and the antibiotic treatment would have had a significant impact on the intestinal microbiome, although the illness and the treatment were common to both dietary groups. In mice and humans, ciprofloxacin has been shown to have a minimal impact on intestinal anaerobes, related to their resistance to this antibiotic and to the binding of these bacteria to faecal matter (Donskey et al., 2004). However, ciprofloxacin partially upset the colonisation dynamics of the commensal microbiota defined in a gnotobiotic pig model (Huang et al., 2018). One could argue that, in this case, the antibiotic treatment was short, and it was observed that the effect of antibiotics on the swine gut microbiota was variable based on duration and that the swine gut microbiota exhibited considerable resilience (Holman and Chénier, 2014). Finally, the present data mimicked what happened on the farm after the necessary antibiotic treatment of the individual pigs. Nevertheless, the trial demonstrated that the addition of xylanase did not favour the development of a gut microbiota able to contrast the infection of ETEC F18. In chickens, the detrimental effect on the growth of experimental infection with Salmonella Typhimurium at 3 days of age was reduced by the combined supplementation of L. plantarum and xylanase when the infection dose was 105 colony forming units (CFU) per chick, but not at 108 (Vandeplas, et al., 2009). These microbiological observations suggested that specific bacterial communities could have demonstrated competitive inhibition against the pathogen when the infection dose was limited. However, no other specifically targeted research has been reported in pigs regarding the efficacy of xylanase during infection with any enteropathogenic agent.

4. Conclusion

The xylanase supplementation of a corn/barley-based diet favoured the persistence of xylose-fermenting *Lactobacillus reuteri* which is considered beneficial. This may explain the mild marginally significant increase in villous length. Supplementation with xylanase did not improve growth and robustness against pathogenic E. coli, but its effect on some beneficial bacterial species merits additional study.

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Tables

Table 1. Composition of the basic diet.

Items	% as fed
Ingredients	
Corn	27.00
Barley	25.10
Biscuit by-product	20.00
Rice protein concentrate	4.20
Dried milk whey	4.00
Wheat middlings	4.00
Soybean meal 50 % CP	3.00
Wheat gluten	2.50
Soy oil	3.50
Brewer's yeast	2.00
Potato, protein concentrate	1.50
Dicalcium phosphate	1.00
Carob pulp	1.00
Miner-vitamin premix[1]	0.50
Calcium carbonate	0.50
Salt	0.20

Calculated chemical composition

Crude protein	16.50
Crude fat	5.80
Crude fiber	3.12
Ash	4.22
Ca	0.60
P, total	0.50
NaCl	0.38
Lysine	1.20
Methionine +Cysteine	0.70
Tryptophan	0.22
Threonine	0.74
Valine	0.79
ME[2] pigs (Kcal/kg)	3144.00

[1]Provided the following per kg of diet: vitamin A (retinyl acetate): 7,500 IU; vitamin D3 (cholecalciferol): 1,000 IU; vitamin E (DL-α_tocopheryl acetate): 80 mg; vitamin K (menadione sodium bisulfite): 1 mg; riboflavin, 2.3 mg; calcium-D-pantothenate: 9.0 mg; niacin: 17.5 mg; pyridoxine hydrochloride: 1 mg; folic acid: 0.5 mg; biotin: 0.10 mg; thiamine: 1.45 mg; vitamin B12: 15 µg; ferrous sulphate monohydrate: 302.8 mg; zinc oxide: 118.2 mg; manganous sulphate monohydrate: 18.48; copper sulphate: 347.6; sodium selenite: 0.55 mg; potassium iodide: 1.31 mg.

[2]ME: Metabolizable energy. The values were estimated using EvaPig® software (Noblet et al., 2008) with information from the INRA-AFZ tables of feedstuff composition.

Table 2. The effect of the addition of xylanase to the diet on growth performance.

	Control	**Xylanase**	**SEM**	**P**
Starting live weight, kg	5.46	5.46	0.31	0.994
At week 1[1]				
-live weight, kg	5.26	5.12	0.37	0.785
-daily live weight gain, g	-57.8	-60.1	15.9	0.918
-feed intake, g/day	86.7	99.3	9.7	0.362
-gain/feed ratio	-1.14	-0.77	0.29	0.362
-faecal score	3.45	3.52	0.22	0.820
At week 2[2]				
-live weight, kg	6.46	6.81	0.53	0.649
-daily live weight gain[3], g	54.6	58.0	24.0	0.890
-feed intake[3], g/day	162.3	185.1	19.8	0.431
-gain/feed ratio[3]	0.34	0.31	0.23	0.330
-faecal score	2.30	2.15	0.18	0.579
At week 3[2]				
-live weight, g	9.192	9.38	0.82	0.875
-daily live weight gain[3], g	166.6	161.0	27.4	0.888
-feed intake[3], g/day	278.9	307.5	27.5	0.477
-gain/feed ratio[3]	0.50	0.50	0.07	0.942
At week 4[2]				
-live weight, kg	12.34	12.51	1.17	0.921
-overall daily live weight gain[3], g	237.3	232.7	33.1	0.922
-overall feed intake[3], g/day	373.6	398.0	38.8	0.666
-overall gain/feed ratio[3]	0.59	0.57	0.04	0.713

[1] On 14 pigs in the Control Group and15 pigs in the Xylanase Group. [2] On 12 pigs in the Control Group and 12 pigs in the Xylanase group. [3]From the starting day.

Figures

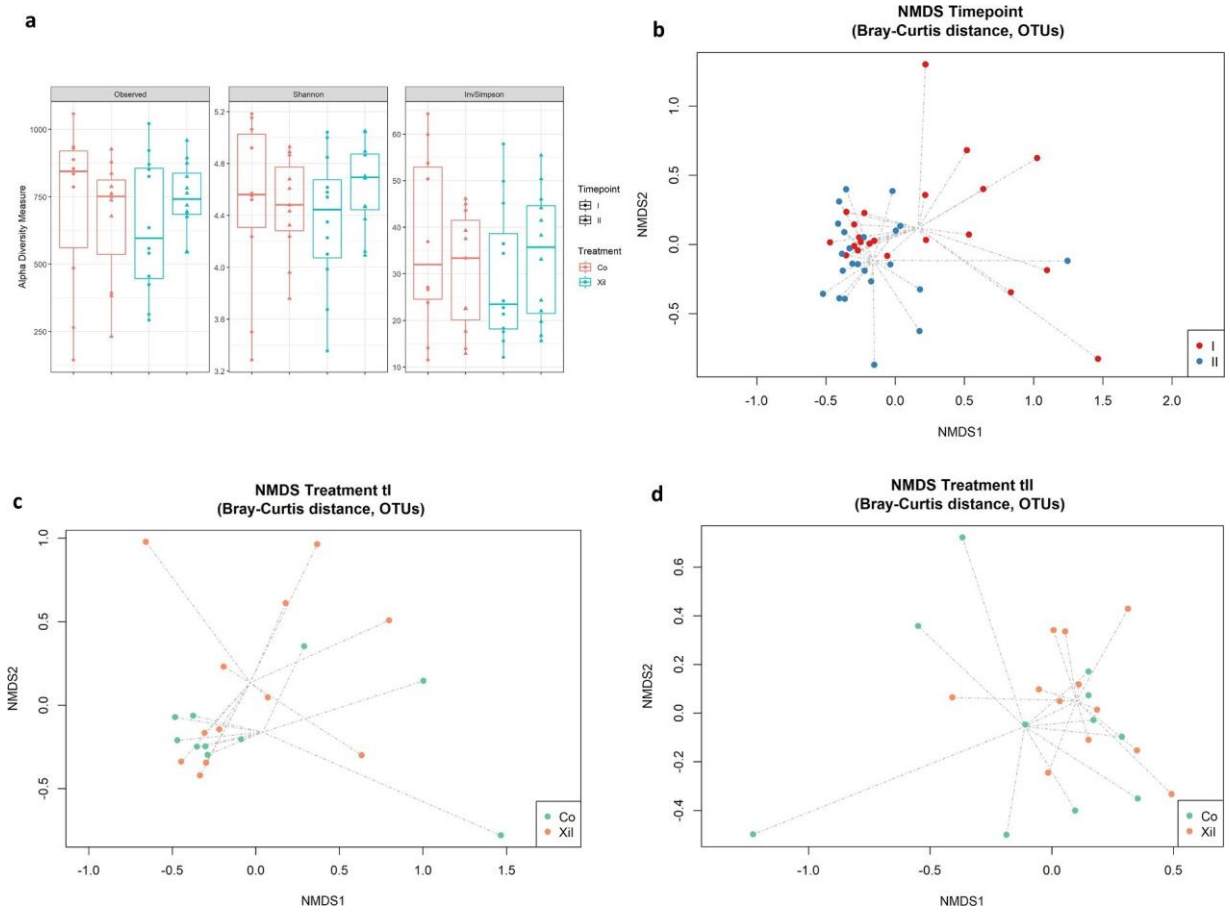

Figure 1. a) Boxplot of observed operational taxonomic unit (OUT) abundances (Observed) and of Alpha diversity indices (Shannon and InvSimpson) values. Co= control; xil= xylanase treatment; 1= day 14 post-weaning; 2= day 28 post-weaning. B) Non-Metric Multidimensional Scaling (NMDS) plot on a Bray-Curtis distance matrix. 1= day 14 post-weaning; 2= day 28 post-weaning. C) Non-Metric Multidimensional Scaling (NMDS) plot on a Bray-Curtis distance matrix at day 14 post weaning d) Non-Metric Multidimensional Scaling (NMDS) plot on a Bray-Curtis distance matrix at day 28 post weaning; co: control; xil: xylanase treatment.

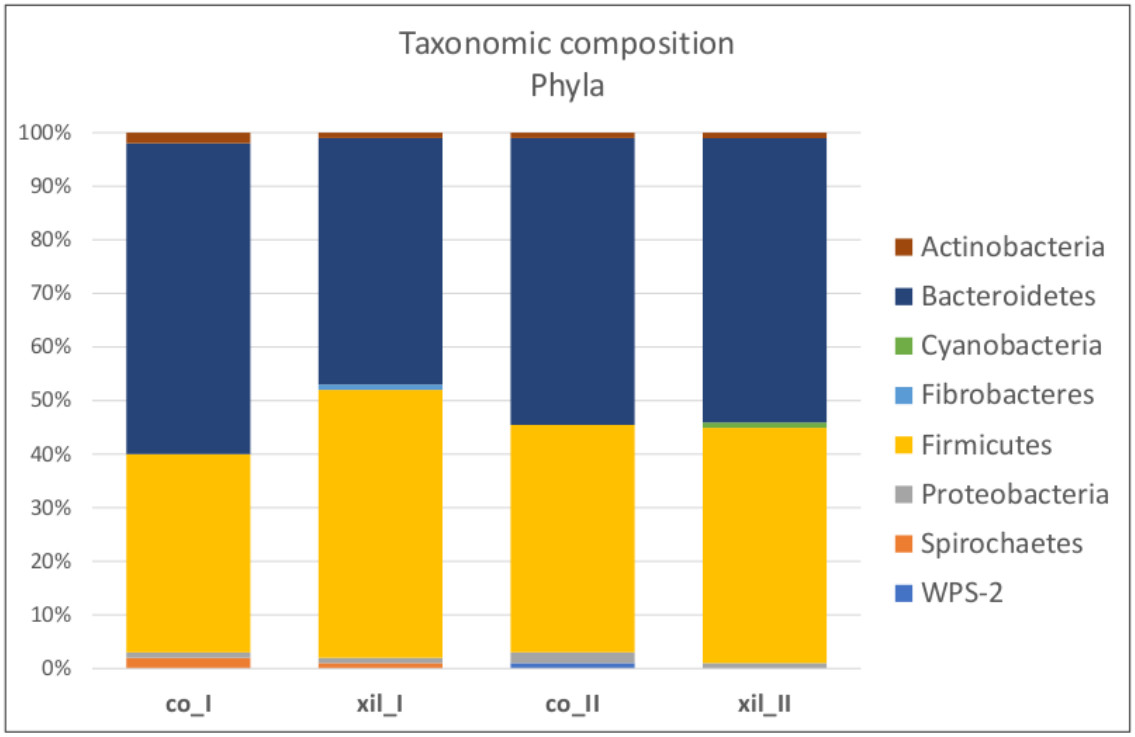

Figure 2. Phyla abundances per treatment and time point co: control; xil: xylanase treatment; 1= day 14 post-weaning; 2= day 28 post-weaning.

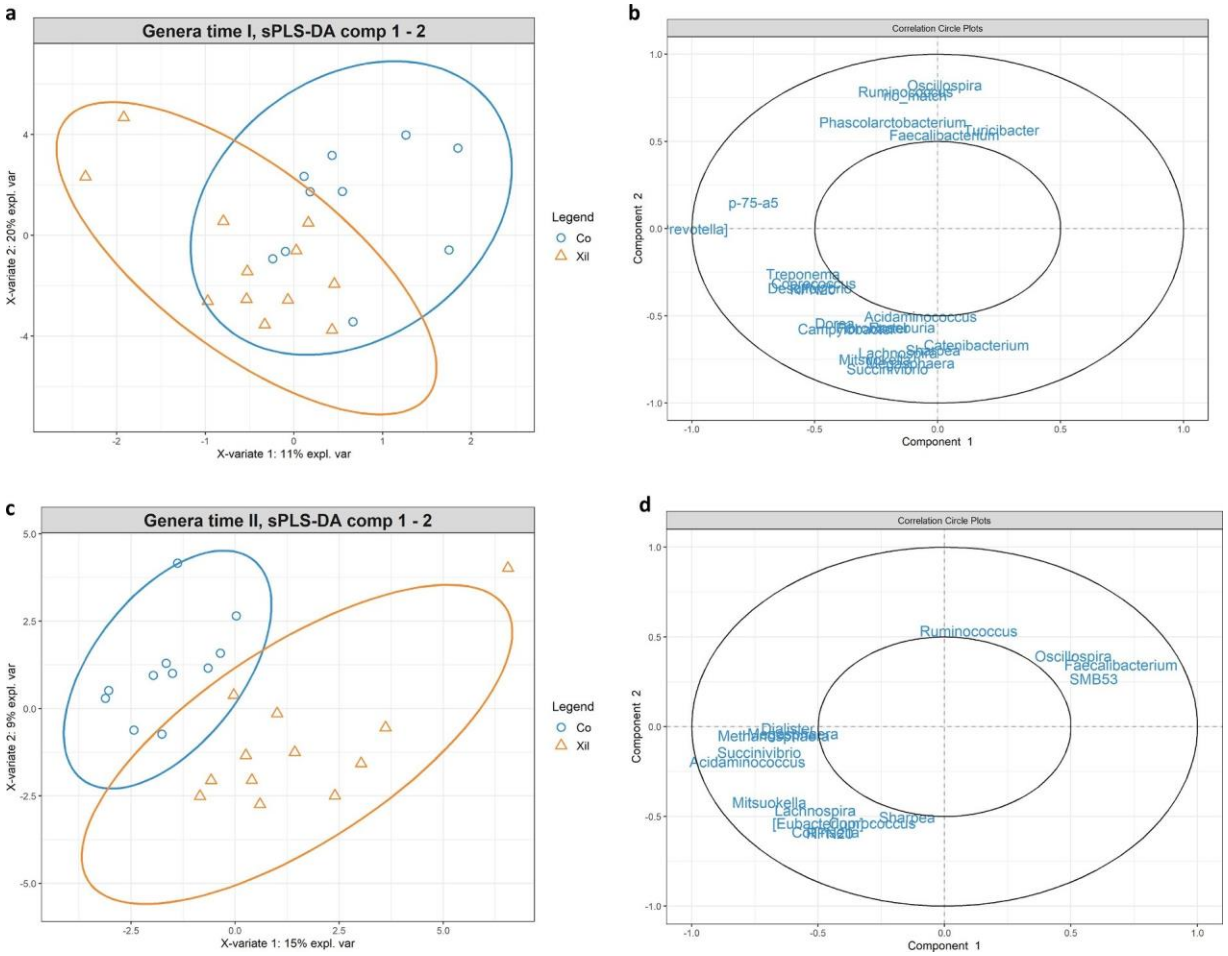

Figure 3. sPLS-DA (sparse Partial Least Squares Discriminant Analysis) on genera aggregated data at time point 1 (a, b) and time point 2 (c, d). a; c) Scatter plot of the samples along the first two components. b; d) Correlation circle plot: the discriminating variables are located in the radius between the ellipses. List of the genera in the plot: *Acidaminococcus*, *Campylobacter*, *Catenibacterium*, *Coprococcus*, *Desulfovibrio*, *Dorea*, *Faecalibacterium*, *Fibrobacter*, *Lachnospira*, *Megasphaera*, *Mitsuokella*, *no_match*, *Oscillospira*, *p-75-a5*, *Phascolarctobacterium*, *RFN20*, *Roseburia*, *Ruminococcus*, *Sharpea*, *Succinivibrio*, *Treponema*, *Turicibacter*. Co: control; xil: xylanase treatment.

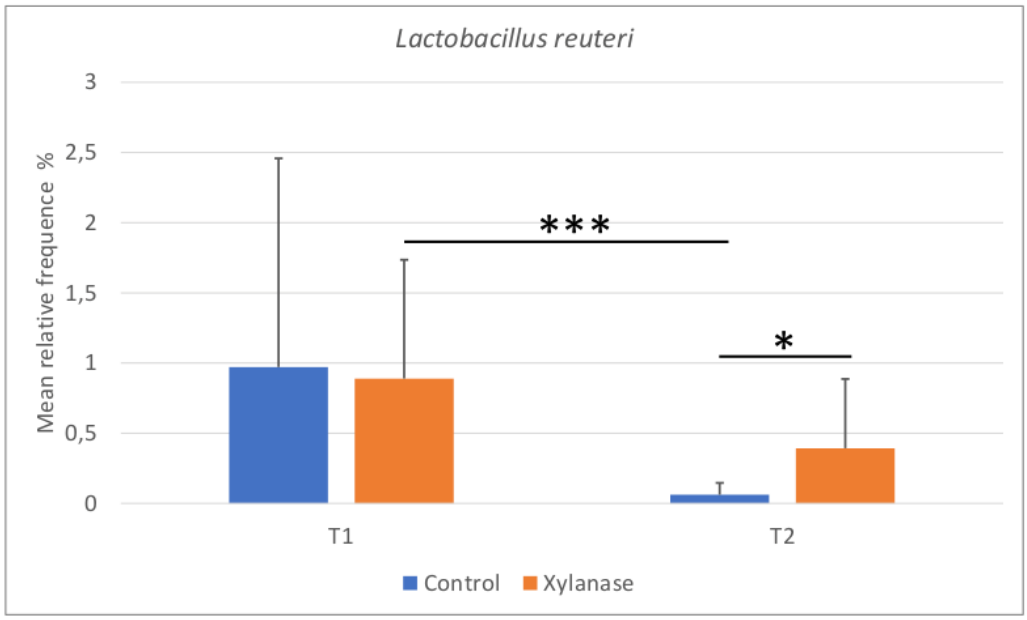

Figure 4. Abundance dynamics of *Lactobacillus reuteri*. T1: day 14 post-weaning; T2: day 28 post-weaning. The differences in relative abundances were tested using the non-parametric Kruskal-Wallis test followed by Welch's post hoc test; the p-values were corrected using the Benjamini-Hochberg method.

Supplementary Materials:

The following are available online at https://doi.org/10.1016/j.livsci.2017.10.026. **Supplementary Table 1:** Litter of origin and sex of the pigs in the two dietary groups (Co = Control; Xil = Xylanase). **Supplementary Table 2**: Per sample information about sequencing depth (reads abundances), OTUs abundances and alpha diversity indices (Shannon and InvSimpson) co= control; xil= xylanase treatment; I= day 14 post-weaning; II= day 28 post-weaning. **Supplementary Table 3**: Genera relative abundances. The data aggregated at genus level were reported for the four experimental groups (co= control; xil= xylanase treatment; I= day 14 post-weaning; II= day 28 post-weaning). The square brackets indicate taxonomic assignment based on genome trees (not verified) in GreenGenes annotation. **Supplementary Figure 1:** PCR results of the genotyping for the isolated F18 ETEC strain (F18 fimbria; Sta and Stb toxins) in the 4 faecal samples taken from two control and two xylanase supplemented pigs with diarrhoea. **Supplementary Figure 2:** PCR results of the genotyping for FUT1 marker on a subset of the subject in the trial (GG is the homozygous ETEC F18 susceptible, AG is the heterozygous ETEC F18 susceptible).

Preliminary Results

Effect of the growth of suckling pigs on the gut microbiota and on the pattern of muscular fiber types

1. Introduction

The gut microbiota is composed by an immense number of microorganisms that colonize the gastro-intestinal tracts of mammals. This symbiotic relationship between microorganisms and host has been linked to several process, most of them rely on the ability to process and utilize components of the diet that are indigestible for the host, promoting host cell differentiation, protecting the host from the colonization of pathogens, and stimulating/modulating the immune system. Hosted microorganisms produce several metabolites that are absorbed and released in the blood stream and participate to several physiological and biochemical reactions at different site, including hormone secretion and nervous system signalling (Festi et al., 2014; Ochoa-Repáraz and Kasper, 2016). The interaction between gut microbiota and host organ systems has been recently an important object of research. However, few studies have been focused on the possible interaction of the microbiota with skeletal muscle development. The possibility that gut microbiota can affect also muscle fibers characteristics is suggested by the observation that the porcine gut microbiota transplanted to germ-free (GF) mice reproduced the myocellular phenotype of the donor (Yan et al., 2016). In addition, muscle atrophy, decreased expression of insulin-like growth factor (IGF), and decreased expression of genes associated to skeletal muscle development and mitochondrial function were reported in GF mice (Sirisinha, 2016). In piglets, Qi et al. (2021) showed that the removal of the gut microbiota leads to muscle loss and muscle atrophy and changes the composition percentage of muscle fiber type. Subsequently, the faecal transplant from healthy piglets partly restored the growth and function of muscle tissue. In piglets, there are cases when subjects with equal birth weight have substantial variation in their growth rates already starting from the suckling period. At this stage, growth rates are closely related to the muscle development that is linked to muscle fibers development. Variations in postnatal development of muscle fibers depends on the primary fiber number and on the ratio of secondary to primary fibers as a lower secondary-to-primary fiber ratio is associated with reduced growth rate (Handel and Stickland, 1987). Most of the research on this topic has been focused on the genetic and epigenetic factors (Estellé et al., 2008; te Pas et al., 1999; Zhao et al., 2011) but scarce are data on the possible link between microbiota composition and muscles fiber development.

This study was designed to investigate the possible connection between gut microbiota and the muscles development in slow and fast-growing piglets during the suckling period.

2. Materials & Methods

A total of 16 multiparous sows (Swiss Large White) were selected after farrowing for the trial. For each litter, two piglets, with a similar body weight at birth, but differing for the average daily gain from birth to 17 days of life, for 96.76 ± 29 g/day were selected.

The day before weaning (25 days of life), piglets were slaughtered (mean BW for fast growing 8.37 ±1.17 and 6.00 ±1.21 for slow growing) and a muscles sample from the semi-tendinous muscles was collected and secured to a labelled flatstick, rolled in talcum powder, and frozen in liquid nitrogen for subsequent analysis. In addition, immediately after slaughter, Ileum contents were collected a in sterile tubes and immediately frozen in liquid nitrogen and stored at -80. Slaughter and dissection procedures were carried out according to the Swiss Pig Performance Testing Station (MLP, Sempach, Switzerland)

For fiber determination, frozen muscle samples were equilibrated to −20°C, and then a section of tissue was cut from the stick and trimmed to facilitate transverse sectioning. Samples were mounted on a cryostat chuck, and sections (10 µm in thickness) were cut using a Cryotome (Shandon, Inc., Pittsburgh, PA) and subsequently mounted on glass microscopic slides. Sections were then treated with the combination succinic dehydrogenase and acid myofibrillar ATPase staining procedure (Solomon and Dunn, 1988). Stained sections were observed at 10× with a Olympus BX50 microscope (Olympus Optical Co., Hamburg, Germany) equipped with a high-resolution charge-coupled device digital camera (ColorView12, Soft Imaging System GmbH, Munster, Germany). Muscle fibers were classified as Type 1 (slow oxidative) and Type 2 (fast glycolytic) based on the stain reaction. The fibers showing the darker staining intensity were classified of Type 1 and those with the lightest, of Type 2. Two slides per muscle samples were assessed, and two random fields at different locations within a slide of each muscle sample were captured as TIFF files. Images were analyzed using Fiji ver 1.8 (Schindelin et al., 2012) and cellpose (Stringer et al., 2021), a mean of 815 muscle fibers were analyzed per sample. The statistical analysis on the muscles fiber, considering number of Type 1, Type 2, Type1:Type2 ratio, mean area of Type 1 and Type 2, was carried out in R using the lme4 package (Bates et al., 2015) and

fitting a linear mixed model considering Group (Fast or Slow growing) as factor and the suckling mother as a random effect.

For the microbiota analysis, bacterial DNA extraction from the ileum content was carried out using HostZERO Microbial DNA Kit (Zymo Research, California, USA) following the manufacturer instruction. DNA concentration and purity (absorbance ratio 260/280 and 260/230) of the isolated DNA were checked by spectrophotometry on the NanoDrop (Fisher Scientific, 13 Schwerte, Germany). The V3-V4 region of the 16S rRNA gene (~460 bp) was amplified, amplicons were produced using the universal primers Pro341F: 5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG-3' and Pro805R: 5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC-3' using the Platinum™ Taq DNA Polymerase High Fidelity (Termo Fisher Scientific, Italy) and sequenced using the Illumina MisSeq platform 300x2bp. The libraries were prepared using the standard protocol for MiSeq Reagent Kit V3 and sequenced on MiSeq platform (Illumina Inc., San Diego, Ca, USA). For the bioinformatics analysis, the DADA2 pipeline (Callahan et al., 2016) was used considering the Silva database (Quast et al., 2013) (version 138) as reference for the taxonomic assignment. In addition, 2 blank samples were included during the extraction and sequencing procedures in order to identify and remove any contaminat sequence as described by Davis et al. (2018)

The statistical analysis on Alpha diversity, Beta diversity and taxonomic composition was carried out with R v3.6. Data were normalized using the variance stabilizing transformation function provided by the DESeq2 package (Love et al., 2014). Bacterial richness was estimated using the R package breakaway v4.7.5(Willis and Bunge, 2015). For the Alpha diversity an ecological network regression models to estimate Shannon and Simpson diversity with the R package DivNet was used (Willis and Martin, 2020). Differences in bacterial richness, Shannon, and Simpson diversity, between slow and fast-growing piglets, were tested using the hierarchical model Betta including subject as random factor, which accounts for incomplete community sampling and allows for multivariable adjustment (Willis et al., 2017). For the Beta diversity a dissimilarity matrix using a UnWeighted Unifranc distances was constructed, results were plotted using a NMDS plot, then a PERMANOVA test was performed to test for any correlation between community composition and growing performance, with 10,000

permutations. The differential abundance analysis was performed using the *metagenomeSeq* function (Paulson et al., 2013) implemented in the wrapper function included in the package microbiomeMarker (Yang, 2020). The correlation analysis between muscle fiber and bacterial genera abundance was performed using Pearson correlation and correcting the P-values for multiple comparing with Bonferroni correction.

3. Results and Discussion

For the muscles fiber typing a total of 25,098 (average of 784 fiber per subject) were included in the analysis. Slow growing piglets had a significant higher average number of fiber compared to fast growing (SG=878 vs FG= 753, P-value<0.05). No differences were found regarding the average number of Type 1, but a tendency for a higher number for Type 2 was observed in Slow growing compared to Fast growing piglets (SG= 628 vs FG=543, P-value=0.09). In addition, no differences were found for the Type1/Type2 ratio, the average fiber area and the mean area of Type 1 and Type 2 fibers. All the average values are reported in Table 1.

For the microbiota analysis, after a first exploratory analysis one sample from the FG piglets was classified as outlier and removed from the analysis. In total, 1,614,140 quality checked reads were included in the analysis that produced a total of 5,800 amplicon sequence variants (ASVs) after the DADA2 analysis. 93 ASVs were classified as contaminants by the decontam function leading to a total of 5,907 ASVs. In total, 14 Phyla (Firmicutes, 97 ± 0.7% abundance, and Bacteroidota, 2 ± 0.1%), 81 Families (Lactobacillaceae, 90.42 ± 9.65% abundance, and Prevotellaceae, 1.73 ± 0.87%) and 175 genera (*Lactobacillus,* 90.42 ± 9.65% abundance, and *Prevotella,* 1.15 ± 1.26%) were identified.

Estimated bacterial richness was significantly lower in slow growing (SG) piglets compared to Fast growing (FG) piglets (SG = 75.4 vs FG = 85.4, P-value <0.01). In addition, SG piglets had lower estimated Shannon diversity values compared to FG (SG = 1.86 vs FG = 1.93, P-value <0.01) . On the other hand, no significant differences were found for the estimated Simpson index (Figure 1).

For the beta diversity, the NMDS plot in Figure 2 shows no evident clustering of the samples. In addition, no differences between SG and FG pigs were found. The DA analysis showed that SG piglets were characterized by a higher abundance of an uncultured strains of the Rhodospirillaceae family ab

by *Millisia*, *Desulfovibrio*, and *Weissella*. Instead, the FG piglets were characterized by a higher abundance of *Staphylococcus* and *Holdemanella* Figure 3.

A correlation analysis between genera relative abundance and morphometric parameters of the muscles fibers to identify possible bacterial genera involved in the muscle fibers development is represented in Figure 4. Several genera were significantly positively associated with the number of Type 2 fibers. Conversely a negative important association was seen for *Lactobacillus* genus. Some positive and negative association of microbiota genus abundances were also seen for the Type 1 to Type 2 ratio and for the areas of fibers.

Pigs with slower growth may be characterized by and higher number of muscles fibers in the semi-tendinous macules, particularly for Type 2, but without effect on their ratio. These pigs also have a less variable microbiota in the small intestine, with some variation in the abundance of some genera. Without considering the pig growth fastness, the muscle fiber pattern, particularly the density of Type 1 fiber, is positively associated to several anaerobe genus, but negatively with abundance of lactobacilli. Assessing if it is a causal or undirect relationship should be a further step of study.

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Tables

Table 1. Mean values for the major morphometric parameters of the muscles fiber from the semi-tendinous muscles of Fast growing and Slow growing piglets.

Muscle fiber parameters	Fast-growing	Slow-growing	SEM	Group, P - value
Type 1 (n)	210	250	20.8	0.12
Type 2 (n)	543	628	35.7	0.09
Type 1/Type2	0.403	0.409	0.0418	0.89
Total number of fiber (n)	753	878	44.1	0.04
Mean area Type 1 (μm^2)	624	796	111	0.27
Mean area Type 2 (μm^2)	701	853	117	0.36
Mean fiber area (μm^2)	682	839	115	0.34

Figures

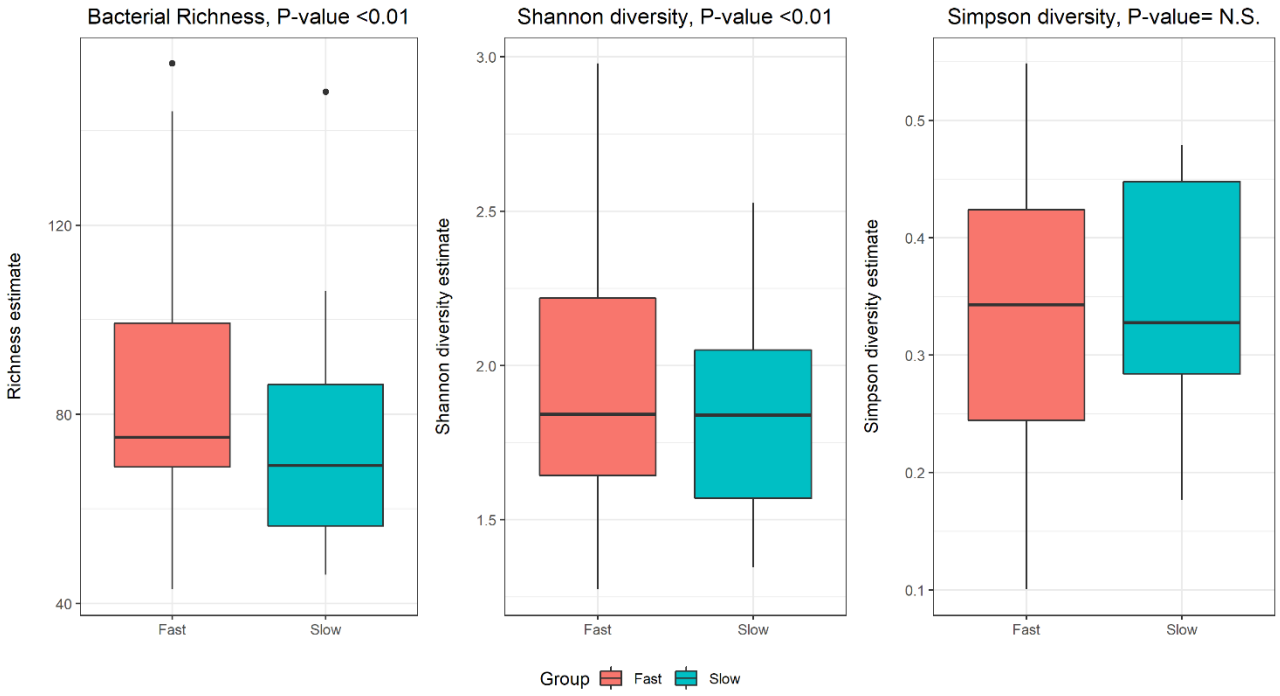

Figure 1. Boxplots showing estimated bacterial richness, Shannon, and Simpson diversity values. Fast= Fast growing piglets, Slow= Slow growing piglets

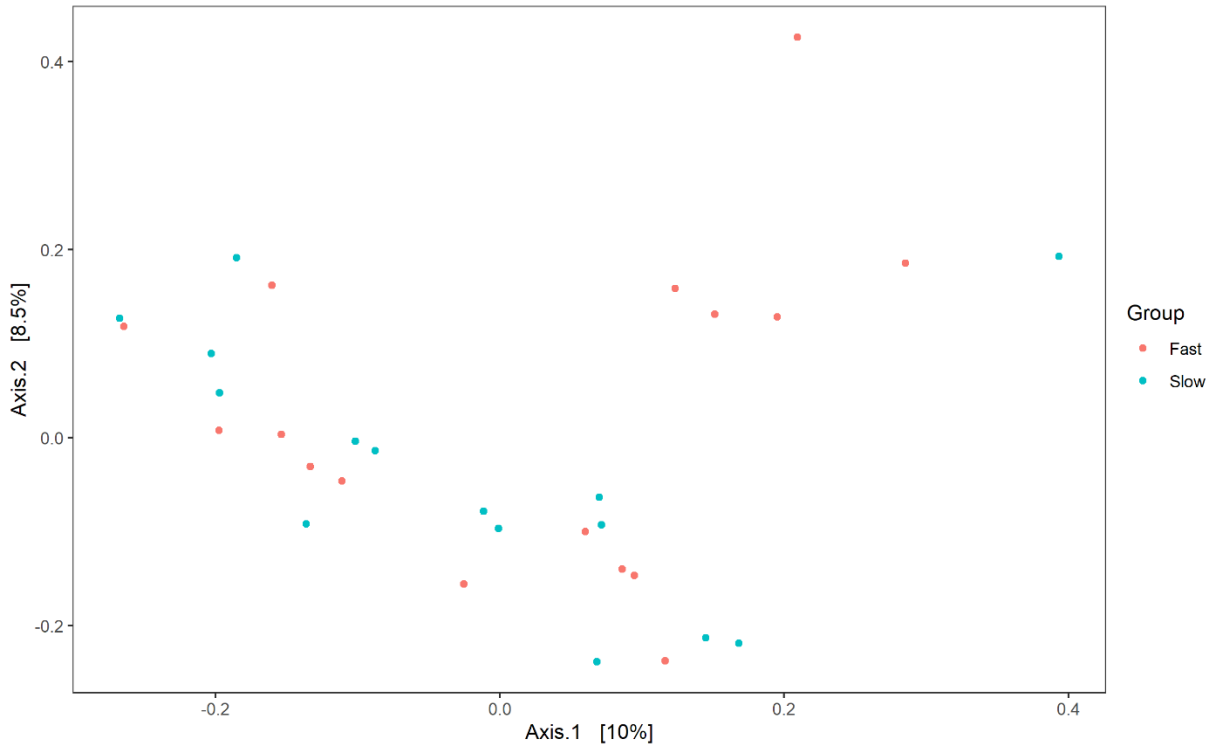

Figure 2. Non-Metric Multidimensional Scaling (NMDS) plot using UnWeighted-Unifrac distance matrix. Fast= Fast growing piglets, Slow= Slow growing piglets.

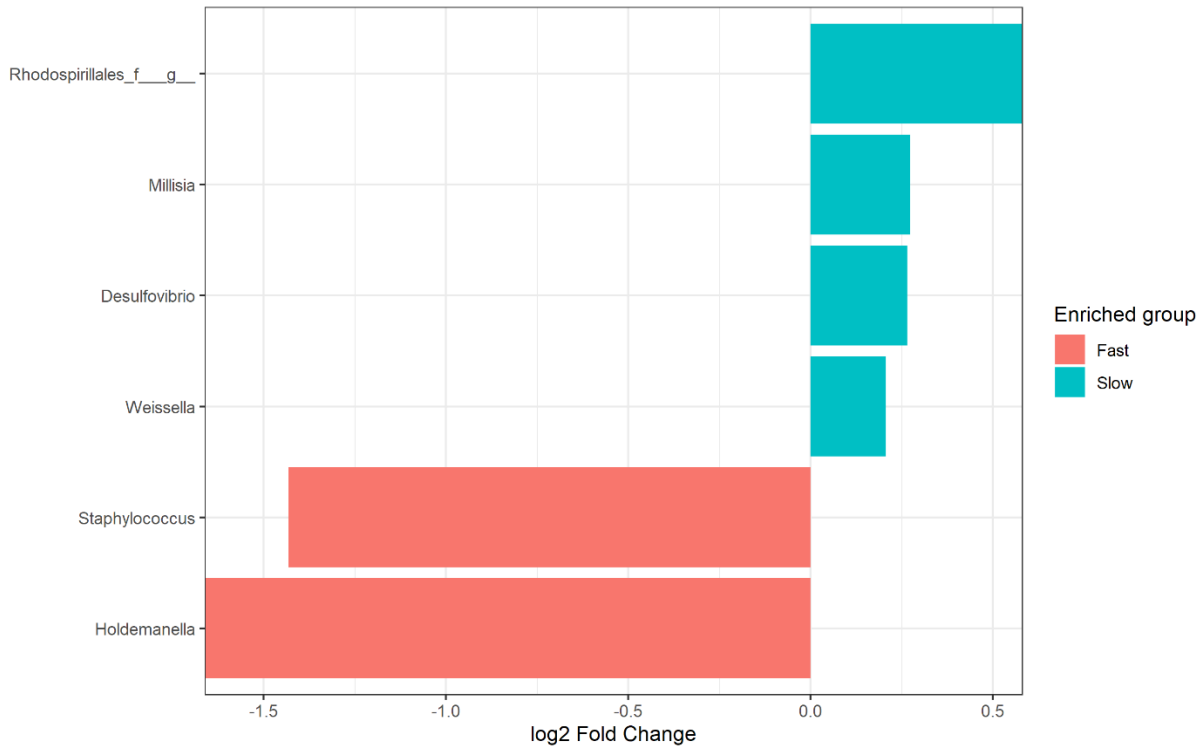

Figure 3. Bar plot showing log2fold change of bacterial Genera associated with Fast growing (Fast) or Slow growing piglets (Slow).

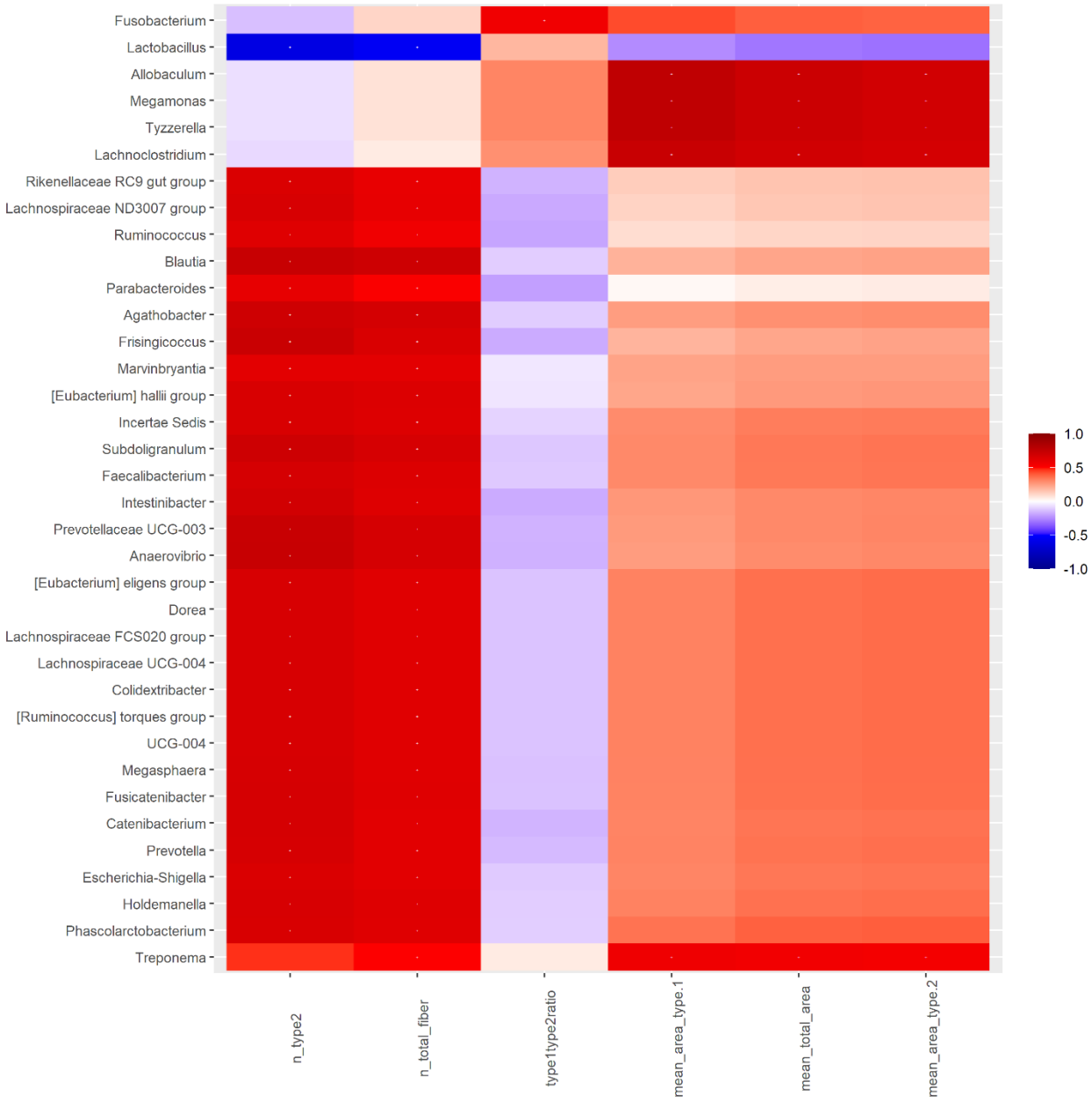

Figure 4. Heat-map showing the correlation coefficient of bacterial genera abundance and morphometric characteristic of muscles fibers.

General Discussion

In the pig industry, weaning is commonly seen as the most stressful event of a pig's life. It happens abruptly and earlier than in the wildlife (3-4 weeks versus 3-4 months) and is associated with social, environmental, and dietary changes. This stressful condition causes a transient anorexia immediately after weaning inducing structural changes of the intestinal epithelium and mucosa. In addition, the cessation of the passive immune protection derived from sow's milk and the immaturity of the immune response can cause the proliferation of pathogenic bacteria that lead to PWD . In the past years, this problem was managed by using in feed antimicrobial substances, but with the emerging global concern of antimicrobial resistance, new strategies are needed for preventing/containing colibacillosis and sustain piglet performance around weaning. Moreover, knowledge about the mechanisms related to PWD needs to be updated, by considering the role that the gut-commensal microbiota and the immune response play in its pathogenesis.

In the first paper, analysed the possible connection between the colonization of the small intestine, the development of the local immunity in the intestine of piglets and their general health. To do so, we compared the jejunal Peyer patches (JPPs) and peripheral blood (PB) transcriptomic profile before and after weaning, in association with the jejunal microbiota. According to the results, in the post-weaning, JPPs were characterized by the enrichment of several biological processes related to the activation of the interferon gamma response and to the antigen processing of MHC class I molecules. Among the weaned pigs, the subjects that clustered for the expression of 44 genes representative of these pathways were associated to the reduced microbial abundance of *Weisella* genus and *Faecalibacterium prausnitzii* in jejunal content, suggesting that the presence of this taxa apparently prevented the maximal activation of these pathways. For PB, weaning was associated to the enrichment of genes related to hemoglobin production and iron metabolism and the upregulation of erythrocytic spectrin beta (SPDB), indicating a higher presence of reticulocytes and immature erythrocytes. In line with the literature, weaning was associated with an increase in alpha diversity and a higher abundance of fibrolytic and/or short chain fatty acids producer bacterial groups, like *Ruminococcus*, *Methanobrevibacter*, *Blautia* and *Subdoligranulum* in the jejunal microbiota (L. Chen et al., 2017;

Choudhury et al., 2020; Guevarra et al., 2019). These results highlighted the immunological and physiological changes that occur with weaning and allowed as to define a set of genes that can be used as potential markers of the immune system maturity in further studies. In addition, given the higher activation of genes involved in the recognition of intracellular microorganism, further studies should consider implementing the use shotgun metagenomics, in order to investigate the possible viruses that may have been involved in the activation of this pathways.

In the second paper, we evaluated the effect of an *E. coli* bivalent vaccine and the potential influence of the host genetic susceptibility to ETEC growth performance and faecal microbial profile of piglets around weaning. Even if, bo effect of vaccination and genetic susceptibility was observed on growth performance, the vaccination modulated the faecal microbial composition, reducing the alpha diversity indices and affecting the beta diversity. Indeed, vaccinated piglets were characterized by the presence beneficial bacteria genera including *Dialister, Prevotella, Blautia, Ruminiclostridium, Parabacteroides* and *Faecalibacterium.* In addition, F4 resistant genotype showed a higher alpha diversity index than the susceptible ones pre- and postweaning. These findings showed the beneficial effect of oral live bivalent F4/F18 *E. coli* vaccine on piglets' gut homeostasis and microbiota eubiosis. Then based on the results of these studies we designed the following two experiment.

In particular, the third study was designed to evaluate the short-term effect of oral administration of nucleotides before and after weaning on growth performance, health, transcriptomic profile of JPPs and PB and microbiome of piglet ETEC susceptible and vaccinated with the same *E. coli* bivalent vaccine. A nucleotide-based product (NU) was orally given four times before weaning and once after to one group of piglets, while a second group was used as a control (CO). No differences were observed regarding the growth performance of the two groups, but the NU group had increased hemoglobin and hematocrit values 12 days post-weaning compared to CO. At weaning, feces of NU piglets had a microbial profile more typical of growing pigs, while those of CO were more representative of suckling pigs. In addition, subjects of the CO group were characterized by an upregulation of genes in PB at weaning that can be indicative of more activation towards an inflammatory response, while genes of erythropoiesis were more active in NU pigs post-weaning. This could be related to a higher

proliferative activity in the JPPs of subject supplemented with nucleotides, that possibly reduced the inflammation in the immediate pre-weaning, and increased the erythropoietic activity in the post-weaning in the PB. However, further studies are needed in order to understand which cell populations were involved in the proliferative activity observed in the JPPS, in this sense the use of flow cytometry to characterize the immune cells involved, can represent a viable strategy.

In the fourth study, we tested the use of bacterial xylanase in weaning diets based on corn and barley, in this study only subject susceptible to ETEC F4 infection were selected. Overall, the xylanase was not particular effective in influencing the growth, the fecal scores, or the reactive oxygen metabolites in the blood, only a minor effect was observed on villi length in the jejunum. For the micoribiota composition, the xylanase treatment favored the persistence of *Lactobacillus reuteri*, we therefore, speculated that xylose released by the action of xylanase may have favored the presence of *Lactobacillus reuteri* which is capable of using xylose (Staudigl et al., 2014; Tannock, 2004). This species is one of the most studied having the aim of providing humans and domestic animals with a probiotic effect. In pigs, several strains were tested with several positive effects on gut health as reviewed by Hou et al. (2015). In addition, a favorable effect on the growth of lactic acid bacteria by the dietary addition of AXOS has already been observed in broilers (Courtin et al., 2008).

In the last preliminary study based on the work carried out during my research period at Agroscope we tried to identify the possible factors that can explain differences in growth performance in piglets with a similar birth weight and coming from the same litter. First, the microbiota results showed that fast growing piglets arbor a richer and more diverse intestinal microbiota compared to slow growing piglets, and we also identified the bacteria genera that were differentially abundant between the two group. Then, the results on the muscle fibers showed that slow growing piglets had a significant higher number of muscles fibers and tended to have an average higher number of fast twitch (Type II) fiber compared to fast growing. In the last part of the analysis, we identified which bacterial genus can be correlated with the muscle's fiber development. This is an aspect that was never considered before, to our knowledge. Currently, the main hypothesis that have been developed to explain if the gut bacteria are able to affect the growth and function of host muscle tissues, are related to the possibility that

microbes can increase the secretion of hormones related to the muscle gorwth (i.e., insulin and IGF1) or by producing functional metabolic compounds (i.e., SCFAs) that act as signaling factors for muscle cells (Maltecchi et al.2020). However, further studies are needed in order to confirm if a richer and more diverse microbiota, established at birth, can increase the development of the muscle fibers or piglets that growth faster may harbor a different microbiota per se.

In the last years, research showed how the microbial gut ecosystem is essential for the pig's appropriate dietary, physiological, and immunological functioning. Any disruption in the microbial ecology allows pathogenic organisms to invade and cause illness. Management techniques in intensive swine production, such as early and abrupt weaning, preventive and subtherapeutic antibiotic usage, and food formulation, can inadvertently disrupt the microbial ecosystem, exposing piglets to illness. In this sense, feeding strategies need to be oriented to promote a stable gut microbial ecosystem. However, little is known about the composition of a healthy pig gut microbiota. Concept like microbial diversity and stability needs to be expanded into microbes that are truly beneficial and fully understood.

Overall, these studies had heterogenous results in terms of modulation of the microbiota composition. For example, the use of an attenuated *E. coli* vaccine seemed to be more effective rather than nucleotides or xylanases. This might be explained by the fact that the two vaccination strains used, have fimbriae that allow them to attach to the piglet's small intestine (Fairbrother et al. 2017, Nadeau et al., 2017). Once attached, they might proliferate and thereby provide a sequence of stimulus signals to the development of the specific immune response, but also to the maturation of the immunological and digestive systems more broadly, that can affect both the morphofunctional maturation of the gut as well as the post-weaning development of the gut microbiota. On the other hand, the limited effect observed for the other additives can be related to the dose used, indeed further studies should be focused on testing different dose levels. In addition, even if no differences in the incidence of diarrhea were found, the maturation of the microbial and the proliferation of some beneficial bacteria observed in these studies, can represent an advantage for the piglets in terms of resistance to further insults.

The results obtained in this studies evidenced how ‘omics’ technologies can be a useful tool to investigate the possible mechanism underling the effect of a specific feed supplement and the role of

the microbiota in this biological and physiological process, and also allowed us to identify specific taxa that can be linked with the intestinal eubiosis or better growth rate. For example, the beneficial effect of taxa like *Prevotella* and *Lactobacillus* already extensively present in the literature (Ramayo-Caldas et al., 2016; Tan et al., 2017) was confirmed. However, some aspects regarding the community ecology still remains controversial, for instance the reduction of alpha diversity has always been associated with intestinal imbalance and in general to an alteration of the intestinal eubiosis. This conclusion are mutated manly from the research conducted on human microbiota (Das and Nair, 2019). However, this was not the case in our experience, as indicated for instance by the reduced diversity of vaccinated pigs, still growing like the control pigs. More recent studies have shown that the concept of microbiota stability may vary depending on the microorganisms present, the different ecosystem, and the host's ability to respond to stress; therefore, these studies argue that increased microbial variability does not always represent a favorable condition for the host (Falony et al., 2018; Glasl et al., 2018). A reduction in alpha diversity can be observed also after probiotic administration, and are related to the colonization of the intestinal tract by the probiotic strains and/or modulation of the microbial ecosystem through the different mechanisms specific to individual probiotics (pH modulation, production of specific metabolites and fatty acids, production of bacteriocins, etc.) (Poulsen et al., 2018; Wang et al., 2012). In addition, a reduction of the bacterial variability has been observed in as the pigs ages but only in the small intestine and not in the large intestine were bacterial richness and diversity continue to advance with age. On the other hand, these measures of ecosystem stability can be valid in macroecology but their application in the microecology can be inappropriate. In addition, there are several limitations related to the technology itself, including sampling point, extraction kit, PCR sequencing and bioinformatic and statistical analyses, that make difficult to compare different studies. For example, bacterial species differ in how easily they are lysed and therefore how much DNA can be obtained during the extraction (Costea et al., 2017), and they also differ in the number 16S rRNA gene copies and thus how much PCR productcan be obtained per cell (Kembel et al., 2012). This led to the fact that different extraction protocols can produce 10-fold or greater differences in the measured proportion of a taxon from the same sample (Costea et al., 2017), and almost every choice in a metagenomic experiment has been implicated as contributing to bias (Sinha et al., 2017; Pollock et al.,

2018). There is, therefore, the need to find new tools that can help us identify markers of the intestinal health and to interpret their biological meaning. The reduction of costs of the deep sequencing of the whole microbiome will provide more opportunities for testing the impact on the swine microbiota, complementing the knowledge on the population composition with that on potential functional properties.

Conclusions

Overall, the studies included in the present thesis contribute to understand how weaning affects the immunological and intestinal microbiota maturation of the piglet and propose some possible nutritional strategies to attenuate its negative effects. In particular, the first study showed that weaning is associated with the activation of MHC response against class I antigens and related to the stimulation to IFN-γ and showed, for the first time, that piglets at weaning remains marked by a relevant quantity of immature blood cells. In the second study, we showed how a vaccinal strain of F4/F18 *E. coli* can affect the microbial composition of piglets, regardless of their genetic susceptibility to ETEC infection. In the third studies we evidenced how a nucleotide supplementation can favor the proliferation of jejunal Peyer patches and anticipate the maturation of the fecal microbiota. In the fourth study we reported how xylanase can favor the proliferation of *Lactobacillus reuteri*. Finally, we showed some first results on the muscles fiber development in fast and slow growing piglets and their relationship with the intestinal microbiota.

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