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UNIVERSITÀ DEGLI STUDI DI SASSARI

CORSO DI DOTTORATO IN SCIENZE AGRARIE

Indirizzo Scienze e Tecnologie Zootecniche

Ciclo XXXIII

THE USE OF DIFFERENT OLIVE OIL WASTEWATER EXTRACTS IN THE DIET OF WEANING PIGLETS

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Docente guida	prof. Gianni Battacone

ANNO ACCADEMICO 2019-2020

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CHAPTER 1

1. General introduction

The antimicrobial resistance (AMR) is the ability of microorganism to challenge antimicrobial compounds and subsequently decreasing the efficacy of treating diseases in humans, animals and plant (FAO, 2021). The AMR is among the most serious problems of the world; in the period between 2014 and 2016 about 1 million of people died because of antimicrobial resistance infection (Vikesland et al., 2019). Moreover, drug-resistant pathogens caused 25,000 deaths per year in the European Union (EU) and 700,000 deaths per year globally (European Commission, 2017).

Explaining AMR and its drivers is quite complex. However, AMR is the outcome of the use and misuse of antimicrobials in animals, environments, and humans; thus, a One Health approach is necessary at all levels. The One Health is an approach which multiple sectors communicate and work together to achieve better public health outcomes. In figure 1 is represented the dynamics of diffusion in humans, animals, and environment of bacterial resistance (Booton et al., 2021). The One Health approach is fundamental to achieve the UN 2030 Agenda for sustainable development, of which one of the goals is: "Ensure healthy lives and promote well-being for all at all ages" (European Commission, 2017).

There are several organizations working together to achieve the goal of reducing antimicrobial resistance: Food and Agriculture Organization of the United Nations (FAO), World Health Organization (WHO), World Organization for Animal Health (OIE), and United Nations Environment Programme (UNEP). According to Chokshi and colleagues (2019), the key contributors of AMR are different between developing Countries and developed ones. In the first: lack of supervision of resistance development, low quality of antibiotics, clinical abuse, and facility of access to antibiotics. In the latter case: low regulation of use in human health and

use in food producing animals. The first step in reducing antimicrobial resistance is the quantification and the tracking of antimicrobial use (AMU).

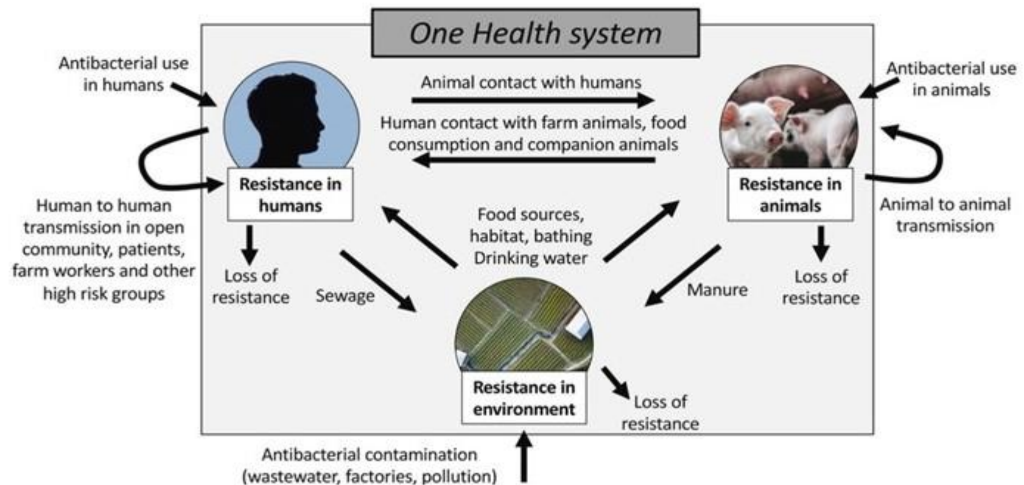

Figure 1. Model to transmission of One Health setting (Booton et al., 2021).

1.1. Antimicrobial use in animal breeding

Several studies associated the use of antimicrobials in livestock and the increase of AMR in both agriculture and humans. Van Boeckel and colleagues (2015) estimated that 73% of antimicrobial sold worldwide are used in animal for food production. The bacterial resistance does not affect only food-producing animal, but also humans who work with animals or live close to the farms because of the environmental contamination.

The use of antimicrobials in the veterinary sector could on of the main problems: they are often used to treat or prevent disease, but in the last decades also to promote growth (Mcewen and Fedorka-Cray, 2002). To invert the trend of AMR increase, the tracking of Antimicrobial Usage (AMU) was necessary. The first European Country to understand the importance of tracking the use of antimicrobials was Denmark. In 1995, the Danish integrated Antimicrobial resistance Monitoring and Research Programme (DANMAP) was founded; it was aimed to monitor the

antimicrobials consumption and to study the AMR. Fifteen years later, the European Medicines Agency (EMA) started the project to collect information on how, and how many, antimicrobials are used in animal. In the first report, only data from eight countries was collected (the Czech Republic, Denmark, Finland, France, Netherlands, Norway, Sweden, and the UK). The name of this project is European Surveillance of Veterinary Antimicrobial Consumption (ESVAC) and it actually includes 30 European Countries plus Switzerland. This type of data is necessary to identify risk factors that could spread the AMR in animals (European Medicines Agency, 2019a). Every year data is collected and analyzed to produce a report. A conversion index has been introduced to allow comparison among all species, a population correction unit (PCU) is applied considering the size of the species. The ESVAC report is based on milligrams of active ingredient sold (mg/PCU). The PCU is calculated by multiplying number of animal livestock and slaughtered animals by the theorical weight at the time of the treatment (European Medicines Agency, 2019b). According to this report the use of classes of veterinary antimicrobial is different in 31 countries (Figure 2). In 2013, the sales of veterinary antimicrobial agents ranged from 3.7 mg/PCU registered in Norway to 425.8 mg/PCU in Cyprus. Two years later, the sales ranged between 2.9 mg/PCU always in Norway to 466.3 in Cyprus. Looking to the data, some countries have reduced their purchase, such as Spain which moved from 317.1 mg/PCU in 2013 to 219.2 mg/PCU in 2018. On the other hand, other countries increased their purchasing: the worst result was registered for Cyprus, which increased its purchasing from 425.8 mg/PCU to 466.3 mg/PCU in five years (table 1). Italy is the second largest purchaser of veterinary antimicrobial agents, despite a downward trend.

Different antimicrobial classes are sold in unequal quantities in European countries, which can partly be explained by difference in animal demographics, dosage regimes, type of data sources, and veterinarians' prescribing habits.

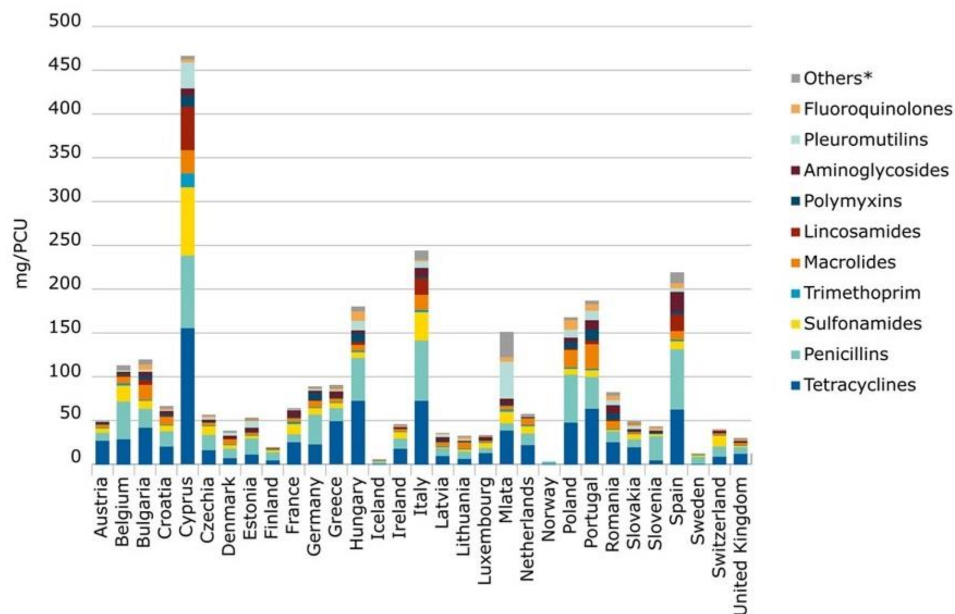

Figure 2. The sales of the different veterinary antimicrobial classes varied between 31 countries (European Medicines Agency, 2019b).

Nevertheless, changes in reported sales (mg/PCU) among the countries are often attributable to variations in animal population sizes, production system and prescriptions guidelines.

Antimicrobials are used in livestock in four different way: treatment, metaphylaxis, prophylaxis and growth promoters (Innes et al., 2019). Treatment is carried out when signs of bacterial infection are clearly visible. Metaphylaxis is the administration of antimicrobials to healthy animals which have been exposed to sick animals. Prophylaxis is used to prevent infection in risky situations: the animals

are healthy and have not been exposed to other sick animals. Growth promoters are used to increase animal growth rates; however, this administering in Europe is banned since the 2000s. The use of antimicrobials, in addition to ensuring animal health, is dictated by the need to reduce economic losses. Tiseo and colleagues (2020) analyzed data from 41 countries to estimate the global consumption of veterinary antimicrobial in 2017. They estimated that AMU in cattle, chicken, and pigs (93.75 % of all total food producing animals), was 93,309 tons of active ingredients in 2017 and they have predicted an increase of 11.5% by 2030. According to this report, pigs will be the specie mostly contributing to the increase in antimicrobials consumption, with + 45% from 2017 to 2030.

Figure 3 reports the the data of the World Organization for Animal Health (OIE) (2018) about 155 countries worldwide: 110 countries did not use antimicrobial promoters (71%), whereas 45 countries used antimicrobial growth promoters (45%).

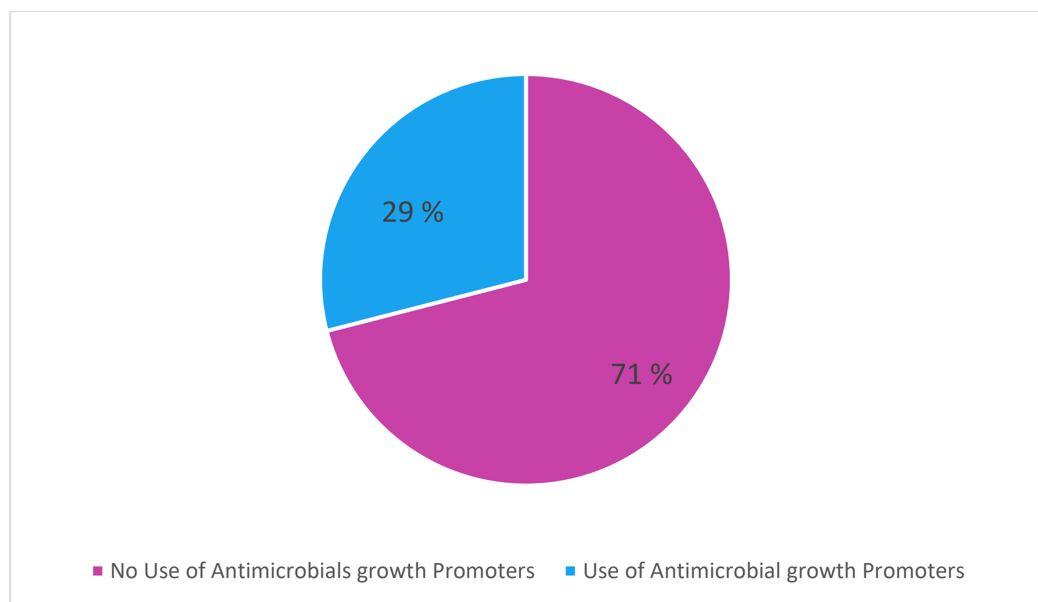

Figure 3. Use of antimicrobial growth promoters in 155 countries in 2017 (World Organization for Animal Health, 2018**)**.

Among the Countries that did not use antimicrobial growth promoters, 45 declared that they do not have a regulatory framework prohibiting their use. On the other hand, among the countries using antimicrobial growth promoters, 60% of those do not have a regulatory framework prohibiting their use (World Organization for Animal Health, 2018). For this reason, the survey of the organization is very important because it allows to have a clear picture of the situation and to improve regulatory gaps.

The intensification of the livestock production, due to the increasing demand for protein, has led to an increase in methaphylaxis and prophylaxis actions to avoid the emergence of unmanageable infection in the farm. In fact, intensively reared animals tend to have less variability in their microbiota (Schokker et al., 2014). The reduction of alpha diversity, i.e., the richness of species present in a given environment, must be avoided because greater alpha diversity is associated to better animal health (Chen et al., 2017). However, the improvement of management factors, as well as biosecurity, would help to reduce the antimicrobial consumption. Nevertheless, the use of antimicrobials has made possible to prevent and treat diseases by reducing morbidity and mortality rates (Xiong et al., 2018).

1.1.1. Antimicrobial use in swine

In food producing animals, pig husbandry is one of the major users of antimicrobial. Based on DANMAP (2019), the 75% of antimicrobials was prescribed for the Danish pig sector, of which 45% in weaners pig. The data from Denmark confirms that pigs are the largest recipients of antimicrobials compared to all other livestock species (Van Boeckel et al. 2015).

Mazurek et al. (2013) found higher level of resistance in *E. coli* isolated in Polish piglets. These numbers are associated to the prophylatic use of antimicrobial in young pigs to prevent respiratory and gastrointestinal diseases. Indeed, during weaning period, gastrointestinal and respiratory infections are very frequent, and *E. coli* is one of the major pathogens (Jabif et al., 2021).

The European Medicines Agency (EMA, 2015) proposed a method to indicate the AMU: Daily dose/population correction unit standardized daily dosages (DDDvet/PCU), calculated as the average concentration of various products sold in EU.

DDDvet/PCU = total administered active substance (mg) / defined daily dosage (mg/kg/d) x n animals x expected weight at treatment (kg).

A study about antimicrobial use in Italian pig farms highlighted a decrease of the total amount of AMU in 36 finishing farms, moving from 24.46 DDDvet/PCU in 2015 to 15.91 DDDvet/PCU in 2017 (Tarakdjian et al., 2020). The same report identified a significantly association between welfare-friendly production system and lower AMU levels, whereas the farm size did not influence antimicrobial use.

1.2. Gut health in weaned piglets

The weaning phase is an extremely delicate period in the piglet's life. The young animals have to deal with various changes, such as separation from the mother, handling, new environment, mixing litters, new hierarchies, and feeding changes (Campbell et al., 2013). Additionally, piglets are often weaned early to optimize productivity. Generally, the weaning occured at around 3-4 weeks of age, whereas in nature it occurs at around 17 weeks of age (Gresse et al., 2017). In the early weaning of piglets the gastrointestinal tract (GIT) is not fully formed (Everaert et

al., 2017). In the first 48h post weaning some piglets do not feed and this fasting phase slows down the growth rate (Lallès et al., 2007a; Sutherland et al., 2014). This temporary anorexia is one the main factors affecting the structural mucosal changes observed in weaned piglets (Marion et al., 2005). This involves increased susceptibility to intestinal disorders, often associated with the development of pathogens, such as *Escherichia coli* and *Clostridium perfrigens*, leading to the development of diarrhea (Lallès et al., 2007a). The low pH of the stomach, due to the secretion of hydrochloric acid (HCl), converts gastric zymogens into active enzymes (Heo et al., 2013b). The acid environment prevents the proliferation of pathogens and their subsequent entry into the intestine (Lallès et al., 2009). The metabolizable energy (ME) intake in the first week post-weaning is between 30–40% lower than in the pre-weaning period when piglets had milk; 2 weeks are needed post-weaning to reach the pre-weaning ME intake levels. The low intake can affect intestinal inflammation, villus height and crypt depth (McCracken et al., 1999). The height of the villi is associated with proper functioning of the small intestine. The host's defense mechanisms are provided by the GIT and they consist of a multilayer system of epithelial cells and components of the enteric nervous and immune system (Moeser et al., 2017). The intestinal epithelium breaks down and absorbs nutrients, and it is responsible for regulating the secretion of Cl^- and HCO^-_3, whereas the tight junctions (TJs) regulate the permeability of the barrier (Turner, 2009). The gastro-intestinal (GI) immune system is the biggest immune organ in the body. Indeed, it provides a defense barrier against inflammatory and pathogenic agents by activating immune response (Moeser et al., 2017). The enteric nervous system is responsible to control motility, secretion and absorption (Fernandez-Cabezudo et al., 2010). Alterations of the enteric nervous system often occur in the

weaning phase and lead to a reduction if the immune response. The early weaning, typical of piggery, is not only stressful for the animals but also occurs when the GIT is not yet formed and many of its functions are lost leading to intestinal dysbiosis.

Several studies highlighted the importance of the gut microbiota on the animals health (Fouhse et al., 2016; Gresse et al., 2017). Indeed, the gut microbiota performs several functions, such as digestion and fermentation, vitamins production, immune response regulation, and protection by pathogenic bacteria. During the weaning period, the microbiota is severely compromised (Lallès et al., 2007b). The weaning age of piglets has an effect on the maturation of microbiota; early weaned piglets show a decreased of the alpha diversity (Hu et al., 2016). In particular, with low values of alpha diversity, i.e., number of species present, the defense against adverse situations is more difficult (Chen et al., 2017). During the weaning phase there is a reduction of obligate anaerobic bacteria and an increase in facultative anaerobic bacteria, such as *Enterobacteriaceae* (Gresse et al., 2017).

Dysbiosis during weaning transition can result in post weaning diarrhea (PWD) (Trevisi et al., 2021). According to the literature, the major pathogen responsible of PWD is *Escherichia coli,* and, in particular, the Enterotoxigenic *Escherichia* coli (ETEC) with fimbriae F4 and F18. ETEC belong to the Enterobacteriaceae family, and it is a gram-negative bacterium. ETEC strains have two ways of manifesting their virulence: colonising the intestine with adhesins and by relasing enterotoxins that cause fluid secretion (Luise et al., 2019b). Often *E. coli* isolated in the piggeries show a wide range of antimicrobial resistance (Fairbrother et al., 2005). This is why alternative solutions to the use of antimicrobials are needed.

1.2.1. Preventive strategies to reduce the use of antimicrobial during the post weaning period

The regulation EC 1831/2003 on additives for use in animal nutrition banned the use of antibiotics as growth promoters from 1 January 2006. The animal health is closely associated with a correct balance of intestinal flora (Vondruskova et al., 2010), therefore protecting its compositions and functions is crucial.

Several studies focused on finding alternative solutions, such as vaccine against ETEC, oral administration of bacteria, Zinc Oxide (ZnO), organic acids, and probiotics. As aforementioned, ETEC manifest their virulence through the fimbriae that allow bacteria to colonize the small intestine. To inhibit colonization IgA response is necessary at the moment of the infection. According to Melkebeek et al. (2013), a live vaccine against F4 is a fine alternative. The vaccine administered with water had a good response, significantly reducing the percentage of diarrhea in piglets. However, its action is not immediate, because several days after weaning occur to see the first advantages. This means that the piglets are not protected during the most critical phase when the ETEC infection takes over. The same outcome was find by Fairbrother et al. (2017), who used a single-dose of non-pathogenic *Escherichia coli* against $F4^+$ ETEC. Another study tested the effect of a bivalent *E.coli* F4/F18 vaccine on the growth performance and microbiota profile (Luise et al., 2020). The study reported a change in the fecal microbiota favoring SCFA producing bacteria and no effect on growth performance of piglets.

Another practice tested was the use of bacteriophages (Lee and Kang, 2016), which are employed to attack a specific bacterium or group of bacteria. Nonetheless this therapy showed disadvantages, including the possible development of resistance.

In literature, several studies use zinc oxide (ZnO) to reduce the negative effects of the weaning. The zinc is an essential mineral (micronutrient) in swine. It is important for the growth and for the enzyme activity, such as DNA and RNA synthetase and transferases (Heo et al., 2013b). In addition, zinc is involved in the metabolism of lipids, proteins and carbohydrates (Li et al., 2006). High levels of zinc have showed a positive effect on the diversity and stability of microbiota, resulting in an improved response on the diarrheal infection and on growth performances (Kaevska et al., 2016).

Due to its antimicrobial properties, zinc oxide has been used for a long time at high level to prevent PWD and control *Escherichia coli* F4 infections (Bonetti et al., 2021). However, this solution against PWD will no longer be usable, as the European Union (EU) will ban it in June 2022. According to communicate of EMA, the benefits of using zinc oxide in piglets do not outweigh the risk related to the environmental pollution (EMA and CVMP, 2017).

The other compounds used in the diet of young piglets to prevent PWD are organic acids (OA), such as formic, fumaric, citric acids, and potassium diformate. These compounds are used because they lower the gastric pH of weaning piglets and, therefore, they reduce enterobacteria to encourage positive bacteria (Hansen et al., 2007). However, OA have antimicrobial direct activity in addition to improve health status (Bonetti et al., 2021). The OA, calcium formate specifically, was tested in weaning pigs infected with pathogenic *Escherichia coli* K88 (F4). The results of the test showed that calcium formate has a growth promoting effect in weaned piglets and a reduction days of diarrhea, fecal score and fecal bacterial excretion (Bosi et al., 2007).

The solution with probiotics envisages the use of live microbial supplements which influences positively the host. The activity of probiotics focuses on caudal segments of ileum, on caecum, and on ascending colon (Vondruskova et al., 2010). The continued administration of *Bacillus subtilis* DSM25841 in challenged piglets with ETEC F4ac 7 days after weaning mitigated negative effect of infection. Additionally, *B. subtilis* improves the gut health of piglets as observed by Luise and colleagues (2019a). Challenged piglets with *Escherichia coli F18*$^{+}$ feed multispecies probiotic (*Lactobacillus acidophilus, Lactobacillus casei, Bifidobacterium thermophilum* and *Enterococcus faecium*) showed enhanced growth performance, decreased gastric pH, intestinal oxidative response, and increased intestinal villi height, as reported by Sun and coworkers (2021).

1.3. Natural bioactive compounds

Bioactive compounds are substances that occur in nature can positively influence human or animals' health. Plant extracts have always been used in the popular medicine due to the presence of bioactive compounds (Aleksic Sabo and Knezevic, 2019). In the recent era, their use has increased to include fields other than medicine, such as cosmetic, food preservation, and animal nutrition (Bhavaniramya et al., 2019). The main bioactive compounds used in animal nutrition are specially polyphenols, such as flavonoid and proanthocyanins (Correddu et al., 2020). Polyphenols have a characteristic structure with several hydroxyl groups on aromatics ring (Manach et al., 2004). Polyphenols can be classified either by their origin or by their chemical structure. According to Di Lorenzo et al. (2021), they are classified into two main groups: flavonoid (flavanols, flavanones, flavonols, flavones, isoflavones, and antocyanins) and non-flavonoid (tannins, lignans,

hydroxycinnamic acids, phenolic acids). The amount of polyphenols in plants is influenced by many factors such as ripeness, time of the harvest, environmental factors (pedoclimatic, agronomic), processing and storage (Manach et al., 2004). These compounds are produced by plants to cope with stressful situations, some of which show antimicrobial or antioxidant properties (Leontopoulos et al., 2017). Several studies showed a correlation between polyphenols consumption and decreasing of chronic disease. According to Ros and colleagues (2014), the Mediterranean diet, characterized by foods rich in polyphenols, is associated with improved cognitive function and reduced cardiovascular risk in humans.

Flavanols and tannins are the most extensively studied polyphenol compounds due to their broad spectrum of antimicrobial action; some of these compounds also inhibit biofilm formation, prevent host linkage and neutralize bacterial toxins (Girard and Bee, 2020).

As mentioned above, polyphenols have also been tested in animal feeding, including pig feed. Among them, many studies tested polyphenols to contrast negative effect associated to the weaning phase by exploiting their antimicrobial and antioxidant capacity (Table 2). Girard and coworkers (2018) used hydrolysable chestnut tannins to evaluate postweaning diarrhea in piglets non-infected and infected with ETEC F4. In this work was tested the supplementation on 18 non-infected and 18 infected piglets for two weeks. Results showed decrease in fecal score and number of day with diarrhoea in piglets fed hydrolysable chestnuts tannins.

The addition of tannins in the diet of piglets has been associated with a reduction in iron. In fact, iron competes with microbial toxins or adhesins for the binding sites of tannins. The tannins reduce the average fecal score, the duration of diarrhea, and

the percentage of piglets in diarrhea. In another study, the supplementation of 2% of chestnut extract has alleviated ETEC infection in weaned piglets by reducing the number of excreted ETEC (Girard et al., 2020). This effect can be explained by a possible bactericidal or bacteriostatic effect.

Polyphenols extracted from grape seed and grape marc meal supplemented in 48 weaned piglets has enhanced the gain:feed ratio in growing pigs and cause an alteration in microbial composition, with a decrease in *Clostridium* and *Streptococcus* (Fiesel et al., 2014). In this study the diet with polyphenols did not affect villus height, therefore better gain feed could be explained by the altered microbial composition as well as the anti-inflammatory properties.

Bruins and colleagues (2011) studied the effect of black tea extracts on piglets infected with ETEC F4. The black tea extract supplementation was used in two different doses 0.4% and 0.8%. Their results showed that this extract reduces diarrhoea but it impaired feed intake and growth performances. Again, the anti-diarrheal effect may be due to the iron binding capacity of phenolic compounds. The same research group analyzed the effects of black tea extracted *in vitro* test and they found a delayed exponential growth of ETEC F4 but they observed a reduction of this delayed with the addition of iron.

However, the identification of the amount with beneficial effects is difficult because of the low bioavailability of polyphenols. The bioavailability is influenced by interaction with the other compounds present in the feed and the interaction mechanisms in the liver, microbiota and intestine (Di Lorenzo et al., 2021). For these reasons, to date, only the polyphenols contained in extra virgin olive oil and cocoa have received approval for a health claim associated to the content of phenolic compounds (Di Lorenzo et al., 2021).

1.3.1. Bioactive compounds from olive oil production

The olive tree is very common in the Mediterranean basin. Today, its cultivation is of great socio-economic importance for the Countries of southern Europe; these Countries together produce 95% of the world's output (Fraga et al., 2021). The oleuropein, tyrosol, hydroxytyrosol and verbascoside are the most common polyphenols in olives (Gorzynik-Debicka et al., 2018).

Research reported important role of olive polyphenols in the anticancer activity, in metabolic syndrome, in type 2 diabetes, carbohydrate adsorption, glucose homeostasis, redox state, autophagy, thermogenesis, adipogenesis, lipid metabolism, energy metabolism (Rigacci and Stefani, 2016).

The process of olive oil production is associated with the generation of by-products, such as olive oil wastewater (OOW) and olive pomace (Berbel and Posadillo, 2018). About 30 million m^3/year of OOW are produced in the Middle Eastern Countries (Azaizeh et al., 2011). The reuse of this agro-industrial by-product can increase the life cycle of the olive and reduce the environmental pollution problems due to the higher hydrophilicity (Servili et al., 2011; Elhag et al., 2017).

Of the polyphenols contained in the olive, only 2% pass into the oil, while 98% remain in the OOW (Azaizeh et al., 2011). Several studies reported antimicrobial activity of this by-product towards both gram positives and gram negative bacteria (Azaizeh et al., 2011; Carraro et al., 2014; Fasolato et al., 2015). As in olives, the variability of the polyphenols in OOW is very high due to the agronomic and genetic factors of olive cultivation and extracting process (Servili et al., 2004).

Servili and coworkers (2011) have developed a system with a membrane filtration to recover the phenolic fraction of OOW with water elimination and stabilization

of the by-product. According to Carraro and colleagues (2014), the result of this extraction process showed repression of chemiotaxis genes and motility of *Escherichia coli* K-12. Similar study with phenolic extraction of OOW has identified minimum bactericidal concentration (MBC) in different gram negative and gram positive bacteria (Fasolato et al., 2015). The range of MBC was between 0.75 mg of phenolic extract per mL of 20% ethanol/water for *Staphylococcus xylosus* and 12 mg/mL for *Salmonella Typhimurium* and *Pediococcus pentosaceus*. In addition to antimicrobial activities, the effects on the oxidative stability of proteins and lipids in chicken meat during storage were also tested. Diets supplemented with 4.8% and 9.9% of OOW in chickens could delay meat protein and lipid oxidation without interfering with the color (Roila et al., 2018).

1.3.2. Hydroxytyrosol, Tyrosol and Verbascoside and their properties

The OOW is rich in hydroxytyrosol, tyrosol and verbascoside, whose chemical structures are represented in Figure 4 (Azaizeh et al., 2011).

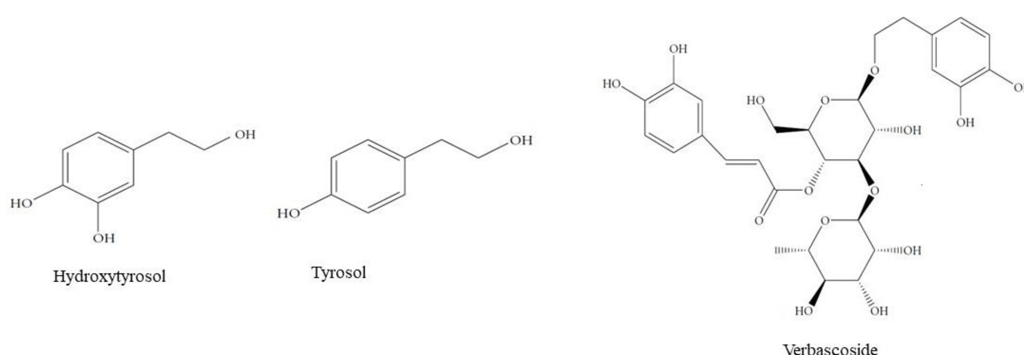

Figure 4. Structure of bioactive compounds present in olive oil wastewater.

Hydroxytyrosol is a phenolic compound with chemical formula $C_8H_{10}O_3$ with catechol structure and it is soluble in water and fat (Wani et al., 2018). This phenolic compound is present in all by-products of *Olea eoropea L.* such as oil, olive pomace, OOW and leaves. According to Mosele (2014), hydroxytyrosol is stable under *in vitro* colon fermentation protecting the colon from bowel disease. Hydroxytyrosol shows antimicrobial property toward *Escherichia coli, Candida albicans, Clostridium perfrigens, Streptococcus mutans,* and *Salmonella enterica* (Medina et al., 2006). Study by Robles-Almazan and coworkers, (2018) underlines the importance of this compound in biofilm-based infection, such as *Escherichia coli* disease. Hydroxytyrosol and its metabolites eradicate intracellular and extracellular production of reactive oxygen species (ROS) (Robles-Almazan et al., 2018). Additionally, positive effects have been recorded against arthritis, colitis cardiovascular disease and cancer (Wani et al., 2018).

Tyrosol is a phenolic compound with a chemical formula $C_8H_{10}O_2$, it is rather stable compound, that makes it less susceptible to autoxidation (Karković Marković et al., 2019). According to Chang and colleagues (2019), tyrosol influences modulation of the upstream pathways regulating LPS-induced inflammatory response. In fact, it could have effects on atherosclerosis, coronary heart disease, hypertension, chronic heart failure, and insulin resistance. Antimicrobial properties of tyrosol were found against *Escherichia coli* and *Staphylococcus aureus.* In addition, in the presence of vitamin Rf and visible light, the compound presented a more pronounced cytotoxic effect on *Staphylococcus aureus* than on *Escherichia coli* (Casadey et al., 2021).

Verbascoside is a phenylethanoid glycosides with chemical formula $C_{29}H_{36}O_{15}$. This bioactive compound in OOW also shows important properties such as anti-

inflammatory, antioxidant, UV-protective, anti-neurodegenerative disease, and anticancer (Alipieva et al., 2014). According to Yang and coworkers (2021), verbascoside may reduce the toxicity of cells infected with *Staphylococcus aureus*, establishing the compound as an excellent antiviral against *S. aureus.*

1.4. Effects of olive oil by-products on gut health in swine

Often the results of *in vitro* analyses do not coincide with *in vivo* evaluations. This is because the *in vivo* model is more complex and not 100% replicable. Likewise, the effect of individual bioactive compounds may differ when administered with others. In the literature, there are not many studies on the effect of feeding OOW in the pig's diet. In one of them, the authors studied the influence of OOW on the gastrointestinal tract alveolar macrophages and blood leukocytes of Casertana finishing pigs (Varricchio et al., 2019). The daily finishing diet for pigs was supplemented with 0.03 g/kg of OOW polyphenols. Their results showed no effect on the intestinal morphology of pigs feed with OOW. This maybe be explained by the short period of administration, or by the time necessary for modifying the epithelial structure. However, the low level of expression of cyclooxygenase-2 (COX-2, a marker of inflammation) in immunoreactive cells could be explained by the anti-inflammatory response of polyphenols in the OOW.

The knowledge about the antioxidant and antimicrobial properties of individual polyphenolics in OOW could be exploited during the post-weaning period.

In addition, the use of olive oil by-products increases the life cycle of the olive making the oil production process more sustainable. Furthermore, olive oil wastewater is a by-product which, if not reused, could cause environmental problems.

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Table

Table 1. Comparison in tonnes of active ingredient, of veterinary antimicrobial agents for food-producing animals' sales in mg/PCU by country for 2013 and 2018 (European Medicines Agency, 2015, 2019b).

	Sales mg/PCU	
Country	Year 2013	Year 2018
Austria	57.2	50.1
Belgium	156.6	113.1
Bulgaria	116.1	119.6
Croatia	-	66.8
Cyprus	425.8	466.3
Czechia	82.1	57.0
Denmark	44.9	57.0
Estonia	62.2	53.3
Finland	24.3	18.7
France	95.0	64.2
Germany	179.1	88.4
Greece	-	90.9
Hungary	230.2	180.6
Iceland	5.3	4.9
Ireland	56.5	46.0
Italy	301.6	244.0
Latvia	37.0	36.1
Lithuania	36.6	33.1
Luxemburg	53.6	33.6
Malta	-	150.9
Netherlands	69.9	57.5
Norway	3.7	2.9
Poland	151.2	167.4

Country	Year 2013	Year 2018
Portugal	187.2	186.6
Romania	-	82.7
Slovakia	62.5	49.3
Slovenia	22.4	43.2
Spain	317.1	219.2
Sweden	12.6	12.5
Switzerland	-	40.2
United Kingdom	62.1	29.5

Table 2. Biological effect observed *in vitro* and *in vivo* studies using by-products rich in polyphenols.

By-products	Level	Species	Infection	Effects	Reference
Hydrolysable chestnut tannins	1% and 2% of hydrolysable chestnut tannins	*In vivo* – Weaned pigs	*E. coli F4* 1×10^8 CFU/ml	• Reduction fecal score • Less number days of diarrhoea	(Girard et al., 2018)
Chestnut extract	1% and 2% of chestnut extract	*In vivo* – Weaned pigs	*E. coli F4* 1×10^8 CFU/ml	• Increase feed intake • Increase body weight • Reduction fecal score • Less number days of diarrhoea • Reduction of ETEC excretion	(Girard et al., 2020)
Grape seed and grape marc meal extract	-	*In vivo* – pigs	No infected	• Microbiota alteration • Equal gut morphology	(Fiesel et al., 2014)
Black tea extract	0.4% black tea extract 0.8% black tea extract	*In vivo* – Weaned pigs	*E. coli F4* 1×10^3 CFU/ml	• Reduction diarrhoea • Reduction growth performance	(Bruins et al., 2011)
Black tea extract	0.15% black tea extract	*In vitro* *E. coli K88* 1×10^3 CFU/ml	-	• 12-hour slowdown in the growth curve	(Bruins et al., 2011)

By-products	Level	Species	Infection	Effects	Reference
Olive oil wastewater	1 mg/ml	*In vitro* *E.coli K-12*	-	• Inhibitory growth effect	(Carraro et al., 2014)
Olive oil wastewater (OOW)	1.5-3 mg/ml OOW	*In vitro*	-	• Minimal Bactericidal Concentration (MBC): *S.aureus, L.monocytogens, E.coli O:157H/NCTC12900*	(Fasolato et al., 2015)
Olive oil wastewater (OOW)	4.8% of OOW and 9.8% OOW	Chickens	-	• Increase antioxidant capacity of meat	(Roila et al., 2018)
Olive oil wastewater (OOW)	0.03 g/kg of polyphenols	Casertana finishing pigs	-	• Equal gut morphology • Reduction of cyclooxygenase-2 (COX-2)	(Varricchio et al., 2019)

OBJECTIVE OF THE THESIS

In recent years, the increasing alarm about antimicrobial resistance has intensified interest in finding viable alternatives. The pig sector is responsible a consuming a large quantity of antimicrobial, most of which are used in the weaned piglets. The main objective of this thesis was to evaluate the effect of a by-products rich in polyphenols in piglets' nutrition to alleviate the negative consequences of post weaning stress in piglets. Olive oil wastewater has been selected because of the antimicrobial and antioxidant property of its polyphenolic compound such as hydroxytyrosol, tyrosol and verbascoside.

The first chapter introduces the problems related the post-weaning phase in pigs and the current strategies studied to solve them are described.

In a second part, olive oil wastewater and its polyphenolic compounds.

Specifically, the objectives of this thesis were:

- To extend the knowledge on the *in vitro* and *in vivo* effects of bioactive substances contained in olive oil wastewater, with the aim of alleviating the severity of post-weaning diarrhoea and assessing the effects on piglet growth performance.
- To analyze the response of the gut microbiota on weaned piglets feed with extracted olive oil wastewater at different doses at different time points.
- To evaluate the effect of extracted olive oil wastewater, known to contain antimicrobial bioactive compounds in weaned piglets infected with ETEC F4ac.

CHAPTER 2

2. Effect of increasing levels of bioactive compounds from olive oil wastewater on post-weaning performances

2.1. Abstract

Weaning is a critical phase in a pig's life associated with high antimicrobial consumption. Vegetal bioactive compounds, such as phenolic compounds (e.g. hydroxytyrosol, tyrosol and verbascoside), have been shown to have antimicrobial activity in vitro. The aim of this study was to test whether substances present in dehydrated olive oil wastewater (OOW) can be used in starter diets for weaned piglets to reduce problems linked to weaning without negatively impacting the growth performance. At 25 ± 1.05 days of age and a body weight (BW) of 7.41 ± 1.16 kg, 64 piglets were assigned to four treatments: control (CON), OOW0.5 (CON + 0.5% OOW), OOW1 (CON + 1% OOW) and OOW2 (CON + 2% OOW). Body weight was determined at days 0 (weaning), 7, 14 and 19, and feed intake per pen was determined daily. Faecal scores were monitored on days 0, 3, 4, 5, 7, 10, 14 and 19. In the mixed linear model, treatment, the experimental day and their interaction were used as fixed effects and animals within the pen as a random effect. Regardless of the OOW inclusion levels, average daily gain tended to be greater ($P = .10$), whereas BW ($P = .52$) and average daily feed intake ($P = .20$) were similar across all groups. Furthermore, the duration of diarrhoea was lower ($P < .0001$) in piglets fed OOW than in the CON group. In conclusion, OOW inclusion had no detrimental impact on growth performance but alleviated the incidence of post-weaning diarrhoea at inclusion levels of 0.5% and 2%.

2.2. Introduction

Antimicrobial resistance (AMR) is a threat faced by humankind. Between 2014 and 2016 on worldwide, approximately 1 million people died due to antibiotic-resistant infections, and current estimations suggest that AMR will cause about 300 million premature deaths by the year 2050 (Vikesland et al. 2019). While the factors leading to the spread of AMR are complex, it is generally thought that one major cause is the excessive use of antimicrobials in both clinical and agricultural settings. The use of antibiotics in the livestock sector contributes to the overall emergence of AMR, and scientific evidence supports the interweaving of a resistant bacterial population in livestock and humans (Landers et al. 2012).

As mentioned in a joint report from European Centre for Disease Prevention and Control (ECDC), European Food Safety Authority (EFSA) and European Medicines Agency (EMA) (2017), the consumption of antimicrobials in 2014 in Europe was generally higher in animals than in humans. The average consumption was 123.7 mg/kg in humans (range 49.9–181.7 mg/kg) and 151.5 mg/kg in food-producing animals (range 3.1–418.8 mg/kg), both expressed in milligrams per kilogram of estimated biomass. According to data from Denmark, approximately 75% of all veterinary-prescribed antimicrobials are used in the pig sector and make them a great reservoir for AMR. Most of these antimicrobials for pigs have been used to treat weaned piglets to control post-weaning diarrhoea (PWD) (DANMAP 2018). Weaning imposes tremendous stress on piglets and is accompanied by marked changes in the gastrointestinal physiology, microbiology, and immunology (Heo et al. 2013). These alterations often result in disturbances in the gastrointestinal environment, including microflora imbalances, and pre-dispose the young piglet to gastrointestinal tract infections and development of PWD (Bruins

et al. 2011). PWD is a major health issue in pig husbandry and is responsible for economic losses due to mortality, morbidity, decreased post-weaning growth and cost of medication. Enterotoxigenic *Escherichia coli* (ETEC) carrying the F4 (K88) or the F18 adhesin is one of the most important causes for PWD in piglets (Luise et al. 2019).

By-products of grapes, myrtle berries (Buffa et al. 2020) and olives (Branciari et al. 2017) are known to contain a considerable number of bioactive substances of the polyphenol class. Polyphenols are recognised to have antimicrobial, anti-inflammatory and antioxidative properties (Varricchio et al. 2019). It should be noted that polyphenols in olive oil wastewater (OOW) represent about 50% of the total polyphenols found in olives (Rodis et al. 2002). Of the polyphenols found in OOW, hydroxytyrosol, tyrosol and verbascoside are of great interest for PWD, as they are known for their anti-inflammatory, antioxidative and antimicrobial activity (El-Abbassi et al. 2012; Carraro et al. 2014; Varricchio et al. 2019).

The current study aims to extend the knowledge on the in-vitro and in-vivo effects of substances contained in OOW, which potentially could prevent or alleviate the severity of PWD and assess the effects of OOW on palatability and growth performance of weaned piglets.

2.3. Materials and methods

The experimental protocol used in this study was approved by the Cantonal Veterinary Office of Fribourg (Switzerland [No.2019_11_FR]).

2.3.1. Animals, housing, experimental design, and diet

To determine the susceptibility or resistance to ETEC F4ac using the protocol provided by Hu et al. (2019), genotyping was performed on ear biopsies of 108 one-

week-old Swiss large white piglets originating from the Agroscope sow herd. Briefly, the piglets' DNA was extracted using Maxwell RSC PureFood GMO and Authentication kits (Promega Italia S.r.l, 20126 Milano, Italy). The KASPTM assay (LGC, Teddington Middlesex, UK) was used to determine the alleles of the markers CHCF1 (RefSNP rs340488770) and ALGA0106330.

At 25 ± 1.1 days of age (mean ± SD), 33 male and 31 female ETEC F4ac-susceptible and -resistant (28 and 36, respectively) piglets with a weaning body weight (BW) of 7.41 ± 1.16 kg (mean ± SD) were selected. For the duration of the experiment, piglets (4 per pen) were housed in pens of 2.7 m^2, which were divided into a concrete floor (2/3) and a galvanised steel floor (1/3). Each pen was equipped with a heated nest, a single feeder, a nipple drinker, and a drinking trough. Throughout the experiment, the piglets were kept at an ambient temperature of > 25˚C and had free access to feed, clean water, and a solution of electrolytes (NaCl hypertonic) added to their drinking troughs for 7 days after weaning.

On the day of weaning, piglets were assigned within litter, sex, genotype, and BW to four dietary treatments for a total of 16 piglets/treatment. The piglets in the control (CON) group were offered a basal starter diet without OOW supplementation (table 1), and those assigned to the OOW0.5, OOW1 and OOW2 experimental groups were fed a basal diet supplemented with 0.5%, 1%, or 2% OOW, respectively. The OOW extract was obtained from the aqueous phase of olive oil processing using enzymatic treatment, subsequent membrane filtration and finally freeze-drying (Servili et al. 2011). The dried powder contained hydroxytyrosol (6059.5 ± 201.9 mg/kg of dry matter [DM]), tyrosol (585 ± 82.6 mg/kg of DM), tyrosol derivatives (690.4 ± 26.7 mg/kg of DM) and verbascoside (1390.5 ± 76.0 mg/kg of DM).

2.3.2. Growth performance and faecal score

The day of weaning was considered as day 0 of the experiment. To determine the individual average daily gain (ADG) for each piglet, BW was measured on days 0, 7, 14 and 19. Daily feed consumption was monitored at the pen level and used to calculate average daily feed intake (ADFI) at the pen level. During the experiment, the faecal score was recorded on days 0, 3, 4, 5, 7, 10, 14 and 19. The faecal score of each pig was assessed visually by trained personnel according to four categories using the following score adapted from Li et al. (2011): 1 = mould faeces, 2 = creamy sloppy appearance, 3 = liquid diarrhoea, 4 = watery diarrhoea. The percentage of piglets with diarrhoea and days of diarrhoea were calculated considering piglets having a faecal score equal to or greater than 3.

2.3.3. Sampling and analysis

Feed samples were collected six times during the study and were then pooled and homogenised to generate different aliquots for laboratory analysis. DM and ash were determined after oven drying for 24 h at 105°C and for 5 h at 550°C, respectively. Crude fibre was determined using the ISO 6865:2000 method. The fibrous fractions (neutral detergent fiber [NDF], acid detergent fiber [ADF] and acid detergent lignin [ADL]) were analysed according to the sequential method (ISO 16472:2006 and ISO 13906:2008) using the filter bag equipment of Ankom (Ankom Technology Corp.). Crude protein was analysed using the procedure described in AOAC International (2000; method 988.05) and fat as proposed in ISO 6492:199. The mineral content was measured according to the European Standard EN 155510:2008. The concentration of total polyphenols in the OOW and the four diets were determined by the Folin-Ciocalteau method as described by Kim et al. (2003). Faecal samples were collected from the rectum of each piglet using a sterile

faecal swab on days 0, 3, 5, 7, 10 and 19, and samples were immediately transported to the laboratory. Samples were processed within 30 minutes after collection. Each faecal swab was homogenised for 2 h at room temperature in 500 µL of phosphate buffered saline. Subsequently, dilutions from 10^0 to 10^{-7} were prepared. For all dilutions, 4 µL were deposited as a droplet on an eosin methylene blue (EMB) agar plate (Oxoid CM0069, UK). Plates were incubated at 37°C for 20 h and colony-forming units (CFU) of *E. coli* were then counted on each droplet using binoculars (Girard et al. 2020).

2.3.4. In-vitro analysis

The strain used for the *in-vitro* study was an ETEC F4 isolated from weaned piglets suffering from acute PWD at the Agroscope piggery (Posieux, Switzerland). The ETEC was grown overnight in Luria-Bertani (LB) broth (BD Difco™ Dehydrated Culture Media: LB Broth, Miller, Luria-Bertani) to obtain a standardised bacterial suspension with approximately 1 x 10^8 CFU/ml. Six dilutions of OOW were performed in tubes of 2% or 5% ethanol/water solutions (160, 80, 40, 20, 10, 5 mg/ml). The starting solution (160 mg/mL) was composed of 50 µL of bacterial suspension, 200 µL of OOW at 160 mg/ml and 1800 µL of sterile LB broth. Subsequent dilutions were obtained by using a conventional two-fold serial dilutions (80, 40, 20, 10, 5 mg/ml). All dilutions were performed in triplicate. In addition, the purity of the LB broth (negative control, LB), the vitality of ETEC (positive control, LB + ETEC) and the effect of antimicrobial activity of ethanol (positive control, LB + ETEC + ethanol 5% or 2%) were tested. The tubes were shaken at 170 revolutions per minute (rpm) for 24 h at 37 C, after which each tube was vortexed. Tubes were observed to evaluate the turbidity of the solution. Turbidity indicates the growth and replication of ETEC; therefore, a clear solution

suggests the antimicrobial effect of OOW. Finally, 100 µL of those with lower turbidity were transferred to EMB agar plates (Oxoid CM0069, UK), supplemented with 50 µg/ml of rifampicin (ref50). The minimal inhibitory concentration (MIC) was defined as the dose that inhibits bacteria growth in the three plates. Since the ethanol/water (5:95) solution had a small effect on ETEC F4 growth, the data were thus discarded.

2.3.5. Statistical analysis

The growth performance data, the faecal score and the CFU of *E. coli* were analysed with the nlme package (2020) of the R software (R Core Team, 2018) as a completely randomised design with repeated measurements. The mixed linear model considered dietary treatments, experimental day and their interactions as fixed factors and piglet nested within pen as random effects, only pen for ADFI. The dose-related effect of supplemental OOW was computed by the emmeans package (version 1.4.1.) using the pairwise contrast command for the linear and quadratic effects. The CFU of *E. coli* was obtained by averaging the results of the three replicates. Due to the non-normality of the value, the CFU parameter was log transformed prior to the statistical analysis. For BW, ADG, faecal score and CFU data, the animal was considered the experimental unit, whereas for ADFI data, the experimental unit was the pen. The percentage of piglets with diarrhoea was fitted using the generalised linear mixed model with a logit link function and binomial distribution with the MASS package (7.3-51.1). The quasi-Poisson distribution with log link was used to assess the treatment effect on the number of days with diarrhoea. The probability of developing diarrhoea was calculated with the logistic regression and was reported as an odds ratio using survey package (Lumley 2020).

Variability in the data was expressed as the standard error of mean (SEM). Differences among treatment means were assessed using the adjusted Tukey's test. Probability values of ≤ 0.05 were considered significant and > 0.05 and ≤ 0.10 were considered tendency.

2.4. Results

Piglets remained healthy for the entire duration of the study, and no antibiotic treatments were necessary. The BW was not affected by the diet (linear, P = 0.52) but increased over time (P < 0.0001). Although the treatment × experimental day interaction was significant (P = 0.04), the post-hoc Tukey mean comparisons showed no (P > 0.05) differences (Figure 1). In agreement with the BW development, experimental days influenced (P < 0.0001) ADFI and ADG (Table 2). With increasing OOW supply, there is a tendency to increase ADFI (P = 0.09) increase.

With increasing OOW inclusion, faecal score decreased linearly (P = 0.01, mean 2.72 and 1.98, respectively) (Figure 2). Especially, the addition of 0.5% and 2% OOW lowered (P = 0.03) the faecal score compared to the CON treatment. Regardless of the dietary treatment (dietary treatments × experimental days interaction, P = 0.13), the faecal score increased (P < 0.001) during the first week and decreased (P < 0.001) in the second week.

The percentage of piglets with diarrhoea decreased linearly (P < 0.001) with increasing OOW inclusion (Figure 3). Independent of the diet offered to the pigs (dietary treatments × experimental days interaction, P = 0.12), during the first seven days, the percentage of piglets with diarrhoea increased (P < .0001), and afterwards, there was a specular decrease. Days with the greatest percentage of piglets suffering

from diarrhoea were the fifth and seventh. As show in figure 4, compared to the CON treatment, piglets fed the OOW-supplemented diets had fewer ($P < .001$) days with diarrhoea (CON = 5 d vs. OOW0.5 = 2.75 d; OOW1 = 3.12; OOW2 = 2.16). Considering a confidence interval of 97.5%, a 1% increase in the incidence of diarrhoea in the CON group resulted in a 0.31%, 0.38% and 0.24% increase in the OOW0.5, OOW1 and OOW2 groups, respectively (Table 3).

Despite the marked treatment effect on the faecal score, the shedding of *E. coli* CFU did not ($P = 0.85$) differ among dietary treatments (Table 4). However, in accordance with the faecal score, the CFU values and, thus, *E. coli* shedding peaked at days 3–5and 7 and decreased from day 7 onwards ($P < 0.0001$).

Regarding *in-vitro* analyses, the tubes of 2% ethanol/water solution with 160, 80 and 40 mg/ml of OOW were less turbid. The *in-vitro* growth inhibition study revealed that the minimal concentration of OOW able to prevent the ETEC F4 growth (MIC) was 80 mg/mL when using ethanol 2% as a solvent.

2.5. Discussion

The anti-inflammatory, antioxidative and antimicrobial effects of different polyphenolic substances have been amply demonstrated (Etxeberria et al. 2013; Zhang et al. 2014; Lipiński et al 2017). Nevertheless, it is difficult to establish an optimum dietary inclusion level of by-products due to the different quantities and compositions of phenolic compounds (Brenes et al. 2016). Indeed, the concentration of polyphenols in plants or fruits varies considerably due to various intrinsic and extrinsic factors. For instance, the phenolic content of olive depends on the cultivar, the climate, the irrigation regimes, the degree of ripeness of the fruit and the processing (Pereira et al. 2006). In the OOW used in this study, the

predominant polyphenols were tyrosol, hydroxytyrosol and verbascoside and coincide with the data presented by Varricchio et al. (2019). Liehr et al. (2017) showed that the addition of olive oil bioactive extract to piglets' diets can reduce subclinical chronic inflammation and consequently have a positive impact on growth performances. In the present study, OOW given for 19 days had no negative effect on growth performances, whereas verbascoside given to piglets for 56 days was able to increase piglets' BW (Corino et al. 2007). In both studies, verbascoside and hydroxytyrosol effects were obtained after long-term administration.

Post weaning diarrhoea is a condition in weaned pigs characterised by frequent discharge of watery faeces during the two weeks after weaning (Heo et al. 2013). The most striking effect of OOW inclusion was the significant reduction in the incidence and length of diarrhoea, resulting from the overall reduction of the faecal scores. However, animals that received OOW tend to eat more and have better growth. This effect could be attributed to the antimicrobial properties of verbascoside and hydroxytyrosol (Pereira et al. 2006; Azaizeh et al. 2011; Yakhlef et al. 2018; Pannucci et al. 2019). Previous studies have suggested that the inclusion of bioactive compounds can reduce the duration and incidence of PWD. For instance, feeding hydrolysable chestnut tannins has reduced the duration of diarrhoea in post-weaning piglets (Girard et al. 2018).

However, the results on CFU and faecal score do not follow the same pattern. This difference could be attributed to the non-selectivity of the EMB agar plates, which allows the isolation of all strains of *E. coli,* including the commensal ones and not only the pathogenic ETEC F4. It is known that one of the major causes of PWD in piglets is ETEC (Lu et al. 2019), specifically the ETEC with adhesive fimbriae F4 and F18 (Luise et al. 2020).

However, PWD is a disease with multiple origins in which pathogens (e.g., ETEC) are not always involved. Indeed, limitation of the potential pathogens (ETEC) contributes only partly to the reduction of the faecal score. The change in diet (from sow milk to solid vegetal feed) and other stressors (hierarchy fights, withdrawal from the mother) lead to a large intestinal dysbiosis, which often results in diarrhoea (Pluske et al. 1997; Lallès et al. 2007).

Fasolato et al. (2015) studied the antimicrobial effect of OOW on *E. coli* O:157 H7 NCTC and *E. coli* LMG 8223. They identified an MBC equal to 3 mg/mL for *E. coli* O:157 H7 NCTC and an MBC equal to 6 mg/mL for *E. coli* LMG 8223. This MBC value is much lower than the present calculated MBC (80 mg/mL).

The higher value is justified because the amount of hydroxytyrosol, tyrosol, and verbascoside in our extract are 10 times lower than the values found by Fasolato and colleagues (2015). With regards to antimicrobial property, it is supposed that verbascoside inhibits leucine absorption in *E. coli* (Funes et al. 2010). As reported by Crost et al. (2003), leucine plays a role in leucine-responsive regulatory protein, an important transcriptional regulator of metabolism in *E. coli*, including the formation of fimbriae. In line with this information, *in-vitro* study using OOW showed a repression of chemotaxis genes and motility phenotypes in *E. coli* K-12 (Carraro et al. 2014). Bacterial motility is fundamental to allow colonisation of *E. coli* in the intestinal walls. To find alternatives to antibiotics for treating PWD in piglets, potential solutions should be tested in a challenge model with ETEC infection (Luise et al. 2019). To evaluate the efficiency of OOW, it is important to use reliable and repeatable experimental models of PWD. Such a model would give more precise answers regarding the usefulness of the new prophylactic measures that are being developed to control PWD.

2.6. Conclusion

Dietary supplementation with OOW did not negatively affect growth performance and reduced adverse post weaning diarrhoea effects. Feeding OOW up to 2% reduced the percentage of piglets with diarrhoea and the number of days with diarrhoea. In addition, these results showed how a by-product of the food industry, if well characterised, could be used in livestock nutrition, enforcing the concept of the circular economy and at the same time helping reduce the use of antimicrobial resistance.

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Tables

Table 1. Ingredient composition (g/kg) and gross chemical content of the basal diet of each treatment.

Ingredient[2]	Basal diet[1]
Barley	8.20
Oats	2.90
Corn	25.00
Wheat	30.00
Cerelac	5.00
Rapeseed oil	0.03
Potato protein	5.49
Soybean meal	16.00
Dried beet pulp	3.00
L-Lysin-HCl	0.25
DL-Methionine	0.02
L-Threonine	0.01
Monocalcium phosphate	0.84
Calcium carbonate	0.79
Sodium chloride	0.40
Ca-Formate	0.53
Pellan[3]	0.30
Vitamin-mineral premix[4]	0.40
Luctarom[5]	0.01
Greencab-70-c[6]	0.20

	Basal diet
Natuphos 5000[7]	0.01
Mikrogrit[8]	0.60
Dry matter (g/kg)	896.50
Ash	51.48
Crude fibre	31.20
Crude fat	29.40
Crude protein	218.00
Ca	7.11
Cu (mg/kg)	8.51
Fe (mg/kg)	165.00
K	8.30
Mg	1.51
Mn (mg/kg)	31.30
Na	2.26
P	5.57
Zn (mg/kg)	90.55
DE, MJ/kg[9]	13.80

[1] Basal diet of each treatment: CON group (basal diet), OOW0.5 (basal diet + 0.5% of OOW), OOW1 (basal diet + 1 % of OOW) and OOW2 (basal diet + 2 % of OOW).
[2] Each diet was formulated according to the Swiss feeding recommendation for pigs (Agroscope, 2018) and was analysed in triplicate to determine chemical composition.
[3] Pellet binding aid: Pellan, Mikro-Technik; Bürgstadt, Germany.
[4] Supplied per kg of diet: vitamin A, 8000 IU; vitamin D3, 1000 IU; vitamin E, 25 mg; menadione, 3 mg; thiamine, 2 mg; riboflavin, 5 mg; biotin, 0.1 mg; niacin, 20 mg; pantothenic acid, 15 mg; iron, 80 mg as iron sulphate monohydrate; iodine, 0.15 mg as calcium iodate; copper, 6 mg as copper sulphate; manganese, 10 mg as manganese oxide; zinc, 75 mg as zinc oxide; selenium, 0.2 mg as sodium selenite.

[5] Luctarom, Lucta; Montornès del Vallès, Spain.
[6] Greencab-70-c = Coated calcium butyrate: Greencab 70- c; Brenntag; Denmark.
[7] Natuphos 5000 = Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 μmol P from mM phytate/min at pH 5.5 and 37°C.
8 Colour marker, which helps make visible the different diets.
[9] Digestible energy content estimated according to the Swiss feed database (Agroscope, 2018), considering the relative amount of each feed ingredient in the diet.

Table 2. Effect of increasing dietary olive oil water (OOW) inclusion on post-weaning growth performance of pigs

	Treatment[1]					Experimental days				P-value			P-value[4]	
	CON	OOW0.5	OOW1	OOW2	SEM	0_7	7_14	14_19	SEM	Trt	D	Trt × D	Linear	Quadratic
ADG[5]	144.00	210.00	180.00	205.00	19.0	49.1[b]	230.4[a]	274.5[a]	14.5	0.10	<0.0001	0.73	0.09	0.29
ADFI[6]	241.00	316.00	315.00	317.00	27.9	125.0[a]	291.0[b]	475.0[c]	14.3	0.20	<0.0001	<0.0001	0.09	0.22

[1]CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW.
[2]0_7: date refer to the period between day 0 to 7; 7_14: data refer to the period between day 7 and day 14; 14_19: data refer to the period between day 14 to 19.
[3]P-values for the main effect of OOW supply (Trt), experimental days (D) and the interaction (Trt × D).
[abc] Within traits, rows with no common superscript differ at $P < .05$.
[4] P-value linear and quadratic contrast referred to treatment.
[5] ADG = Average daily gain, individually
[6] ADFI = Average daily feed intake for pen

Table 3. The onset of diarrhoea in experimental treatments. Conditional odds ratio in 97.5% confidence interval.

Treatment[1]	Odds ratio[2]	CI 97.5%[3]
OOW0.5	0.31	0.18 - 0.52
OOW1	0.38	0.23 - 0.63
OOW2	0.24	0.14 - 0.40

[1] OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW
[2] An odds ratio < 1 indicates that OOW treatment has lower odds of developing post-weaning diarrhea than the control group.
[3] CI = confidence interval is a range of values restricted by a lower and upper limit in which the true value lies with a 97.5% degree of probability. Likelihood of error 2.5%.

Table 4. Effect of increasing dietary olive oil water (OOW) inclusion on *Escherichia coli* shedding as determined using plate counting in faeces samples collected from all piglets at days 0, 3, 5, 7, 10, and 19 post weaning. Data are expressed as log 10 colony formant units.

	Treatment					P-value		
	CON	OOW0.5	OOW1	OOW2	SEM	Trt	T	Trt×T
0	12.61^{A}	12.07^{A}	12.08^{A}	12.24^{A}	0.32	0.85	<0.0001	0.97
3	11.92^{B}	11.38^{B}	11.39^{B}	11.56^{B}	0.32			
5	11.84^{B}	11.30^{B}	11.31^{B}	11.48^{B}	0.32			
7	13.17^{A}	12.63^{A}	12.64^{A}	12.81^{A}	0.32			
10	10.45^{C}	9.91^{C}	9.92^{C}	10.09^{C}	0.32			
19	7.31^{D}	6.77^{D}	6.78^{D}	6.95^{D}	0.32			

[1] CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW.
[2] P-values for the main effect of OOW supply (Trt), time (T) and the interaction (Trt × T).
^{ABCD} Referred to time, columns with no common superscript differ at $P < .05$.

Figures

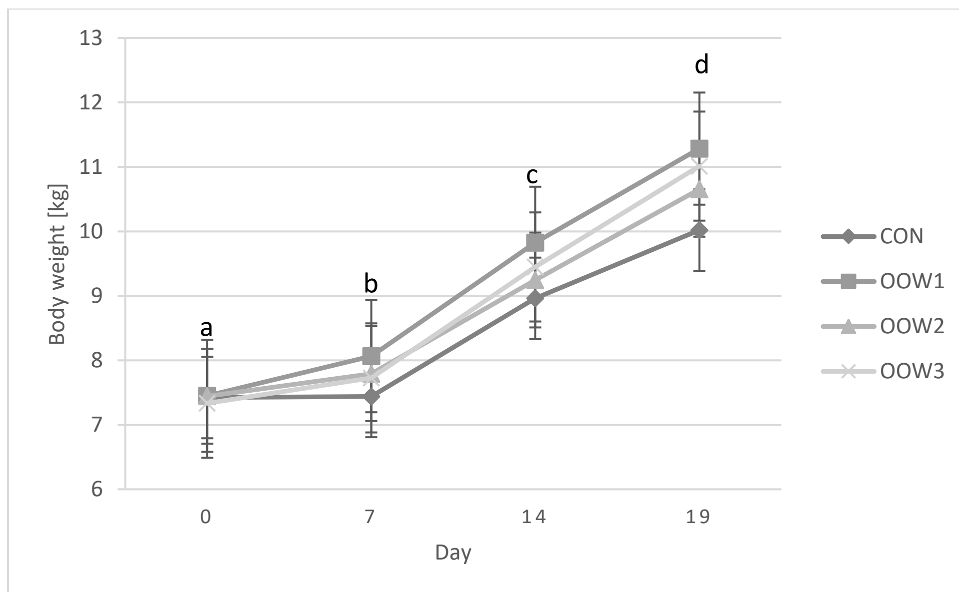

Figure 1. Effect of increasing dietary olive oil water (OOW) inclusion on piglets' body weight. CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW. Results are presented as least square means ± standard error of the means (SEM). P-values for the fixed effect of dietary treatments: P = .52, experimental days = <.0001 and dietary treatments × experimental days interaction P = .04. [ABCD] Referred to time no common superscript differ at P < .05.

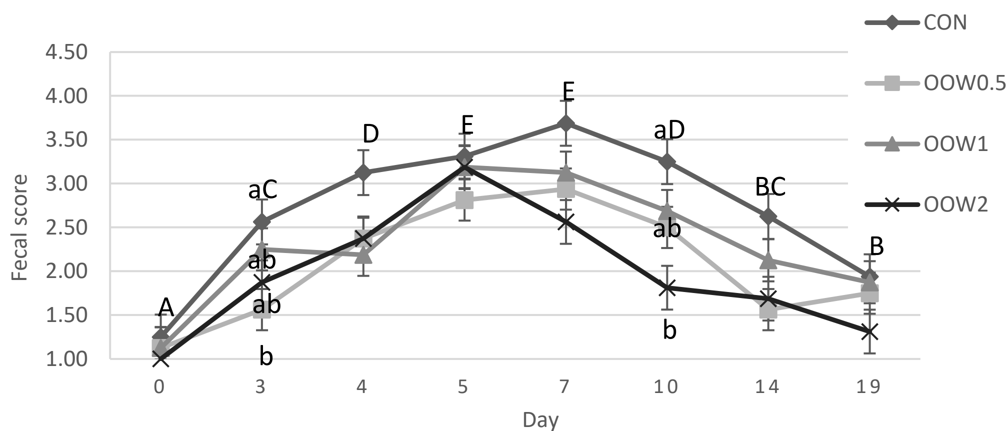

Figure 2. Effect of increasing dietary olive oil water (OOW) inclusion on faecal score. CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW. Faecal score 1 = mould faeces; faecal score 2 = creamy sloppy appearance; faecal score 3 = liquid diarrhoea; faecal score 4 = watery diarrhoea. Results are presented as least square means ± standard error of the means (SEM). P-values for the fixed effect of dietary treatments: P = .03, experimental days = <.001 and dietary treatments × experimental days interaction P = .13. Different lowercase letters differ at P < .0001. Different capital letters differ at P < .03.

3

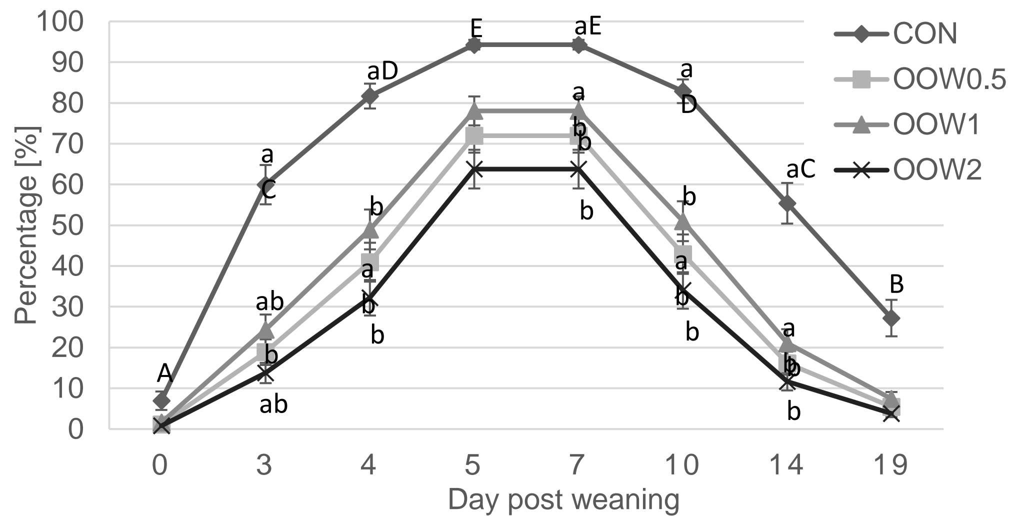

Figure 3. Effect of increasing dietary olive oil water (OOW) inclusion on the percentage of piglets with diarrhoea. When a piglet had a faecal score ≥ 3, it was considered to have diarrhoea, CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW. Faecal score 1 = mould faeces; faecal score 2 = creamy sloppy appearance; faecal score 3 = liquid diarrhoea; faecal score 4 = watery diarrhoea. Results are presented as least square means ± standard error of the means (SEM). P-values for the fixed effect of dietary treatments: $P = .03$, experimental days = $<.0001$ and dietary treatments × experimental days interaction $P = .12$. Different lowercase letters differ at $P < .0001$. Different capital letters differ at $P < .03$.

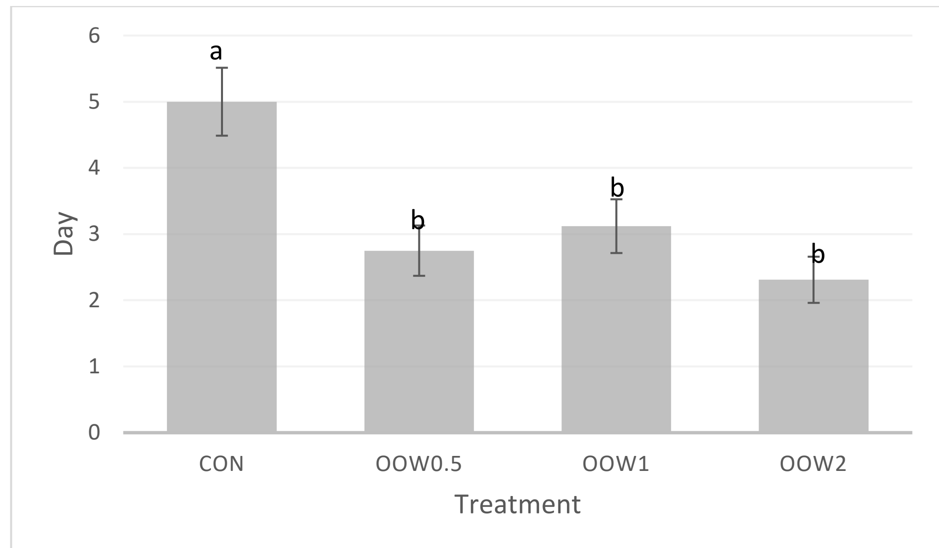

Figure 4. Effect of increasing dietary olive oil water (OOW) inclusion on the number of days with diarrhoea. When a piglet had a faecal score ≥ 3, it was considered to have diarrhoea, CON = starter diet with 0% OOW; OOW0.5 = starter diet with 0.5% OOW; OOW1 = starter diet with 1% OOW; OOW2 = starter diet with 2% OOW. Results are presented as least square means ± standard error of the means (SEM). P-values for the fixed effect of dietary treatments: P < .0001. Bars with different letters differ at P < .05.

CHAPTER 3

Response of weaned piglet's gut microbiota to the dietary inclusion of extracted olive oil wastewater at different doses

3.1. Abstract

During the weaning transition, intestinal immaturity, and many life changes can compromise pigs health. These risk factors involve the alteration of microbiota equilibrium. Understanding the evolution of microbiota in the intestine during the first period of weaning is important to contrast the negative effects of the weaning. Enterotoxigenic Escherichia coli (ETEC) is the main pathogen involved in post-weaning diarrhoea. Therefore, the intake of bioactive compounds with antimicrobial properties could reduce antimicrobial use at this period. Often enterotoxigenic Escherichia coli (ETEC) cause postweaning diarrhoea (PWD), therefore intake bioactive compound with antimicrobial properties could reduce the antimicrobial use in this period. Moreover, polyphenols have the capacity to modulate gut microbiota promoting beneficial bacteria. The aim of this study was to evaluate the effects of olive oil wastewater (OOW) on the growth performances and feces microbiota during the weaning transition. A total of forty-eight Large White piglets (25 ± 1 d of age) were randomly assigned to three groups with different diets, balanced for litter, sex, and genotype for Escherichia coli F4 susceptibility. The three groups were: CON (control diet), OOW1 (control diet + 0.05% of OOW) and OOW3 (control diet + 2% of OOW). Body weight (BW) was recorded at day 0 (at weaning) 7, 14, and 19 days post weaning. The fecal score was observed at 0, 3, 4, 5, 7, 10, 14, and 19 days post weaning. The feces were recorded at 5, 10, and 19 days post weaning. The 16S rRNA V3 – V4 amplicon was amplified and sequenced with the Illumina MiSeq platform. The BW was not affected by the

dietary treatment (P = 0.29); ADFI and ADG of OOW1 group were higher (P = 0.02 and P = 0.06, respectively) compared to CON from d 8 to d 19. The overall fecal score of the OOW3 group was lower than the CON group (P = 0.03). Groups fed OOW3 showed higher bacterial alpha diversity indices for the overall period of the study (P = 0.009). However, when each day was analyzed alone, alpha diversity was not influenced by treatment at day 5; moreover, a tendency increases in alpha diversity for treatment OOW1 and OOW3 at d 10 (P = 0.08) and d 19 (P = 0.05) was observed. Generally, treatment (P = 0.002), day of sampling (P = 0.001) and genotype (P = 0.01) influenced the microbial composition. Considering the whole period, OOW treatment reduced the Escherichia-Shigella (OOW1 vs CON, P -adj < 0.001, Log2 FC = -4.22; OOW3 vs CON, P-adj = 0.01, Log2 FC = -2.80). A greater abundance of Campylobacter was recorded in CON group (OOW1 vs CON, P-adj < 0.001, Log2 FC = -4.85; OOW3 vs CON, P-adj < 0.001 and Log2 FC = -4.71). In conclusion, the supplementation of OOW3 in piglets' diet had a positive effect on ADFI and fecal score. Additionally, OOW reduced the relative abundance of some negative bacterial genera frequently associated with PWD of piglets.

3.2. Introduction

The weaning is one the most critical periods in the life of pigs, because it is often associated with transient anorexia, inflammation of intestinal tract, and gut microbiota alteration. The piglets are subject to gastrointestinal tract, disorders such as post weaning diarrhoea (PWD), mainly caused by *Escherichia coli* enterotoxigenic (ETEC) (Luise, et al. 2019). The ETEC use F4 fimbriae to colonize the small intestine and to produce enterotoxin heat-labile and/or heat-stable (Verhelst et al., 2014). A region on chromosome 13 has been related to the resistance to ETEC F4 (Hu et al., 2019): piglets with different allelic combinations in that region can show resistance (RR) or not to the ETEC F4. Some plant extracts, containing polyphenols, are widely used as additives in various feed, based on the supposed positive effects, attributed to their anti-oxidative (Stagos, 2020), and anti-bacterial properties (Daglia, 2012). The phenolic compounds present in the extracted olive oil wastewater (OOW), such as hydroxytyrosol, tyrosol, and verbascoside, have antimicrobial activities in vitro. Previous study highlighted the inhibitory effects of OOW (1 mg/ml of Luria Bertani) on E.coli (Carraro et al., 2014).

In order to contrast PWD, the best choice is to contrast harmful bacteria without disrupting beneficial ones (Singh et al. 2019). The composition of the gastrointestinal microbiota is influenced by different factors, such as the age (Motta et al., 2019), the supplements in the diet (Girard et al. 2021), the genotype (Lu et al., 2018; Luise et al., 2020), the orally administration of beneficial bacteria (Luise, et al. 2019), and the environment (Patil et al., 2020). Patil et al. (2020) reported that the first factor influencing the microbiota of piglets is the passage through vagina of the sow. However, during pig's life the bacterial composition is dynamic and

subject to changes. Generally, during the weaning transition, the alpha diversity increases (Motta et al., 2019), which is influenced by the age at weaning; indeed, piglets early weaned showed decreased alpha diversity (Chen et al., 2017).

Since bacteria play important roles in the pig's health, understanding the composition of the microbial community and its functional capacity is very important. The aim of this study was to test the response of the gut microbiota of weaned piglets to the dietary inclusion of extracted OOW at different doses in different time point.

3.3. Material and methods

The experimental protocol used in this study was approved (No.2019_11_FR) by the Cantonal Veterinary Office of Fribourg (Switzerland). The trial was carried out at the piggery of the research station Agroscope-Posieux.

3.3.1. Animal use and care

At weaning (26 ± 1 day of age and 7.40 ± 1.16 kg of weight) forty-eight Large White piglets were allocated into 3 equal groups, in a completely randomized design (CRD), based on litters, genotype, sex, and body weight. The piglets were genotyped at one week old to determine susceptibility or resistance to ETEC F4ac using two flanking markers: CHCF1 and ALGA0106330 (Hu et al., 2019). The piglets were allocated in pens of four piglets each. The dietary treatments included: control (control diet, CON), OOW1 (CON + 0.05% of OOW), and OOW3 (CON + 2% of OOW). Each treatment group had four replicates. Both feed and water were offered *ad libitum*. To have an adequate number of animals, the trial was performed in two runs: two consecutive experiments with 24 piglets each. The trial lasted from the day of weaning until 19-days post weaning.

3.3.2. Experimental procedure and sampling

Body weight (BW) was recorded individually at the weaning day (d 0), and other three times (7, 14, and 19 d). The feed intake was recorded daily for pen and average daily gain (ADG) was calculated. During the experiment, the fecal score was recorded at day 0, 3, 4, 5, 7, 10, 14, and 19 by trained personnel. The fecal score was evaluated using 4 classes adapted by Li et al. (2011): 1 = mold feces; 2 = creamy sloppy appearance; 3 = liquid diarrhea; 4 = watery diarrhea. Individual fecal samples were collected from the rectum of the piglets at day 5, 10, and 19 post weaning. The fecal sample were stocked at -20°C until bacterial DNA extraction.

3.3.3. Bacterial DNA extraction

From a total of one hundred and forty-four samples of feces (16 per treatment at three time points), total bacterial DNA was extracted with FastDNA SPIN for soil (MP Biomedicals, Santa Ana, Ca, USA) following the manufacturer's instruction. DNA concentration and purity (absorbance ratio 260/280 and 260/230) of the isolated DNA were checked by spectrophotometry using NanoDrop (Fisher Scientific, 13 Schwerte, Germany). The 16S rRNA V3-V4 amplicon was amplified using Pro341F and Pro805R (Table 1) (Takahashi et al., 2014) and sequenced with the Illumina MiSeq 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).

3.3.4. Bioinformatic and statistical Analysis

The data related to growth performance and fecal score were analyzed by ANOVA using a linear mixed model with repeated measurements using *nlme package* (Pinheiro; 2020) of R software (version 4.0.2; R Core Team, 2019). The treatment, genotype, run and day were considered as fixed effects, and animal nested within

pen was the random effect, for the ADFI was considered pen as random effect. The differences between treatments were calculated using a Tukey test and level of significance for all analysis was declared for $P < 0.05$, whereas statistical tendency was reported with $P < 0.10$.

The Bioinformatic analysis was performed using DADA2 1.14.0 (Callahan et al., 2016); for the taxonomic assignment, the Silva database release 138.1 (Quast et al., 2013) was used as reference.

Analysis of Alpha diversity, Beta diversity and taxonomic composition were carried out in using phyloseq (McMurdie and Holmes, 2013) and Vegan (Dixon, 2003) packages.

The statistical analysis, on Alpha diversity, Beta diversity and taxonomic composition, was carried out firstly considering all days of sampling together. For the Alpha diversity, the Shannon, InvsSimpson and Chao1 indices were calculated. To test the differences among the treatments for the Alpha diversity a Multifactorial ANOVA (MANOVA) with repeated measures was fitted, considering sequencing depth, treatment, time, run, and genotype as fixed effects and animal nested within pen as random effect. For the Beta diversity, the Bray Curtis distance was calculated, and the differences among treatments were tested using a non-parametric PERMANOVA (Adonis) model, with 999 permutations, using treatment, time, run and genotype as factors. In addition, to test the homogeneity of dispersion among treatments, the BETADISPER and PERMDISP tests were used (Anderson, 2001). The differences for the taxonomic composition among treatments were tested using DESeq2 (Love et al., 2014) aggregating the data at Genus level, including time and genotype in the model. Subsequently, to investigate the effect of the treatment at each timepoint, the analysis was repeated considering

separately each day of sampling applying ANOVA for Alpha diversity indices using car R package (John et al., 2020). The effect of treatment, run, and genotype for each time point was calculated using the Adonis function with 999 permutations. The Beta diversity was assessed with the Non-metric Multi-dimensional Scaling (NMDS) using Bray-Curtis distance matrix. The time factor was removed from the models when analyzing each day of sampling separately.

The P-values were adjusted for multiple comparisons using the False Discovery Rate (FDR) method. Significance was declared for $P\text{-value} < 0.05$ and a trend was considered when $0.05 < P\text{-value} < 0.10$.

3.4. Results

3.4.1. Growth performance and fecal score

The growth performances of piglets are reported in Table 2. Piglets BW did not differ significantly among treatments ($P = 0.29$) or runs ($P = 0.77$), whereas tended to be higher for RR genotype ($P = 0.06$). ADG tended to differ among treatments ($P = 0.06$). From interaction treatment for time, in the second week ADFI increased for OOW1 compared to CON (320.60 and 241.70 g, respectively); within the last week, OOW1 (511.60 g) and OOW3 (497.60 g) showed larger feed ingestion compared to CON (392.30 g). However, considering the whole period ADFI tended to be higher among OOW groups ($P = 0.07$).

As shown in Table 3, fecal score was significant different among treatments ($P = 0.03$) and time ($P < 0.001$). In particular, fecal score was lower ($P = 0.05$) in OOW3 compared to CON on day 7 and 10, whereas no differences were observed between OOW1 and OOW3.

3.4.2. Fecal microbiota

From a total of 144 fecal samples, 6,352,664 quality checked reads were obtained that resulted in 4930 different amplicon sequence variant (ASVs). The rarefaction curves of each sample are shown in Figure 1. Overall, all samples reached a plateau point suggesting a good sequencing efficiency. The results showed 4 dominant phyla, representing ~96% of the total sequence, whereas subsequent level analysis identified 28 families (~95% of the total sequence on average). The bacterial composition at Phylum levels was characterized by Firmicutes 74.45%, Bacteroidota 19.04%, Proteobacteria 1.96%, and Actinobacteriota 1.48%; at Family level by Lactobacillaceae 24.26%, Lachnospiraceae 13.93%, and Prevotellaceae 13.24%; at Genus levels by *Lactobacillus* 24.26%, *Prevotella* 8.36%, *Blautia* 3.27% and *Faecalibacterium* 2.89%. Exploring the taxonomic composition, a total of 228 genera were identified from the fecal samples at the three-time points, however around 14% of total sequence on average was characterized by unclassified genera.

3.4.3. Diversity index and taxonomy

The Alpha diversity tended to be (Chao1 and InvSimpon) or was significantly (Shannon) influenced by treatment (table 4). In addition, Chao1 tended to be influenced by the day of sampling and significantly influenced by run and genotype. The effect of genotype was also confirmed by Shannon index while InvSimpson index was not influenced by genotype. Table 4 shows means, standard errors and P-values for Chao1, Shannon and InvSimpson indices. OOW3

group showed significant higher Shannon and InvSimpson indices (i.e., higher microbial diversity) compared to the CON group (Table 5).

The Figure 2 shows Alpha diversity boxplots for Chao1, Shannon and InvSimpson indices.

For the Beta diversity, even if the NMDS plot based on Bray-Curtis dissimilarity matrix (Figure 3) did not present a clusterization among group , the Adonis test showed that the microbial composition was significantly influenced by the experimental treatment (R^2 = 0.02, P = 0.002), the run (R^2 = 0.02, P = 0.001), the day of sampling (R^2 = 0.06, P = 0.001) and genotype (R^2 = 0.01, P = 0.01). The post-hoc analysis by pairwise comparison revealed significant differences between OOW1 vs CON (P_{adj} = 0.03) and OOW3 vs CON (P_{adj} = 0.009), whereas OOW1 and OOW3 were not different (P_{adj} = 0.13). Moreover, BETADISPER indicated that homogeneity of dispersion between treated group did not vary (P = 0.20), meaning that the results of the Adonis test are consistent. However, dispersion changes according to the day of sampling (P = 0.002).

In order to identify the bacterial taxa that contribute to those differences, differential expression analysis using DESeq 2 was carried out. Results are reported in the Supplementary Table 1. The abundance of 48 genera were found to be significantly different between OOW1 and CON: 39 genera were less expressed in OOW1. On the contrary, only 28 genera showed a significant different abundance OOW1 and OOW3, of which 23 more abundant in OOW3. Finally, 41 genera had a significant different expression between OOW3 and CON. Analyzing the data reported in Supplementary Table 2, genus *Escherichia-Shigella* (P-adj < 0.001 Log2FC = -4.41), *Anaerovibrio* (P-adj < 0.001 Log2FC = -3.91), *Clostridium sensu stricto 6* (P-adj < 0.01 Log2FC = -2.87) and *Dialister* (P-adj < 0.001 Log2FC = -3.07), were higher in OOW1 respect to the CON group. Whereas *Turibacter* (P-adj < 0.01 Log2FC = 2.75), and *Succinivibrio* (P-adj < 0.001 Log2FC = 2.78), were more

abundant in OOW3 respect to CON. *Faecabacterium prausynitzii* was lower the OOW1 compared with in the OOW3 and CON groups (OOW1 vs OOW3 P-adj < 0.01 Log2FC = -1.40; OOW1 vs CON, P-adj < 0.001 Log2FC = -2.09).

3.4.4. Results at day 5

Alpha diversity indices (Chao1, Shannon and InvSimpson) were not affected by treatment (P > 0.10) (Figure 4). However, the run significantly influenced Alpha diversity values for Chao1 (P <0.001), Shannon (P < 0.0001), and InvSimpson (P = 0.02). Genotype significantly influenced Chao1 index. All values are reported in Table 6. The Adonis test showed that treatment (R^2 = 0.06, P = 0.003), run (R^2 = 0.06, P = 0.001), and genotype (R^2 = 0.03, P = 0.034) significantly affected the microbial composition at day 5. Both treatments with OOW were different compared to the control groups (OOW1 vs CON P = 0.04, OOW3 vs CON P = 0.05). BETADISPER showed that microbiome community dispersion between treatment was not homogenous, in particular OOW1 was significantly different (P = 0.03) compared to CON, whereas OOW3 tended to be significant (P = 0.06) compared to CON. NMDS plot using Bray-Curtis distance between treatment on day 5 is reported in Figure 5.

DESeq2 output, for day 5, is reported in Supplementary Table 2. Results showed that the CON group had and higher abundance of bacteria belonging to the genera *Faecalibacterium* (OOW1 vs CON, P-adj < 0.001 Log2FC = -3.84), *Escherichia-Shigella* (OOW1 vs CON, P-adj = 0.04 Log2FC = -2.67), *Campylobacter* (OOW1 vs CON, P-adj < 0.001 Log2FC = -4.85), *Parabcteroides* (OOW1 vs CON, P-adj = 0.02 Log2FC = -3.22), *Anaerovibrio* (P-adj < 0.001 Log2FC = -4.29), and different genera from the family Prevotellaceae (*Prevotella, Alloprevotella* etc.) compared to OOW1. A similar trend was observed comparing OOW3 and CON:

Faecalibacterium (P-adj = 0.02 Log2FC = -2.06), *Campylobacter* (P-adj < 0.001 Log2FC = -4.71), and genera from family Prevotellaceae were more abundant in the CON group. In addition, *Fusicatenibacter* (OOW3 vs CON, P-adj = 0.03 Log2FC = 2.61) and *Lachnospira* (OOW3 vs CON, P-adj = 0.02 Log2FC = 3.19) were also more prevalent in the CON group compared to OOW3.

3.4.5. Results at day 10

The Alpha diversity was not affected by treatment for Chao1, Shannon and InvSimpson indices. The genotype tended to influence Chao1 and Shannon indices (P = 0.07 and P = 0.05). All values are reported in Table 7.

In Figure 6 boxplots of Alpha diversity values for Chao1, Shannon and InvSimpson indices for the three treatments.

Result of Adonis test highlighted differences in microbial composition between treatment (R^2 = 0.06, P = 0.03); however, the contrasts did not show differences among the dietary groups. Also the run influenced the bacterial composition (R^2 = 0.04, P = 0.02). BETADISPER showed that the microbiome community dispersion among treatment was not significant (P = 0.59). Figure 7 shows NMDS plot using Bray-Curtis distance among treatments on day 10.

The differential abundant taxa at day10 are reported in the supplementary Table 3. The *Clostridiaceae* family was more present in OOW1 and OOW3 compared to CON (OOW1 vs CON, P-adj = 0.01 Log2FC = 3.61; OOW3 vs CON, P-adj < 0.01 Log2FC = 4.78), whereas *Alloprevotella* (OOW3 vs CON, P-adj = 0.01 Log2FC = -2.16), *Dialister* (OOW1 vs CON, P-adj = 0.04 Log2FC = -2.78, OOW3 vs CON, P-adj = 0.02 Log2FC = -3.02) and *Peptococcus* (OOW1 vs CON, P-adj = 0.02 Log2FC = -2.90, OOW3 vs CON, P-adj = 0.02 Log2FC = -2.91) were less abundant in both OOW1 and OOW3 compared to CON.

3.4.6. Results at day 19

As reported in Table 8, Alpha diversity analysis using Shannon and InvSimpson indices showed a larger microbial diversity in OOW3 compared to CON ($P = 0.03$). However, Chao1 did not show any differences among treatments ($P > 0.10$). Table 9 reports mean, standard error, and contrasts using Chao1, Shannon and InvSimpson indices.

The boxplots of Alpha diversity analysis by Chao1, Shannon, and InvSimpson indices for the three treatments are reported in Figure 8.

The Adonis test yielded a P-value of 0.03, indicating a significantly different microbial composition among treatments; the comparison between OOW3 and CON group tended to confirm this difference ($R^2 = 0.06$, $P = 0.05$). The same analysis revealed no significant differences between OOW1 and CON ($R^2 = 0.05$, $P = 0.18$) or between OOW1 and OOW3 ($R^2 = 0.02$, $P = 1.00$). Figure 9 shows NMDS plot using Bray-Curtis distance between treatment in day 19.

The differential abundant taxa at day 19 are reported in Supplementary Table 4 The prevalence of *Methanospaera* (OOW1 vs CON, P-adj < 0.001 Log2FC = 6.88), *Oribacterium* (OOW1 vs CON, P-adj = 0.04 Log2FC = 1.53), and *Lachnospiraceae* (OOW1 vs CON, P-adj < 0.001 Log2FC= 3.76) families was higher in CON compared to OOW1 group. However, the *Methanospahaera* genus was more abundant in CON compared to OOW1 and OOW3 (OOW1 vs CON, P-adj < 0.001 Log2FC = 6.88, OOW3 vs CON, P-adj < 0.001 Log2FC = 5.75).

3.5. Discussion

The gut microbiota plays an important role during the weaning transition, because it contributes to the physiological and immunological development of pigs (Foditsch et al., 2014). The weaning period is a particular event for piglets and it is a challenge for the gut physiology (Lallès et al., 2007a). The present study analyzed the effect of different doses of OOW in feed supplementation during the post-weaning period of piglets. Our results showed that polyphenols supplementation significantly increased bacterial Alpha diversity for the overall period of the trial; it appears that the higher dose (i.e., OOW3 diet) had the largest effect on bacterial diversity. However, if we analyzed days individually, an increase in Alpha diversity for treatments OOW1 and OOW3 in day 10 and 19 was observed. Alpha diversity at day 5 was not influenced by polyphenols supplementation; these differences could be explained by the normal loss in bacterial diversity that occurs during the critical weaning period (Gresse et al., 2017). Moreover, the decrease of bacterial composition could be associated to the duration of the administration. Indeed, piglets received polyphenols starting from the day of weaning. A lost in bacterial diversity has been associated to gut disease in human (Nistal et al., 2012; Sha et al., 2013; Fei et al., 2016). In piglets, a higher Alpha diversity is associated with the maturation of the gut microbiota during weaning (Chen et al., 2017). Our results are in accordance with Marcelino et al. (2019), who observed that consumption of EVOO (Extra Virgin Olive Oil) in human was associated to increasing biodiversity of intestinal bacterial and promoting of gut health.

In addition to the increased bacterial diversity, Beta diversity showed that the overall bacterial composition is also influenced by day of sampling and polyphenols supplementation, regardless of the dose used. In fact, the polyphenols increased the

abundance of bacteria of the Clostridiaceae family as observed by Deiana et al. (2014), who administered EVOO to human. Considering similarity on anatomy, genetics, and physiology between human and pigs (Meurens et al., 2012), and that the pigs are used for understanding human diseases.

Ingestion of 50 mg/kg/day of EVOO in male mice, reduced the abundance of Proteobacteria and Rikenella and increased the abundance of Lactobacillus (Liu et al., 2019). According to Marcelino et al. (2019) polyphenols influence the microbiota composition by acting as prebiotics, i.e. by inhibiting pathogenic bacteria growth, such as *Escherichia coli*, and by stimulating the probiotic bacteria, such as Bifidobacterium. The reduction of Escherichia-Shigella in OOW1 and OOW3 evidenced at day 5 could confirm the antimicrobial proactivity known for verbascoside and hydroxytyrosol towards E.coli (Pereira et al., 2006; Carraro et al., 2014). Indeed, the pathogens associated to post weaning diarrhea (PWD) in piglets are enterotoxigenic Escherichia coli (Luise et al., 2019b). At day 5, OOW treatments had lower abundance of Campylobacter compared to the CON group: these bacteria are frequently isolated in piglets and children under 5 years with diarrhea (Yang et al., 2020).

In addition, OOW1 decreased the abundance of Faecalibacterium prausnitzii; low quantity of this bacteria has been associated with chronic inflammatory disease of the gastrointestinal tract (Foditsch et al., 2014). Faecalibacterium prausnitzii is a gram positive bacteria and it is a major producer of butyrate, a short chain fatty acid (SCFA) that is a source of energy for the colonic epithelial cells and has anti-inflammatory and epithelial barrier preserving effects (Plöger et al., 2012). Overall, the differences in bacterial composition between CON and OOW1-OOW3 are more evident at the first 5 day after weaning, then these differences tend to be reduced

until the day 19. This may indicate that, after weaning, the microbiota composition tend to stabilize with the development of the piglets, as also evidenced by Zhao et al. (2015).

3.6. Conclusion

The OOW supplementation in the diet of piglets has improved Alpha diversity index, which is desirable because a wide bacterial diversity helps to cope periods of stress, such as the weaning transition. Between the two levels of inclusion, OOW3 increased bacterial diversity more than OOW1. The supplementation with OOW decreased *Escherichia-Shigella* at day 5, but that effect was not found for days 10 and 19.

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Table

Table 1. Primers V3-V4 regions of 16S rRNA gene. Ref: (Takahashi et al., 2014)

Tailed primers for 16S rRNA V3-V4
Pro341F: 5′-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGCCTACGGGNBGCASCAG -3′
Pro805R:5′-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGGACTACNVGGGTATCTAATCC -3’

Table 2. Effect of the experimental diets with different level's extracted olive oil wastewater on growth performance of weaned piglets.

	Treatments[1]				P-value[2]					
	CON	OOW1	OOW3	SEM	trt	T	trt x T	Run	G	G x trt
Body weight, kg										
d 0	7.21	7.14	7.12	0.35						
d 7	7.22	7.70	7.51	0.35	0.29	<0.0001	0.02	0.77	0.06	0.86
d 14	8.75	9.53	9.23	0.35						
d 19	9.80	11.06	10.80	0.35						
ADG[3], g/d										
d 0 to 7	16.10	82.70	77.20	25.50						
1d 8 to 14	205.40	272.00	266.50	25.50	0.06	<0.0001	0.66	0.03	0.66	0.89
d 15 to 19	239.60	306.20	300.70	25.50						
d 0 to 19	147.00	212.00	204.00	21.20	0.06	-	-	0.02	0.65	0.85
ADFI[4], g/d										
d 0 to 7	89.60	141.60	148.10	18.60						
d 8 to 14	241.70[a]	320.60[b]	303.80[ab]	18.60	0.02	<0.0001	0.0002	0.002	1.00	0.99
d 15 to 19	392.30[a]	511.60[b]	497.60[b]	18.60						
d 0 to 19	160.00	209.00	194.00	18.60	0.07	-	-	0.03	0.64	0.86

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW; [2] P-values for the main effect of OOW supply (trt), experimental days (T) and the interaction (trt × T), the run (Run), genotype (G) and interaction (G x trt). [abc] Within traits, rows with no common superscript differ at $P < .05$.
[3] ADG = Average daily gain, individually; [4]ADFI = Average daily feed intake for pen.

Table 3. Effect of Extracted olive oil wastewater on fecal score in weaned piglets.

Day post weaning	Treatments[1]				P-value[2]					
	CON	OOW1	OOW3	SEM	Trt	T	Trt × T	run	G	G x trt
0	1.25	1.12	1.00	0.05	0.03	<0.001	0.06	0.46	0.72	0.91
3	2.56	1.56	1.88	0.13						
4	3.12	2.38	2.38	0.13						
5	3.31	2.81	3.19	0.12						
7	3.69[b]	2.94[ab]	2.56[a]	0.14						
10	3.25[b]	2.50[ab]	1.81[a]	0.14						
14	2.62	1.56	1.69	0.12						
19	1.94	1.75	1.31	0.10						

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW;
[2] P-values for the main effect of OOW supply (trt), experimental days (T) and the interaction (trt × T), the run (Run), genotype (G) and interaction (G x trt)
[ab] Rows with diverse superscript to differ at $P < 0.05$.

Table 4. Mean alpha diversity for treatment using Chao1, Shannon, InvSimpson indices, with standard error (SE) and contrast.

Alpha diversity index	Treatments[1]						P-value			
	CON	SE	OOW1	SE	OOW3	SE	trt	day	genotype	run
Chao1	307	14.3	334	14.3	353	14.2	0.08	0.07	<0.0001	<0.0001
Shannon	3.92	0.11	3.97	0.11	4.33	0.11	0.009	0.19	0.003	0.14
InvSimpson	19.4	2.96	23.3	2.96	29.6	2.94	0.05	0.34	0.03	0.23

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW.

Table 5. Contrast of treatment by Chao1, Shannon and InvSimpson indices.

Alpha diversity index	Contrast, p		
	CON vs OOW1	OOW1 vs OOW3	CON vs OOW3
Chao1	0.64	0.80	0.31
Shannon	0.93	0.03	0.01
InvSimpson	0.63	0.27	0.04

Table 6. Mean alpha diversity for treatment on day 5 using Chao1, Shannon, InvSimpson indices, with standard error (SE) and contrast.

Alpha diversity index	Treatments[1]						P value		
	CON	SE	OOW1	SE	OOW3	SE	trt	Run	genotype
Chao1	316	27.2	308	27.1	365	27.1	0.33	0.001	0.03
Shannon	3.90	0.21	3.80	0.21	4.34	0.21	0.15	0.0002	0.17
InvSimpson	23.1	6.15	21.0	6.13	25.2	6.13	0.84	0.02	0.18

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW.

Table 7. Mean alpha diversity for treatment on day 10 using Chao1, Shannon, InvSimpson indices, with standard error (SE) and contrast.

Alpha diversity index	Treatments[1]						P-value		
	CON	SE	OOW1	SE	OOW3	SE	trt	Run	genotype
Chao1	292	36.4	317	35.7	341	35.2	0.52	0.12	0.007
Shannon	3.98	0.24	3.72	0.23	4.27	0.23	0.08	0.25	0.01
InvSimpson	3.98	0.24	3.72	0.23	4.27	0.23	0.20	0.91	0.31

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW.

Table 8. Mean alpha diversity for treatment on day 19 using Chao1, Shannon, InvSimpson indices, with standard error (SE) and contrast.

Alpha diversity index	Treatments[1]						P-value		
	CON	SE	OOW1	SE	OOW3	SE	trt	Run	Genotype
Chao1	312	33.3	383	33.2	356	33.2	0.12	0.16	0.08
Shannon	3.9	0.19	4.4	0.19	4.4	0.19	0.03	0.13	0.35
InvSimpson	4.01	0.19	3.68	0.18	4.24	0.18	0.03	0.29	0.32

[1] CON = starter diet with 0% OOW; OOW1 = starter diet with 0.5% OOW; OOW3 = starter diet with 2% OOW.

Table 9. Contrast of treatment at day 19 by Shannon and InvSimpson indices.

Alpha diversity index	Contrast, p		
	CON vs OOW1	OOW1 vs OOW3	CON vs OOW3
Shannon	0.06	1.0	0.05
InvSimpson	0.06	1.0	0.05

Supplementation table 1. The difference of abundance of genera within groups in the total of 144 genes.

Contrast	Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW1	% CON
OOW1 vs CON	2959.52	-1.85	0.41	0	Faecalibacterium	0.75	0.96
	15103.2	-2.4	0.47	0	Prevotella	2.26	3
	2184.88	-0.89	0.36	0.05	Subdoligranulum	0.82	0.84
	1446.78	-4.22	0.91	0	Escherichia-Shigella	0.29	0.31
	982.23	3	1.09	0.02	Sarcina	0.28	0.09
	775.75	2.31	0.65	0	Catenibacterium	0.35	0.07
	1269.37	-3.86	0.54	0	Anaerovibrio	0.08	0.33
	787.62	-1.09	0.41	0.03	Holdemanella	0.22	0.3
	311.5	1.87	0.46	0	Fusicatenibacter	0.21	0.11
	1064.83	-3.05	0.64	0	Dialister	0.21	0.41
	2881.68	-1.83	0.52	0	Prevotellaceae NK3B31 group	0.39	0.72
	273.06	-1.55	0.58	0.03	Collinsella	0.11	0.13
	469.44	-1.69	0.44	0	Intestinimonas	0.11	0.15
	183.25	-1.37	0.44	0.01	[Ruminococcus] torques group	0.11	0.1
	305.19	-1.92	0.66	0.02	Roseburia	0.08	0.2
	459.38	-1.73	0.58	0.01	Olsenella	0.09	0.21

Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW1	% CON
2554.12	-1.44	0.57	0.04	UCG-002	0.38	0.3
139.65	-2.22	0.72	0.01	Streptococcus	0.03	0.13
862.57	-1.72	0.52	0.01	Colidextribacter	0.12	0.1
228.62	-2.18	0.5	0	Peptococcus	0.04	0.09
2400.49	-2.13	0.42	0	Alloprevotella	0.48	1.04
450.21	-1.17	0.47	0.04	Prevotellaceae UCG-003	0.15	0.2
66.89	-2.59	0.74	0	Mucispirillum	0.02	0.05
587.55	-3.65	0.78	0	Mitsuokella	0.06	0.2
715.64	-2.52	1	0.04	Clostridium sensu stricto 6	0.03	0.05
414.86	3.39	0.79	0	UCG-008	0.11	0.04
35.93	-10.71	2.74	0	Erysipelotrichaceae UCG-002	0	0
77.89	-2.91	0.86	0	Veillonella	0.02	0.08
163.62	-2.33	0.68	0	Acidaminococcus	0.04	0.05
150.09	9.4	1.28	0	Methanosphaera	0.04	0
168.46	-3.93	0.58	0	Lachnospiraceae UCG-010	0.02	0.11
39.54	-1.67	0.62	0.03	Sutterella	0	0.03
57.11	-2.95	0.83	0	Senegalimassilia	0.01	0.02
294.85	-2.29	0.85	0.03	Candidatus Soleaferrea	0.02	0.03

	Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW1	% CON
	253.4	-1.53	0.54	0.02	Monoglobus	0.05	0.05
	19.47	4.75	1.67	0.02	Lachnospiraceae XPB1014 group	0.01	0
	53.16	-4.45	1.36	0.01	Lachnospiraceae UCG-003	0	0.01
	19.45	-3.32	0.86	0	Slackia	0	0.01
	49.82	-5.27	1.12	0	Ralstonia	0	0.01
	57.03	-2.23	0.74	0.01	Helicobacter	0.01	0.03
	15.98	-5.32	1.75	0.01	Odoribacter	0	0.01
	113.68	3.43	1.16	0.01	Anaerostipes	0.03	0.01
	11.76	6.9	2.11	0.01	CAG-56	0.01	0
	34.02	-9.4	1.42	0	Fusobacterium	0.01	0.01
	10.19	-4.62	1.32	0	Howardella	0	0.01
	7.46	3.15	1.05	0.01	[Anaerorhabdus] furcosa group	0	0
	16.58	-5.58	2.26	0.05	Elusimicrobium	0	0
	5.54	-24.19	3.56	0	Sphingomonas	0	0
	Base mean	**log2 Fold Change**	**lfcSE**	**padj**	**Genus**	**% OOW1**	**% OOW3**
OOW1	1011.78	-1.45	0.47	0.02	Megasphaera	0.53	0.76
Vs OOW3	2959.52	-1.44	0.41	0.01	Faecalibacterium	0.75	1.18

Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW1	% OOW3
962.79	-1.11	0.36	0.02	Agathobacter	0.55	0.68
1269.37	-2.22	0.54	0	Anaerovibrio	0.08	0.44
1122.24	-1.55	0.44	0.01	Phascolarctobacterium	0.25	0.48
787.62	-2.04	0.41	0	Holdemanella	0.22	0.31
1135.04	-2.31	0.65	0.01	Succinivibrio	0.17	0.39
469.44	-1.36	0.44	0.02	Intestinimonas	0.11	0.14
183.25	-1.29	0.44	0.02	[Ruminococcus] torques group	0.11	0.09
351.52	-1.19	0.41	0.02	[Eubacterium] hallii group	0.16	0.2
459.38	-2.05	0.58	0.01	Olsenella	0.09	0.21
139.65	-2.36	0.72	0.01	Streptococcus	0.03	0.1
136.72	-2.69	1.02	0.05	Frisingicoccus	0.05	0.13
226.96	-1.81	0.58	0.02	Lachnospiraceae NK4A136 group	0.07	0.08
124.8	-2.76	0.69	0	Bifidobacterium	0.08	0.11
66.89	-1.98	0.74	0.04	Mucispirillum	0.02	0.05
587.55	-2.25	0.78	0.02	Mitsuokella	0.06	0.13
414.86	3.74	0.79	0	UCG-008	0.11	0.05
163.62	-2.36	0.68	0.01	Acidaminococcus	0.04	0.07

	Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW1	% OOW3
	39.54	-1.8	0.61	0.02	Sutterella	0	0
	19.47	5.25	1.67	0.01	Lachnospiraceae XPB1014 group	0.01	0.01
	67.05	-4.26	1.3	0.01	Selenomonas	0.02	0.03
	15.98	-5.9	1.75	0.01	Odoribacter	0	0
	11.76	7.84	2.11	0.01	CAG-56	0.01	0
	12.13	-6.3	1.72	0.01	Erysipelotrichaceae UCG-009	0.01	0.01
	5.83	7.52	2.05	0.01	Lachnospiraceae UCG-007	0	0
	86.7	2.94	0.95	0.02	Prevotellaceae UCG-004	0.02	0.01
	16.58	-6.79	2.26	0.02	Elusimicrobium	0	0.01
Contrast	Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW3	% CON
OOW3	10648.1	-4.05	0.94	0	Turicibacter	0.93	0.44
Vs	2143.4	1.17	0.39	0.01	Blautia	1.15	0.84
CON	15103.2	-1.47	0.47	0.01	Prevotella	3.25	2.86
	962.79	1.12	0.37	0.01	Agathobacter	0.68	0.46
	1446.78	-2.8	0.91	0.01	Escherichia-Shigella	0.37	0.31
	982.23	3.61	1.09	0.01	Sarcina	0.42	0.09
	775.75	2.39	0.65	0	Catenibacterium	0.29	0.07
	1269.37	-1.64	0.54	0.01	Anaerovibrio	0.44	0.33

Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW3	% CON
1135.04	2.13	0.65	0.01	Succinivibrio	0.39	0.11
311.5	2.4	0.46	0	Fusicatenibacter	0.21	0.11
3653.86	-1.88	0.75	0.05	Parabacteroides	0.16	0.5
1064.83	-2.48	0.64	0	Dialister	0.24	0.41
2881.68	-1.86	0.52	0	Prevotellaceae NK3B31 group	0.42	0.72
351.52	1.8	0.41	0	[Eubacterium] hallii group	0.2	0.14
2554.12	-1.54	0.57	0.03	UCG-002	0.37	0.3
862.57	-2.23	0.52	0	Colidextribacter	0.1	0.17
2400.49	-2.12	0.42	0	Alloprevotella	0.46	1.04
226.96	2.47	0.59	0	Lachnospiraceae NK4A136 group	0.08	0.04
91.23	1.48	0.46	0.01	Lachnospiraceae FCS020 group	0.06	0.04
213.3	1.63	0.54	0.01	Lachnospiraceae ND3007 group	0.09	0.05
2183.7	-2.1	0.55	0	UCG-005	0.29	0.32
147.27	1.65	0.54	0.01	Oribacterium	0.08	0.03
715.64	-3.04	1	0.01	Clostridium sensu stricto 6	0.04	0.05
150.09	9.47	1.29	0	Methanosphaera	0.03	0
84.18	1.94	0.6	0.01	Lachnospira	0.03	0

Base mean	log2 Fold Change	lfcSE	padj	Genus	% OOW3	% CON
168.46	-3.42	0.58	0	Lachnospiraceae UCG-010	0.04	0.11
294.85	-3.04	0.85	0	Candidatus Soleaferrea	0.02	0.03
52.16	-2.91	0.78	0	Candidatus Saccharimonas	0.01	0.01
26.96	2.57	1.02	0.05	Lachnospiraceae UCG-004	0.02	0.01
253.4	-1.64	0.54	0.01	Monoglobus	0.05	0.05
37.47	1.38	0.47	0.02	Family XIII UCG-001	0.02	0.01
67.05	3.93	1.3	0.01	Selenomonas	0.03	0.01
49.82	-3.89	1.12	0	Ralstonia	0	0.01
44.28	-9.99	2.73	0	Actinobacillus	0	0.02
34.02	-9.35	1.42	0	Fusobacterium	0	0.01
12.13	6.59	1.72	0	Erysipelotrichaceae UCG-009	0.01	0
5.83	-9.45	2.04	0	Lachnospiraceae UCG-007	0	0
86.7	-2.71	0.95	0.02	Prevotellaceae UCG-004	0.01	0.01
18.17	-4.44	1.5	0.01	possible genus Sk018	0	0
6.68	2.69	1.01	0.03	Defluviitaleaceae UCG-011	0	0
5.54	-24.13	3.56	0	Sphingomonas	0	0

Supplementation table 2. The difference of abundance of genera within groups at day 5.

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW1 vs CON	2099.45	-3.84	0.71	0	0	Faecalibacterium
	542.56	-3.32	1.29	0.01	0.03	Clostridium sensu stricto 1
	10398	-4.53	0.83	0	0	Prevotella
	1734.26	-3.55	0.58	0	0	Subdoligranulum
	2382.02	-2.67	1.1	0.02	0.04	Escherichia-Shigella
	385.7	-2.57	0.79	0	0.01	[Ruminococcus] gauvreauii group
	1050.12	-2.72	0.73	0	0	Dorea
	3538.28	-4.85	1.02	0	0	Campylobacter
	1034.43	-4.29	1.01	0	0	Anaerovibrio
	1021.5	-3.43	0.68	0	0	Phascolarctobacterium
	652.17	-3.36	0.68	0	0	Holdemanella
	1117.09	-3.48	1.08	0	0.01	Succinivibrio
	2286.35	-3.22	1.13	0	0.02	Parabacteroides
	166.99	-3.03	0.95	0	0.01	Collinsella
	275.56	-3.11	0.65	0	0	Intestinimonas
	194.32	-3.06	0.77	0	0	[Ruminococcus] torques group

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	161.63	-3.21	0.98	0	0.01	Butyricicoccus
	129.89	-4.64	1.14	0	0	Olsenella
	2993.22	-2.86	0.83	0	0	UCG-002
	257.61	-3.25	0.69	0	0	Peptococcus
	452.59	-3.51	0.75	0	0	Prevotellaceae UCG-003
	2157.1	-3.07	0.95	0	0.01	Prevotellaceae NK3B31 group
	143.73	-2.06	0.79	0.01	0.03	Marvinbryantia
	3412.98	-4.29	0.8	0	0	Alloprevotella
	1170.28	-3.19	0.76	0	0	Rikenellaceae RC9 gut group
	1109.66	-3.52	0.85	0	0	Treponema
	80.51	-1.79	0.68	0.01	0.03	Incertae Sedis
	927.14	-4.66	1.21	0	0	Prevotellaceae UCG-001
	304.63	-6.64	1.41	0	0	Mitsuokella
	610.88	-3.08	0.79	0	0	Colidextribacter
	36.97	-3.81	1.42	0.01	0.03	Sharpea
	247.7	-2.94	1.19	0.01	0.04	UCG-008
	129.6	-2.83	1.05	0.01	0.02	[Eubacterium] eligens group
	107.89	-3.32	1.09	0	0.01	Veillonella

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	2419.69	-2.58	0.9	0	0.02	UCG-005
	65.09	-3.02	0.72	0	0	Mogibacterium
	70.74	-1.89	0.72	0.01	0.03	Shuttleworthia
	526.2	-2.66	0.72	0	0	Family XIII AD3011 group
	124.59	-4.48	1.21	0	0	dgA-11 gut group
	59.7	-4.93	1.75	0	0.02	Senegalimassilia
	66.21	-3.01	1.08	0.01	0.02	Catenisphaera
	245.76	-5.17	0.85	0	0	Lachnospiraceae UCG-010
	40.59	-3.91	1.52	0.01	0.03	Candidatus Saccharimonas
	132.03	-3.49	1.21	0	0.02	Oscillospira
	246.52	-3.22	0.96	0	0	Monoglobus
	71.87	-3.51	1.13	0	0.01	Lachnoclostridium
	1151.9	-3.27	1.18	0.01	0.02	Bacteroides
	514.31	-5.53	1.23	0	0	Candidatus Soleaferrea
	72.22	-2.56	1.05	0.01	0.04	Desulfovibrio
	100.48	-4.99	1.42	0	0	Acidaminococcus
	85.64	-4.11	1.72	0.02	0.05	Negativibacillus
	29.66	-5.74	1.88	0	0.01	Slackia

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	33.94	-8.46	1.82	0	0	Ralstonia
	84.17	-6.92	1.27	0	0	Helicobacter
	27.51	-5.77	2.26	0.01	0.03	Odoribacter
	106.12	-4.4	1.11	0	0	[Eubacterium] siraeum group
	64.19	-3.79	1.12	0	0	Sphaerochaeta
	71.09	-5.07	2.07	0.01	0.04	Fusobacterium
	17.92	-6.65	2.13	0	0.01	Howardella
	47.62	-3.94	1.51	0.01	0.03	UCG-009
	21.3	-3.79	1.48	0.01	0.03	[Eubacterium] nodatum group
	2.15	-27.54	3.58	0	0	28-apr
	78.9	-5.03	1.26	0	0	UCG-004
	89.09	-4.1	1.51	0.01	0.02	Alistipes

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW1 vs OOW3	1021.5	-2.18	0.68	0	0.02	Phascolarctobacterium
	652.17	-2.44	0.67	0	0.01	Holdemanella
	1117.09	-3.94	1.08	0	0.01	Succinivibrio
	129.89	-4.21	1.14	0	0.01	Olsenella
	123.22	-2.38	0.68	0	0.01	[Eubacterium] hallii group

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	1109.66	-2.55	0.85	0	0.04	Treponema
	36.97	-4.92	1.41	0	0.01	Sharpea
	41.75	-3.43	1.06	0	0.02	Lachnospira
	100.48	-4.08	1.41	0	0.05	Acidaminococcus
	1530.08	-4.84	1.56	0	0.01	Turicibacter
	2099.45	-2.06	0.71	0	0.02	Faecalibacterium

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW3 vs	10398	-3.68	0.83	0	0	Prevotella
	1734.26	-2.03	0.58	0	0	Subdoligranulum
CON	3538.28	-4.71	1.02	0	0	Campylobacter
	1034.43	-3.76	1.01	0	0	Anaerovibrio
	123.57	2.61	0.99	0.01	0.03	Fusicatenibacter
	2286.35	-5.56	1.13	0	0	Parabacteroides
	606.09	-3.14	0.88	0	0	Fournierella
	275.56	-1.68	0.65	0.01	0.03	Intestinimonas
	194.32	-2.57	0.77	0	0.01	[Ruminococcus] torques group
	578.18	-1.91	0.72	0.01	0.03	Ruminococcus
	2993.22	-3.7	0.83	0	0	UCG-002

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	257.61	-3.29	0.69	0	0	Peptococcus
	452.59	-2.08	0.75	0.01	0.02	Prevotellaceae UCG-003
	2157.1	-3.81	0.94	0	0	Prevotellaceae NK3B31 group
	1060.43	-2.95	0.92	0	0.01	NK4A214 group
	3412.98	-3.58	0.79	0	0	Alloprevotella
	1170.28	-2.01	0.76	0.01	0.03	Rikenellaceae RC9 gut group
	927.14	-4.3	1.2	0	0	Prevotellaceae UCG-001
	968.14	-2.89	0.94	0	0.01	Christensenellaceae R-7 group
	610.88	-3.42	0.79	0	0	Colidextribacter
	2419.69	-4.46	0.9	0	0	UCG-005
	65.09	-2.33	0.71	0	0.01	Mogibacterium
	526.2	-2.63	0.72	0	0	Family XIII AD3011 group
	41.75	3.19	1.09	0	0.02	Lachnospira
	124.59	-3.08	1.21	0.01	0.04	dgA-11 gut group
	59.7	-6.25	1.76	0	0	Senegalimassilia
	245.76	-5.06	0.85	0	0	Lachnospiraceae UCG-010
	40.59	-4.99	1.53	0	0.01	Candidatus Saccharimonas
	132.03	-3.01	1.2	0.01	0.04	Oscillospira

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	246.52	-2.86	0.95	0	0.01	Monoglobus
	89.01	-4.31	1.61	0.01	0.03	Lachnospiraceae AC2044 group
	1151.9	-4.9	1.18	0	0	Bacteroides
	514.31	-5.19	1.23	0	0	Candidatus Soleaferrea
	85.64	-4.34	1.73	0.01	0.04	Negativibacillus
	29.66	-5.29	1.88	0	0.02	Slackia
	33.94	-8.62	1.83	0	0	Ralstonia
	84.17	-4.07	1.26	0	0.01	Helicobacter
	106.12	-3.6	1.11	0	0.01	[Eubacterium] siraeum group
	71.09	-5.25	2.07	0.01	0.04	Fusobacterium
	53.34	-4.1	1.54	0.01	0.03	Prevotellaceae UCG-004
	99.94	-3.69	1.37	0.01	0.03	Oscillibacter
	78.9	-4.01	1.26	0	0.01	UCG-004
	89.09	-4.85	1.51	0	0.01	Alistipes
	35.21	-9.16	2.25	0	0	Pyramidobacter

Supplementation table 3. The difference of abundance of genera within groups at day 10.

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW1 vs CON	290.34	3.61	1.04	0.00	0.01	Clostridium sensu stricto 1
	551.69	-2.78	0.90	0.00	0.04	Dialister
	143.27	-2.90	0.87	0.00	0.02	Peptococcus
	167.71	5.87	1.47	0.00	0.00	Clostridium sensu stricto 6
	58.43	-25.71	3.34	0.00	0.00	Erysipelotrichaceae UCG-002
	67.40	-6.41	1.52	0.00	0.00	Veillonella
	16.77	28.05	2.83	0.00	0.00	Methanosphaera
	2.28	21.85	2.90	0.00	0.00	Denitrobacterium
	0.53	-19.63	3.59	0.00	0.00	[Eubacterium] ventriosum group
	2.48	17.54	3.60	0.00	0.00	[Eubacterium] fissicatena group
	0.29	-18.43	3.60	0.00	0.00	Nosocomiicoccus

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW1 vs OOW3	635.97	-3.87	0.82	0.00	0.00	Anaerovibrio
	58.43	-26.24	3.34	0.00	0.00	Erysipelotrichaceae UCG-002

	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	0.53	-17.70	3.60	0.00	0.00	[Eubacterium] ventriosum group
	2.48	23.85	3.60	0.00	0.00	[Eubacterium] fissicatena group
	0.98	20.85	3.59	0.00	0.00	Enterobacter
	1.73	-30.14	3.60	0.00	0.00	Angelakisella
	290.34	4.78	1.04	0.00	0.00	Clostridium sensu stricto 1

	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW3 Vs CON	551.69	-3.02	0.90	0.00	0.02	Dialister
	143.27	-2.91	0.87	0.00	0.02	Peptococcus
	100.65	-1.82	0.51	0.00	0.01	Solobacterium
	1472.84	-2.16	0.61	0.00	0.01	Alloprevotella
	16.77	27.64	2.83	0.00	0.00	Methanosphaera
	2.28	25.39	2.88	0.00	0.00	Denitrobacterium
	0.98	-22.98	3.58	0.00	0.00	Enterobacter
	1.73	16.93	3.60	0.00	0.00	Angelakisella
	0.29	-18.76	3.60	0.00	0.00	Nosocomiicoccus

Supplementation table 4. The difference of abundance of genera within groups at day 19.

Contrast	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
OOW1 Vs CON	2545.88	-9.26	1.24	0.00	0.00	Turicibacter
	192.20	-1.89	0.59	0.00	0.01	Anaerovibrio
	703.84	-1.59	0.57	0.01	0.04	Dialister
	147.43	-6.40	1.59	0.00	0.00	Romboutsia
	313.33	-3.29	0.84	0.00	0.00	Intestinibacter
	110.12	1.53	0.54	0.00	0.04	Oribacterium
	259.02	3.14	0.98	0.00	0.01	Treponema
	153.51	-4.07	1.14	0.00	0.00	Clostridium sensu stricto 6
	63.87	6.88	1.29	0.00	0.00	Methanosphaera
	86.97	3.76	0.96	0.00	0.00	Lachnospiraceae NK3A20 group
	309.26	-2.35	0.81	0.00	0.03	Mitsuokella
	55.01	3.41	0.88	0.00	0.00	UCG-008
	34.07	2.74	0.98	0.01	0.04	[Eubacterium] ruminantium group
	63.73	-5.91	1.21	0.00	0.00	Lachnospiraceae UCG-010

	baseMean	log2FoldChange	lfcSE	pvalue	padj	Genus
	43.15	2.34	0.76	0.00	0.02	Catenisphaera
	9.85	-27.68	2.27	0.00	0.00	Ralstonia
	45.93	-13.16	3.56	0.00	0.00	Actinobacillus
OOW1 Vs OOW3	2545.88	-4.34	1.24	0.00	0.02	Turicibacter
	259.02	3.74	0.98	0.00	0.01	Treponema
	55.01	3.25	0.88	0.00	0.01	UCG-008
	9.85	-24.58	2.28	0.00	0.00	Ralstonia
	1.90	-20.21	3.59	0.00	0.00	Pyramidobacter
OOW3 Vs CON	2545.88	-4.92	1.24	0.00	0.01	Turicibacter
	440.14	-1.35	0.42	0.00	0.05	Alloprevotella
	63.87	5.75	1.29	0.00	0.00	Methanosphaera
	63.73	-4.23	1.19	0.00	0.02	Lachnospiraceae UCG-010
	45.93	-12.13	3.56	0.00	0.03	Actinobacillus

Figure

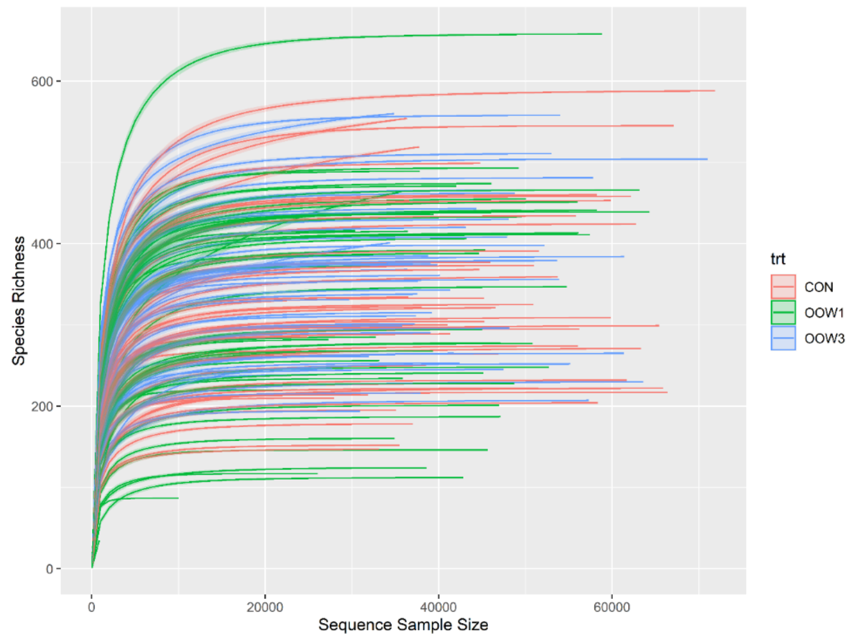

Figure 1. Rarefaction curve of each sample constructed based on observed ASVs.

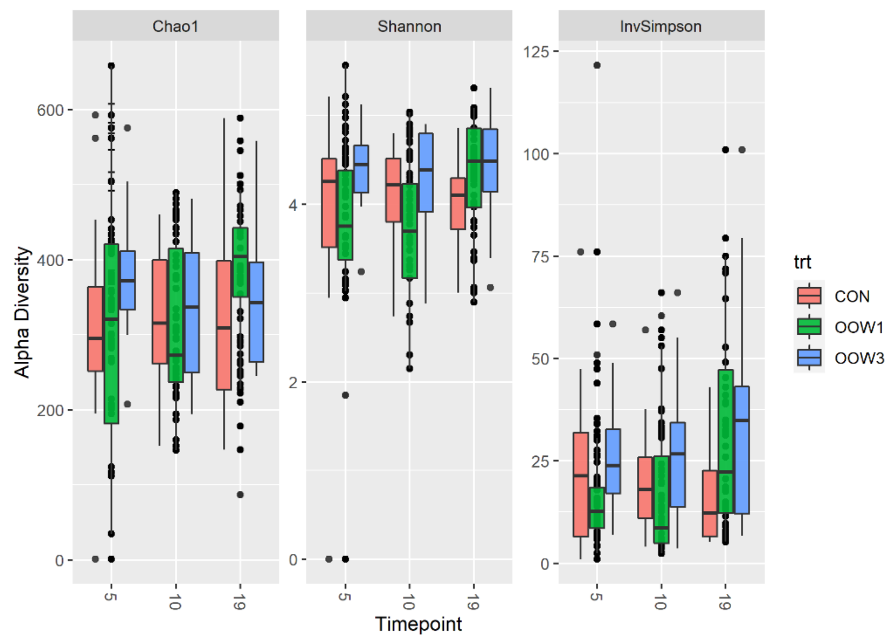

Figure 2. Boxplots showing alpha diversity for Chao1, Shannon, InvSimpson indices (CON = control, OOW1 = low-dose and OOW3 = high-dose).

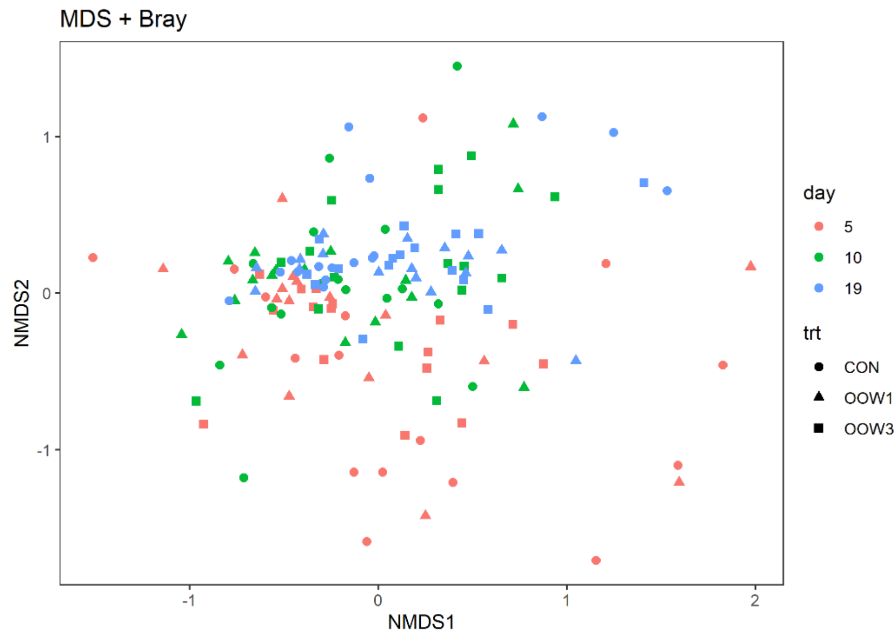

Figure 3. NMDS plot using Bray-Curtis distance metrics for treatment (CON = control, OOW1 = low-dose and OOW3 = high-dose) and days of sampling (day 5, day 10 and day 19).

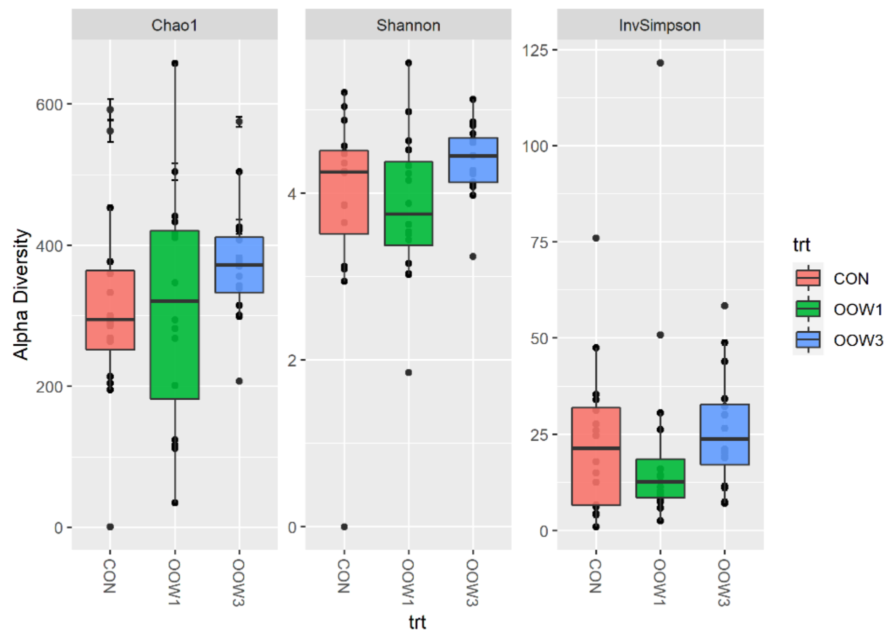

Figure 4. Boxplot with different alpha diversity index: Chao1, Shannon and InvSimpson at day 5.

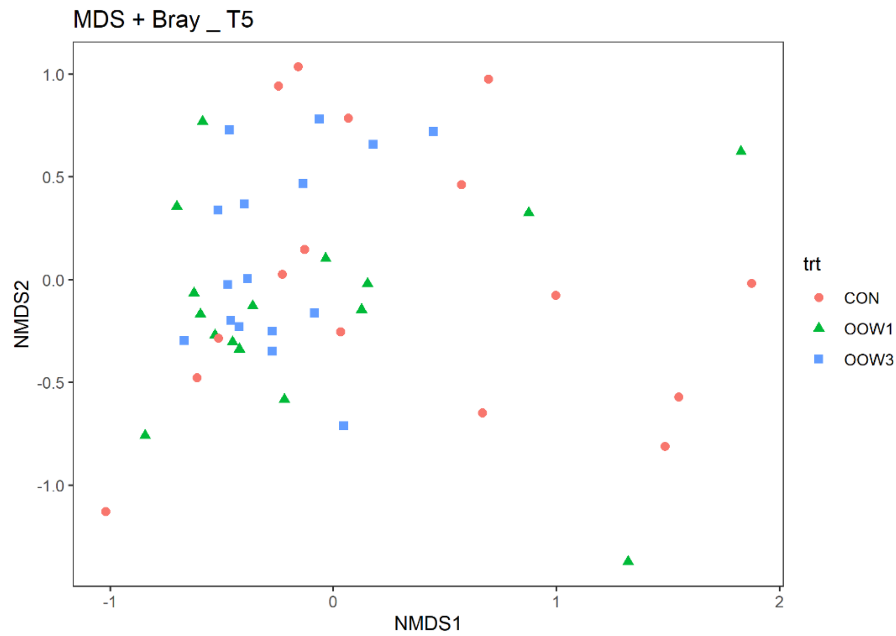

Figure 5. NMDS plot using Bray-Curtis distance metrics for treatment (CON = control, OOW1 = low-dose and OOW3 = high-dose) on day 5 of fecal sampling.

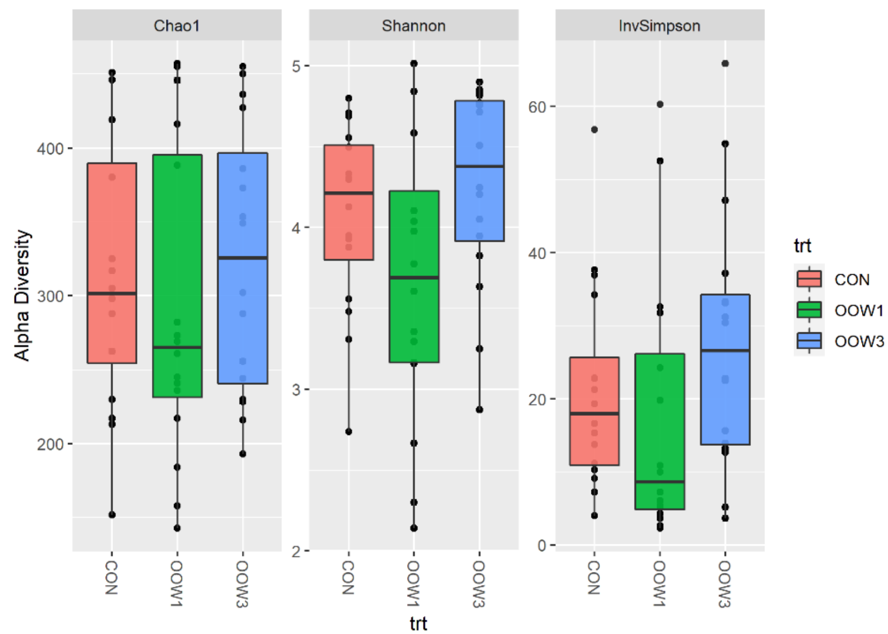

Figure 6. Boxplot with different alpha diversity index: Chao1, Shannon and InvSimpson to day 10.

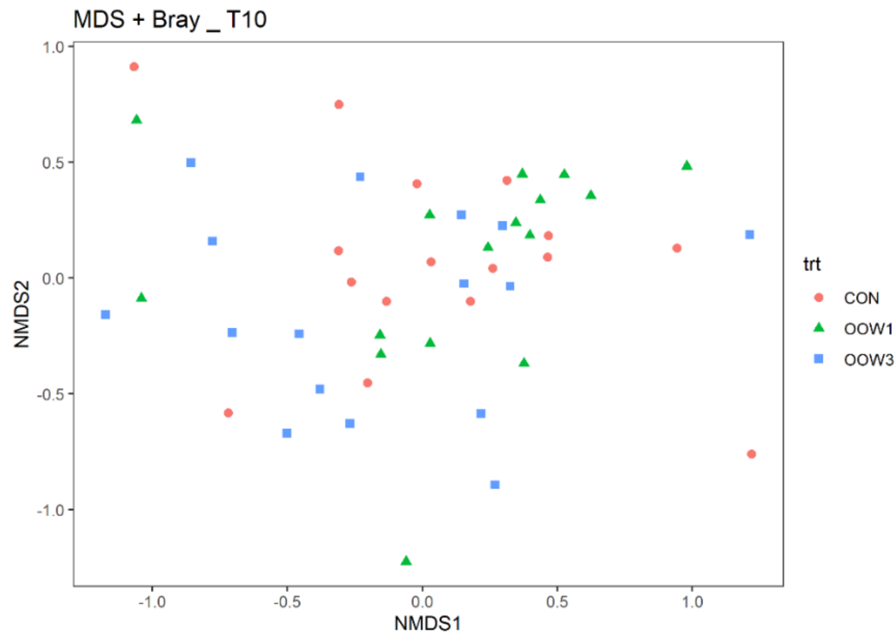

Figure 7. NMDS plot using Bray-Curtis distance metrics for treatment (CON = control, OOW1 = low-dose and OOW3 = high-dose) on day 10 of fecal sampling.

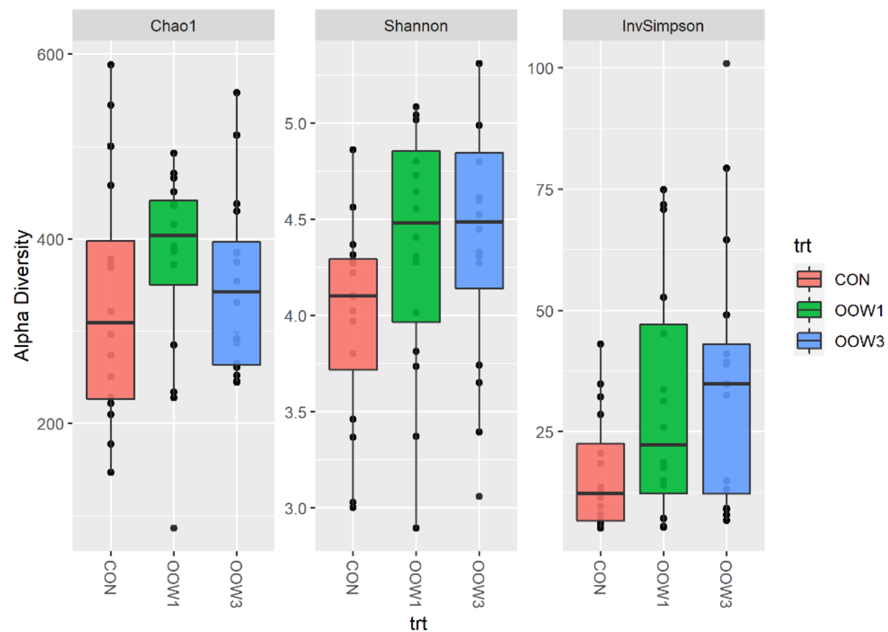

Figure 8. Boxplot with different alpha diversity index: Chao1, Shannon and InvSimpson to day 19.

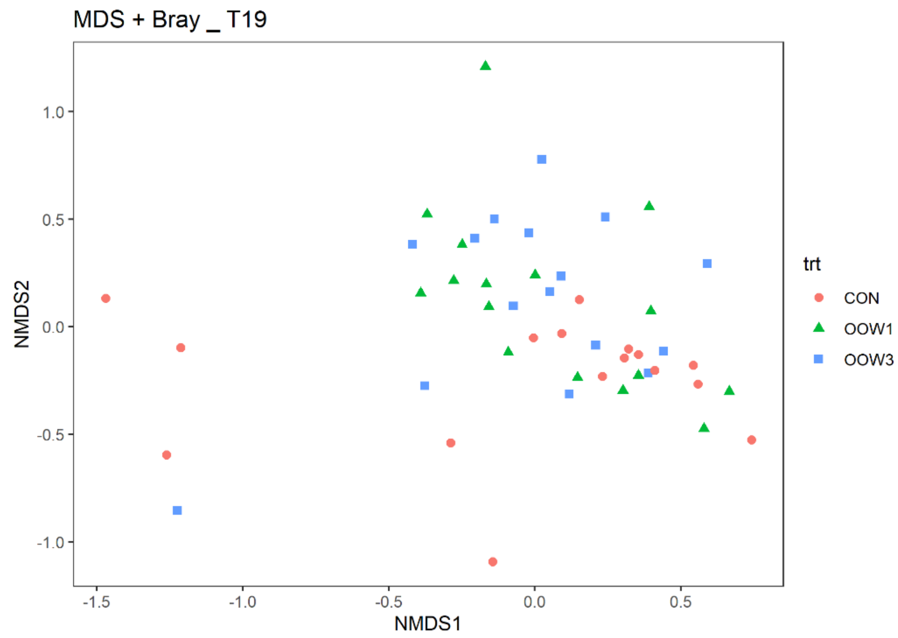

Figure 9. NMDS plot using Bray-Curtis distance metrics for treatment (CON = control, OOW1 = low-dose and OOW3 = high-dose) on day 19 of fecal sampling.

CHAPTER 4

4. Effect of olive oil wastewater in weaned piglets challenged with enterotoxigenic Escherichia coli F4.

4.1. Abstract

Weaning is a delicate phase in the pig's life, animals face different changes at the same time, and this affect their performance and their health. The aim of this study was to evaluate the effect of extracted olive oil wastewater (OOW), known to contain antimicrobial bioactive compounds in weaned piglets infected with ETEC F4. A total of 64 Swiss Large White piglets (weaned at 26 ± 1 d of age, 7.83 ± 1.35 kg of weight) were randomly assigned to two dietary treatments (CON = control diet + 0 % of OOW; OOW = control diet + 2 % of OOW) balanced for litter, sex, body weight, age at weaning and ETEC F4 susceptibility (SS and SR genotype). The body weight (BW), and average daily gain (ADG), were recorded on the day of weaning (d -4), the day of infection (d 0), on the second- and seventh-day post infection (d 2 and d 7). Colony forming unit of ETEC (CFU) were observed after infection on day 0-, 1-, 2-, 3-, and 6-day post weaning, while fecal score (fs) were observed on the same day as well as on day 4(d-4) and 1(d-1) pre infection. The data were analyzed using a model with repeated measure contemplating dietary treatments and using principal components analysis (PCA).

The OOW inclusion were not influence negative effects of post-weaning period. All traits were affected by the age at weaning ($P < 0.02$). Regardless of the dietary treatment, the piglets of the S/S had higher CFU values ($P < 0.01$) than those of the S/R genotype (10.9 vs 9.4 log CFU). These results highlighted the impact of age at weaning and the genotype of piglets on the assessed traits. However, supplementing the starter diet with 2% OOW did not alleviate the ETEC-induced diarrhea.

4.2. Introduction

Weaning is one of the most stressful period in the life of a commercial pig. In the wild, piglets wean themselves at approximately 17 weeks of age, whereas in modern pig production systems weaning occurs 12 to 14 weeks earlier (Barba-Vidal et al., 2018). Thus, at this age pigs are at a very early developmental stage and physiologically not yet prepared for weaning. They have to face various environmental and social stressors cause anorexia, intestinal inflammation and microbial dysbiosis (Lallès et al., 2007a). Ultimately this can result in a condition known as post-weaning diarrhea (PWD), that is mainly caused by enterotoxigenic *Escherichia coli* (ETEC) expressing either F4 or F18 fimbriae (Melkebeek et al., 2013; Luise et al., 2020). The fimbriae allow the bacteria to adhere to the small intestinal epithelium and release of heat labile and/or heat stable toxins, causing water and electrolyte hypersecretion (Lu et al., 2019), which is the main characteristic of PWD. To prevent this and thereby limit important economic losses caused by increased post-weaning mortality rate and impaired growth, prophylactic antimicrobial treatments are used (Kyung-hyo et al., 2020). However, the emergence of antimicrobial resistance (WHO, 2021) pushes the pig industry to find alternative ways to solve this issue. The potential of bioactive compounds from a variety of herbs and spices known for their antimicrobial and antioxidant properties have already been explored (Bruins et al., 2011b; Fiesel et al., 2014; Di Giancamillo et al., 2015; Rossi et al., 2019; Girard et al., 2020). Olive oil wastewater, a by-product from the olive oil production, the disposal of which represent a major environmental issue (Gerasopoulos et al., 2015), is also very rich in bioactive molecules. Approximately 53% of the phenolic compounds present in olive fruit remain in the water phase (Zbakh and El Abbassi, 2012) and some of these phenolic

compounds have bactericidal activity against different type of gram-negative and gram-positive bacteria (Pereira et al., 2006; Abu-Lafi et al., 2017; Pannucci et al., 2019). Instead of being a waste product, olive oil wastewater could be used as a feed additive to overcome PWD.

To test the potential and effectiveness of OOW on reducing the severity or even preventing PWD, a well-established in vivo ETEC challenge model was used Girard and colleagues (2018).

4.3. Material and methods

This study was approved by the Cantonal Veterinary Office of Fribourg (Switzerland [National no.32379_2020_20_FR]).

4.3.1. Animals, dietary treatments and housing

One hundred-thirty one-week-old Swiss Large White piglets were genotyped to determine their susceptibility or resistance to ETEC F4ac following the protocol described by Hu et al. (2019). Briefly, ear biopsies were taken for DNA analysis and to determine the alleles of the CHCF1 (RefSNP rs340488770) and ALGA0106330 markers. Out of the 130, 64 homozygote or heterozygote ETEC F4ac sensible piglets (SS = 21 and SR = 43, respectively) were then selected.

The piglets were weaned at 26 ± 1 day of age (mean ± SD) and an average body weight (BW) of 7.83 ± 1.35 kg (mean ± SD). The 64 piglets were equally allotted within litter, sex, genotype, and BW into two experimental groups. One group was fed a control starter diet (CON) and the other group was offered the same control starter diet supplemented with 2% OOW. The OOW was extracted from the aqueous phase of the processed olive oil according to the method of Servili et al. (2011).

Piglets were raised in 16 small group pens (4 pigs/pen and 8 pens/treatment). Each pen had a total surface of 2.7 m^2, 2/3 of which was a concrete floor equipped with a heated nest and 1/3 was a galvanized steel floor equipped with a single feeder, a nipple drinker, and a drinking trough. Feed and water were provided *ad libitum*. To the drinking water an electrolyte (NaCl hypertonic) was added. During the experiment, an ambient temperature higher than 25° C was guaranteed. Health status of the piglets was observed daily.

4.3.2. Infection

Four days after weaning, piglets were orally challenged with a 1 ml ETEC solution containing 10^8 CFU/ml. The solution was administrated in a gelatin capsule deposited on the tong, using a capsule applicator. The ETEC strain used was isolated from a weaned piglets at the piggery of research center Agroscope-Posieux (Switzerland) and prepared following the method described by Girard et al. (2018).

4.3.3. Growth performance Data

Feed consumption per pen was determined daily (ADFI, kg/day) as the difference between feed offered and leftovers. Piglets were weighted individually at weaning (-4 d prior to infection), on day of infection (d 0), and two and seven days later. Average daily gain (ADG, kg/day) was calculated for each individual pigs for the different time periods.

4.3.4. Slaughtering of the animals

Thirty-two animals (16 for treatment) were slaughtered 7 day post infection for blood samples and enteric contents, small and large intestine specifically. Piglets were euthanized by electronarcosis and bleed by personal trained.

4.4. Sampling and analysis

4.4.1. Feed sampling and analysis

During the experiment feed samples were regularly collected and pooled to 2 samples per treatment for gross chemical analysis. Dry matter (DM) and ash were determined after oven-drying for 24 h at 105° C and 5 h at 505° C, respectively. The crude fiber, crude protein, fat and mineral content were analyzed using the ISO 6865:2000, AOAC International 2000 (method 988.05), and ISO 6492:199 and European Standard EN 155510:2008 procedures, respectively. The ingredients and the chemical composition of the two diets are shown in Table 1.

4.4.2. Analysis of the fecal and intestinal content and fecal score

Feces of each piglets were taken from the rectum using a sterile swab on 0, 1-, 2-, 3- and 6-day post infection. Samples were processed for 2 h at room temperature in 500 μL of Phosphate Buffered Saline 1× (PBS). Each sample was diluted in triplicate to a final concentration of 10^{-7}. Subsequently 4 μL of the dilution was distributed as a droplets on an Eosin Methylene Blue (EMB) agar plate (Oxoid CM0069, UK) supplemented with 50 μg/ml of rifampicin. Before counting the colony forming units (CFU) of ETEC F4ac, the agar plates were incubated for 18 h at 37° C. The consistence of feces of all piglets were assessed on day -4, -1, 0, 1, 2, 3, 6 using a 4-scale fecal score sheet (1 = mold feces, 2 = creamy feces, 3 = liquid diarrhea, 4 = watery adapted from Li et al. (2011)). The results of the fecal score were used to calculate the days on diarrhea and the percentage of piglets with diarrhea, considering diarrhea when the fecal score was equal to or greater than 3.

The dry matter content of the intestinal content (small intestine = SI and large intestine = LI) was determined after freeze drying. The CFU of ETEC F4ac were determined in the SI and LI as previously described for the feces.

The metabolites present of the intestinal content was identified. One hundred mg of samples were extracted using 0.5 mL a methanol and chloroform mixture (1:1, v/v). Before the extraction, 10 μL of a 1 mg mL^{-1} succinic acid 2,2,4,4-d4 aqueous solution were added in each sample as internal standard. Samples were then vortexed 3 times for 1 min every 15min. Following the addition of 760 μL chloroform and 90 μL aqueous 0.2 M KCl, samples were vortexed again and then centrifuged for 15 min at 15,294× *g* (Eppendorf 5810R, Milan, Italy) at 4 C. Two hundred microlitres of the hydrophilic supernatant were dried in glass vials using a gentle nitrogen stream and then derivatised using 50 μL methoxamine chloride dissolved in pyridine at 10 mg mL^{-1}. After 17 h, 100 μL MSTFA were added to the samples and then after 1 h, 800 μL hexane were added.

Derivatised samples were analysed with a Thermofischer TRACE 13300 gas chromatograph and a TSQ9000 mass selective detector. One μL of derivatized samples were injected in split-less mode and separated using a 30 m × 0.25 mm × 0.25μm DB-5MScolumn (Agilent Technologies, Palo Alto, CA). The temperatures for the inlet, interface, and ion source were 200, 250 and 230 °C, respectively. The oven temperature was set at 50 °C for 10 min programmed to increase to 300 °C over 35 min and kept at this temperature for 4 min. The mass range was set between 50 and 550m/z. The identification of metabolites was performed using a co-chromatography approach with analytical standards and by comparison of their mass spectra with the NIST08 library of the National Institute of Standards and Technology (Gaithersburg, MD, USA), and a library developed at the Max Planck Institute of Golm

4.4.3. Determination of blood barrier oxidant

Thirty-three samples of porcine serum were analyzed to evaluate blood antioxidant barrier by means of the Oxy-Adsorbent test (Diacron International- Grosseto, Italy). The antioxidant power of the barrier in the serum consisting of albumin, bilirubin, uric acid, thiol groups, vitamins, glutathione, glutathione peroxidase, superoxide dismutase, catalase by measuring the ability of such barrier to oppose the massive oxidant action of hypochlorous acid (HCLO) to oxidize the complete pool of antioxidant. Control is a serum with known concentration (340 µmol HClO/L), standard were scalar dilutions (1:2) starting from known concentration serum.

According to protocol each serum sample, standard and control was diluted 1:100 and 2 µl of them plus a blank were put on microplate wells. A calibration curve was built to calculate final concentration ($R^2 = 0.98$). Then 200 µl of chromogen were used to fill each well and so the plate was incubated at 37°C for 10 minutes. Finally, 2 µl of the second alkaline chromogen stopped the reaction and the microplate were read at 505nm. Results are expressed as µmol HClO/ml. Intra- and inter-assay coefficient of variation (CV) were 2.7% and 5.10%, respectively.

4.5. Statistical analysis

The body weight, ADG, fecal score, CFU of ETEC F4ac and antioxidant power were analyzed as a completely randomized design with nlme (2020) of R software (R Core Team, 2020). The mixed linear model included treatment, experimental day, genotype, sex, and the following interactions: sex x treatment, experimental day x treatment and genotype for treatment as fixed effect and animal within pen as random effect. The same model was used for ADFI using pen as random effect. Weaning age was used in the model as a covariate. To normalize the data, ETEC F4ac CFU count was log transformed before the statistical analysis. Generalized

linear mixed model with a logit link function and binomial distribution, using the MASS package (7.3-51.1), was applied to calculate the percentage of piglets with diarrhea. Number of days with diarrhea was obtained with the Quasi-Poisson distribution. Odd ratios from logistic regression were used to estimate the probability of developing diarrhea. Means were separated using Tukey's test, and variability was expressed as the standard errors of the means (SEM). Mean difference were considered significant when $p < 0.05$ and defined as a trend at $p < 0.10$.

Difference between intestinal content on small and large intestine were analyzed using Principal Component Analysis (PCA). The PCA was carried with the *prcomp* function of R software using all samples (n= 62), the small intestine only (n = 31), or the large intestine only (n = 31). Variables were scaled to have unit variance before the analysis.

4.6. Results

After the ETEC F4ac challenge, one CON piglet died and was excluded from the data analysis. The dietary treatment had no influence on piglets BW (P = 0.95; Table 2). As expected, the BW increased from 0 to 7 d (P < 0.05). However, from day of infection (d 0) to day 2 post infection the BW remained unchanged. Weaning age, genotype and sex had no effect on BW development (P = 0.13, P = 0.86, P = 52, respectively). The OOW supplementation had no effect on ADG (P = 0.68) and ADFI (P = 0.59), while ADG and ADFI were affected over time (P < 0.001). In both case, ADG and ADFI, were greater from day 2 to day 7 post infection compared to day -4 to day 0 and day 0 to day 2. (Table 3 and 4). In figure 1 is represented feed consumption per pen over day. Weaning age influenced ADG (P = 0.02) as pigs weaned at 27 days of age grew faster than pigs weaned at 25 or 26

days of age. (70.66, 24.50, 24.82 g/d, respectively). The number of ETEC F4ac CFU was influenced by time of sampling (P <0.0001), genotype (P = 0.02) and weaning age (P = 0.01; Table 5). Regardless of dietary treatment, ETEC F4ac excretion was maximal on day 1 and 2 post-infection and then decreased on day 3 and on day 6. The ETEC F4ac CFU was lower in SR than SS piglets (P = 0.02). Fecal score was influenced by the time of sampling (P < 0.0001), as it increased from the day of weaning reaching the greatest scores at 3 days post-infection (Table 6). Weaning age affected the fecal score (P <0.0001), being lower in piglets weaned at 27 days of age (2.26) than in piglets weaned at 25 days of age (2.88). The number of days on diarrhea was not influenced by the dietary treatment (P = 0.17), but weaning age influenced the duration of diarrhea (P = 0.0001; Table 7). Piglets weaned at 25 and 26 d had more days with diarrhea (4.4 and 4.10) than piglets weaned at 27 d (2.8 days). The ETEC F4ac CFU determined in the SI (Table 8) and LI (Table 8) were not influenced by the OOW inclusion (SI; P = 0.36; LI; P = 0.17). However, in the SI a tendency for a treatment × sex interaction existed (P = 0.06) for the ETEC CFU; the CFU in the feces of castrates was lower than in females (48 vs 345) in the OOW group, while in the CON group the ETEC CFU in the feces of castrates was greater than in females (131 vs 65).

Oxy concentration was equal in both treatment groups, 319 µmol of HClO/ml in CON pigs and 313 µmol of HClO/ml in OOW pigs (P = 0.31; Table 10). When age at weaning increases, there is an increase in the plasma antioxidant barrier (P = 0.0009). The PCA of data from intestinal components present in the SI and LI identified fifteen principal component accounting for 80% of the variance. Principal component (PC) 1 and PC 2 explained 28 % and 10 % of variance, respectively, and other PC less than 7 %. As shown in Figure 2, SI and LI were clearly

discriminated ($P < 0.001$). The firsts 10% of compounds discriminated SI and LI: L-proline, methyl-galactose, glycine, D-sorbitol, maltose (isomer 2), 2α-mannobiose (isomer 1), D-gluccose and β-gentibiose. PCA was not discriminated to treatment, sex, and age at weaning.

4.7. Discussion

In this study using the ETEC challenge model we tested the effect of OOW in the diet on PWD, growth performance and antioxidant status. Our results showed no effects of dietary OOW inclusion on growth performance traits like BW development, ADG and ADFI. This is in contradiction to other studies using the same ETEC infection model where hydrolysable tannins, a specific group of polyphenols, positively affected ADG (Girard et al., 2020; Tretola et al., 2019). Nevertheless, Mohammadi Gheisar and Kim (2018) explain how effects of action of bioactive compounds plant extract towards pigs and chicken can be different.

For instance, the bioactive compound can stimulate the secretion of digestive enzymes, or improve palatability of feed, or also increasing feed intake and increasing antimicrobial activity. The explanation could be attributed to the location of functional hydroxyl or alkyl groups in bioactive compounds (Yang et al., 2015). The reason for choosing OOW in this study was based on *in vitro* results published by Carraro et al. (2014) regarding the antimicrobial properties of this agro industrial by-products towards biofilm formation of *Escherichia coli*. However, under *in vivo* condition the supplementation of 2% OOW to the starter diet did not reduce the number of ETEC CFU and had no effect on the fecal score, the latter being a proxy for ETEC induced diarrhea. This outcome confirms the difficulty of extrapolating *in vitro* to *in vivo* responses, one of which could be simply due to the required

concentration of polyphenols having a different effect *in vivo* and *in vitro* (Tretola et al., 2020).

One could question whether the ETEC infection model *per se* worked or not.

The increase of ETEC CFU value within the first two days post infection demonstrates the effectiveness of ETEC infection model. In accordance with the lower fecal ETEC shedding, ADFI was lower in the first 2 days post infection and increased afterwards which coincided as reported by others (Pastorelli et al., 2012; Girard et al., 2018). There is consensus that weaning age affects the incidence of health-related issues of weaning. This is confirmed also in this study as feces of younger animals had greater ETEC CFU values, greater fecal score and more days on diarrhoea. Surprisingly, the maximum difference of age was only 2 days and still significant differences were observed. Untrained gastrointestinal tract is more susceptible to increased permeability of enterotoxin, furthermore higher gastric pH contribute to development of pathogenic bacteria (Heo et al., 2013a). In young piglets the production of hydrochloric acid in the stomach are inadequate, subsequently gastric pH is higher (Ravindran and Kornegay, 1993). This promotes the development of negative bacteria as reported to Heo and coworkers (2013). Weaning stress and immaturity of intestinal tract make younger animals more vulnerable to infection disease (Chen et al., 2017). Moeser and colleagues (2017) detect the first 12 week age as critical windows of postnatal gastrointestinal barrier development. They identify as critical aspect the intestinal barrier permeability, intestinal immune barrier, and immature enteric nervous system. In spite of that in the European Union, piglets are usually weaned about at 28 days of age (Huting et al., 2019), whereas in nature, weaning is around 10-12 weeks of age, gradual process which near to complete maturation of gastrointestinal tract (Moeser et al.,

2017). Our results are in line with Huting and colleagues (2019), according to which increasing weaning age reduced the potential negative effects of weaning.

Oxidative status is given to equilibrium between pro- and antioxidant molecules in organism while their imbalance is known as oxidative stress. The latter it can cause damage of cell constituents including lipids, DNA, proteins and carbohydrates thus leading to tissue damage (Gessner et al., 2017). Recently Corino et al. (2021) showed that weaning can cause oxidative stress in piglets, thus increase susceptibility to disease and reduce growth. Several studies indicated that polyphenols have antioxidant properties (Corino et al., 2007; Zhang et al., 2014; Brenes et al., 2016; Stagos, 2020). Among the polyphenols present in OOW, hydroxytyrosol is considered as one of the most powerful antioxidant presents among *Olea europea* (Pannucci et al., 2019). Gerasopoulos et al. (2015) reported that feeding a 40% liquid permeate or retentate of OOW supplemented diet to broilers improved the redox status of blood. In our results, feeding the OOW supplemented diet had no effect on the antioxidant power of the barrier in serum. Nevertheless, the period of feeding with OOW might have been not sufficiently long to reduce the post-weaning induce oxidative stress. The different age at weaning, associated to a different maturity of the intestinal immune system, can help to understand the differences in the antioxidant capacity: animals weaned later are more developed and therefore they show an increased ability to respond to stress.

In this study, all piglets were genotyped, and only susceptible piglets were taken into account. However, it was not possible to have just SS pigs and so also SR pigs were included. When differentiating between SS and SR pigs, we found that ETEC CFU of ETEC values were greater in the SS genotype, which confirms earlier

findings of Rasschaert et al. (2007). These authors showed that increasing of F4ab ETEC is positive correlated to SR/SS genotype, which could be due to a greater number of receptors.

The PCA analysis highlighted a quite clear separation between large and small intestine, which can be due to a different bioavailability of compounds. Plant polyphenolic compounds, also present in OOW, are absorbed only on 5 – 10% on small intestine. Many polyphenols present in plants are linked to the cell wall constituents (Gessner et al., 2017) and therefore their hydrolysis and liberation are limited in the SI. Thus, the majority of such compounds are absorbed in the large intestine.

4.8. Conclusion

The present study shows that a supplementation of 2% of OOW fed to weaned piglets had no influence on growth performance traits and the incidence of diarrhea. By contrast, small differences in weaning age impacted ADG, ETEC CFU, fecal score and antioxidant capacity of the barrier in serum. Therefore, the maturity of the gastrointestinal tract seems to be of greater importance in countering the negative effects of post-weaning.

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Table

Table 1. Ingredient composition (g/kg) and gross chemical content of the experimental diets[1]

	Treatment[1]	
Ingredient[2]	CON	OOW
Barley	10.00	10.00
Oats	5.00	5.00
Corn	21.39	19.28
Wheat	30.00	30.00
Cerolac	5.00	5.00
Rapeseed oil	0.00	0.83
Extract soya	20.00	20.00
Dried beet pulp	3.00	0.37
L-Lysin-HCL	0.38	0.37
DL-Methionin	0.06	0.06
L-Threonine	0.09	0.09
Monocalcium phosphate	0.76	0.77
Carbonic acid	1.55	1.26
Sodium chloride	0.40	0.37
Ca-Formate	0.66	0.10
Pellan[3]	0.30	0.30
Vitamin-mineral premix [4]	0.40	0.40
Luctarom[5]	0.01	0.01
Globamax performant[6]	0.20	0.20
Natuphos 500[7]	0.01	0.01
Mikroglit[8]	0.60	0.60
OOW[9]	0.00	2.00
Analysed chemical composition (g/kg DM)		

Dry matter (g/kg)	897.40	897.30
Ash	63.00	55.90
Crude fiber	32.90	32.00
Crude fat	29.70	36.10
Crude protein	183.00	184.00
Ca	10.50	8.20
Cu (mg/kg)	13.20	12.20
Fe (mg/kg)	214.00	211.00
K	8.46	8.91
Mg	1.58	1.55
Mn (mg/kg)	38.70	41.80
Na	2.23	2.20
P	5.56	5.72
Zn (mg/kg)	90.90	98.50
DE, MJ/kg[10]	13.80	13.80

[1] CON = starter diet with 0% olive oil wastewater (OOW); OOW = starter diet with 2% OOW.
[2] Each diet was formulated according to the Swiss feeding recommendation for pigs (Agroscope, 2020) and was analysed in duplicate to determine chemical composition.
[3] Pellet binding aid: Pellan, Mikro-Technik; Bürgstadt, Germany.
[4] Supplied per kg of diet: vitamin A, 8000 IU; vitamin D3, 1000 IU; vitamin E, 25 mg; menadione, 3 mg; thiamine, 2 mg; riboflavin, 5 mg; biotin, 0.1 mg; niacin, 20 mg; pantothenic acid, 15 mg; iron, 80 mg as iron sulphate monohydrate; iodine, 0.15 mg as calcium iodate; copper, 6 mg as copper sulphate; manganese, 10 mg as manganese oxide; zinc, 75 mg as zinc oxide; selenium, 0.2 mg as sodium selenite.
[5] Luctaron, Lucta; Montrone del Vallès, Spain.
[6] Globamax performant
[7] Natuphos 5000 = Phytase; 500 units of aspergillus niger phytase/kg diet; 1 phytase unit corresponds to the amount of enzyme that releases 1 µmol P from mM phytate/min at pH 5.5 and 37°C.
8 Colour markers, which helps make visible the different diets.
[9] OOW = Extracted olive oil wastewater.
[10] Digestible energy content estimated according to the Swiss feed database (Agroscope, 2020), considering the relative amount of each feed ingredient in the diet.

Table 2. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on body weight development of the weaned pigs[1].

Treatment	Means, kg	SEM	P-value
CON[1]	8.08	0.35	0.95
OOW[1]	7.78	0.35	
Time			
-4	7.92[b]	0.25	<0.0001
0	7.79[a]	0.25	
2	7.78[a]	0.25	
7	8.25[c]	0.25	
Genotype			
SR[2]	7.93	0.27	0.86
SS[2]	7.94	0.32	
Sex			
F[3]	7.78	0.29	0.52
C[3]	8.08	0.28	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
The results are presented as least squares means ± SEM;
[abc] Within traits, columns with no common superscript differ at $P < 0.05$
Age at weaning (25, 26, 27 d of age) = 0.13;
Treatment × time = 0.77
Treatment × genotype = 0.02
Treatment × sex = 0.76

Table 3. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on average daily gain of weaned pigs[1].

Treatment	Means, g	SEM	P-value
CON[1]	13.8	13.4	0.68
OOW[1]	21.3	13.4	
Time, d			
-4 to 0	-30.32[a]	16.2	<0.0001
0 to 2	-7.42[a]	16.2	
2 to 7	90.41[b]	16.2	
Genotype			
SR[2]	30.74	11.4	0.21
SS[2]	4.38	15.9	
Sex			
F[3]	15.8	12.9	0.92
C[3]	19.3	14.1	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
The results are presented as least squares means ± SEM;
[abc] Within traits, columns with no common superscript differ at P < 0.05
Age at weaning (25, 26, 27 d of age) = 0.02
Treatment × time = 0.91
Treatment × genotype = 0.24
Treatment × sex = 0.38

Table 4. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex ADFI per pen of weaned pigs[1].

Treatment	Means, kg	SEM	P-value
CON[1]	0.353	0.04	0.59
OOW[1]	0.384	0.04	
Time			
-4 to 0	0.255[a]	0.032	<0.0001
0 to 2	0.295[a]	0.032	
2 to 7	0.555[b]	0.032	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
The results are presented as least squares means ± SEM
[abc] Within traits, columns with no common superscript differ at $P < 0.05$

Table 5. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on ETEC F4ac colony forming unit determined in the feces of weaned pigs[1].

Treatment	Means	SEM	P-value
CON[1]	9.86	0.54	0.91
OOW[1]	9.86		
Time		0.54	
0	-	-	<0.0001
1	11.30[c]	0.48	
2	11.14[c]	0.48	
3	9.66[b]	0.48	
6	7.32[a]	0.48	
Genotype			
SR[2]	9.1[a]	0.44	0.02
SS[2]	10.6[b]	0.57	
Sex			
F[3]	9.91	0.50	0.90
C[3]	9.80	0.48	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
the feces collection on day 0 was performed before ETEC F4 infection. Prior to data analysis, the ETEC CFU values were log transformed.
The results are presented as least squares means ± SEM;
[abc] Within traits, columns with no common superscript differ at $P < 0.05$
Age at weaning (25, 26, 27 d of age) = 0.01
Treatment × time = 0.12
Treatment × genotype = 0.21
Treatment × sex = 0.87

Table 6. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on the fecal score of weaned pigs[1].

Treatment	Means	SEM	P-value
CON[1]	2.77	0.09	0.58
OOW[1]	2.69	0.09	
Time			
-4	1.01[a]	0.10	<0.0001
-1	1.64[b]	0.10	
0	1.88[b]	0.10	
1	3.44[c]	0.10	
2	3.61[cd]	0.10	
3	3.79[d]	0.10	
6	3.74[d]	0.10	
Genotype			
SR[2]	2.69	0.07	0.28
SS[2]	2.76	0.08	
Sex			
F[3]	2.71	0.07	0.75
C[3]	2.75	0.07	
Age at weaning[4]			
25	2.88[b]	0.09	<0.0001
26	2.70[b]	0.09	
27	2.26[a]	0.18	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
The results are presented as least squares means ± SEM;
[abc] Within traits, columns with no common superscript differ at $P < 0.05$
[4] Age at weaning (25, 26, 27 day of age)
Treatment × time = 0.90
Treatment × genotype = 0.23
Treatment × sex = 0.27

Table 7. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on number of days in diarrhea, considering a fecal score ≥ 3 as diarrhea.

Treatment	Means	SEM	P-value
CON[1]	3.97	0.35	0.49
OOW[1]	4.16	0.35	
Genotype			
SR[2]	3.98	0.17	0.38
SS[2]	4.24	0.25	
Age at weaning			
25	4.44[b]	0.21	0.0001
26	4.10[b]	0.19	
27	2.75[a]	0.30	
Sex			
F[3]	4.15	0.22	0.60
C[3]	4.00	0.18	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
[abc] Within traits, columns with no common superscript differ at $P < 0.05$
For fecal score we used this scale: 1 = mold feces, 2 = creamy feces, 3 = liquid diarrhea, 4 = watery.
The results are presented as least squares means ± SEM.

Table 8. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on ETEC F4 CFU determined in the small intestine of pigs slaughtered at 39±1 days of age.

Treatment	Means	SEM	P-value
CON[1]	28.1	99.4	0.36
OOW[1]	138.1	98.0	
Genotype			
SR[2]	109	84.4	0.37
SS[2]	57	115.7	
Sex			
F[3]	145.1	101.1	0.36
C[3]	21.1	89.1	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
Results on the gut content are from16 OOW and 15 CON pigs.
The results are presented as least squares means ± SEM;
Age at weaning (25, 26, 27 d of age) = 0.25
Treatment × genotype = 0.27
Treatment × sex = 0.06

Table 9. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on ETEC F4 CFU determined in the large intestine of pigs slaughtered at 39±1 days of age.

Treatment	Means	SEM	P-value
CON[1]	30.6	332	0.17
OOW[1]	685.8	327	
Genotype			
SR[2]	321	288	0.85
SS[2]	396	403	
Sex			
F[3]	291	352	0.75
C[3]	425	308	

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
[2] SR = heterozygote sensible/resistant; SS = Homozygote sensible.
[3] F = female; C = castrates.
Results on the gut content are from16 OOW and 15 CON pigs.
The results are presented as least squares means ± SEM;
Age at weaning (25, 26, 27 d of age) = 0.46
Treatment × genotype = 0.80
Treatment × sex = 0.97

Table 10. Effect of 2% olive oil wastewater (OOW) extract supplementation, measuring time, genotype, and sex on plasma antioxidant barrier using the Oxy-Adsorbent test determied in pigs slaughtered at 39±1 days of age[1].

	Treatment			P-value				
	CON	EOOW	SEM	Trt	Sex	Gen	Trt × sex	Trt × Gen
Oxy Conc. [2]	319	313	25	0.31	0.82	0.60	0.03	0.58

[1] CON = starter diet with 0% OOW; OOW = starter diet with 2% OOW.
The results are presented as least squares means ± SEM; Weaning age was considered as a covariate. P = 0.0009.
[2] Oxy absorbent concentration is expressed as µmol of HClO/ml of sample.

Figure

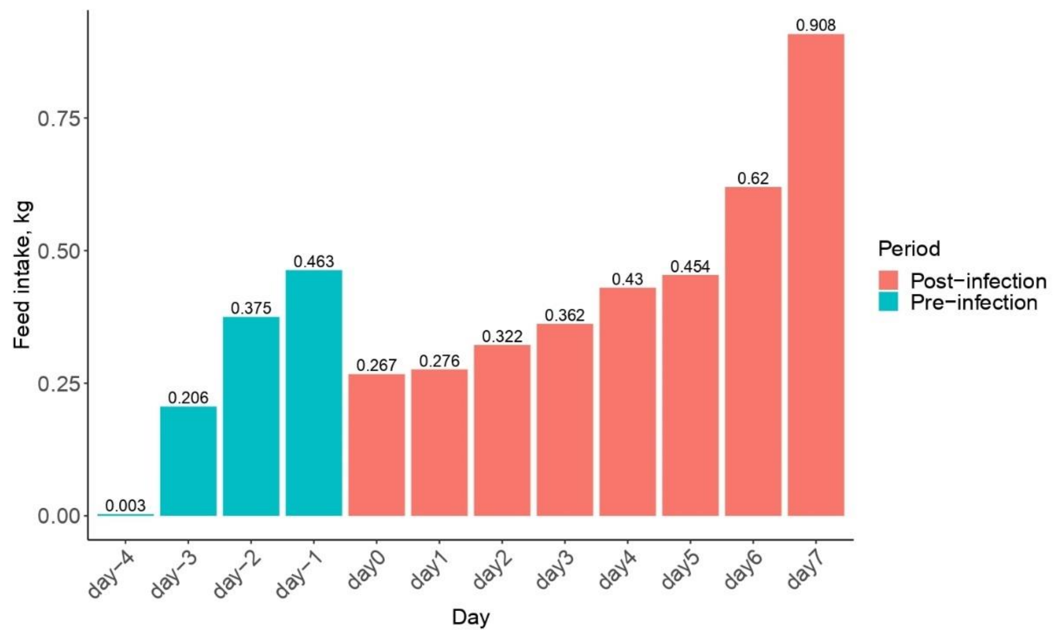

Figure 1. Daily feed consumption for pen blue bars indicate pre-infection average daily intake and red bars indicate average daily consumption post-infection.

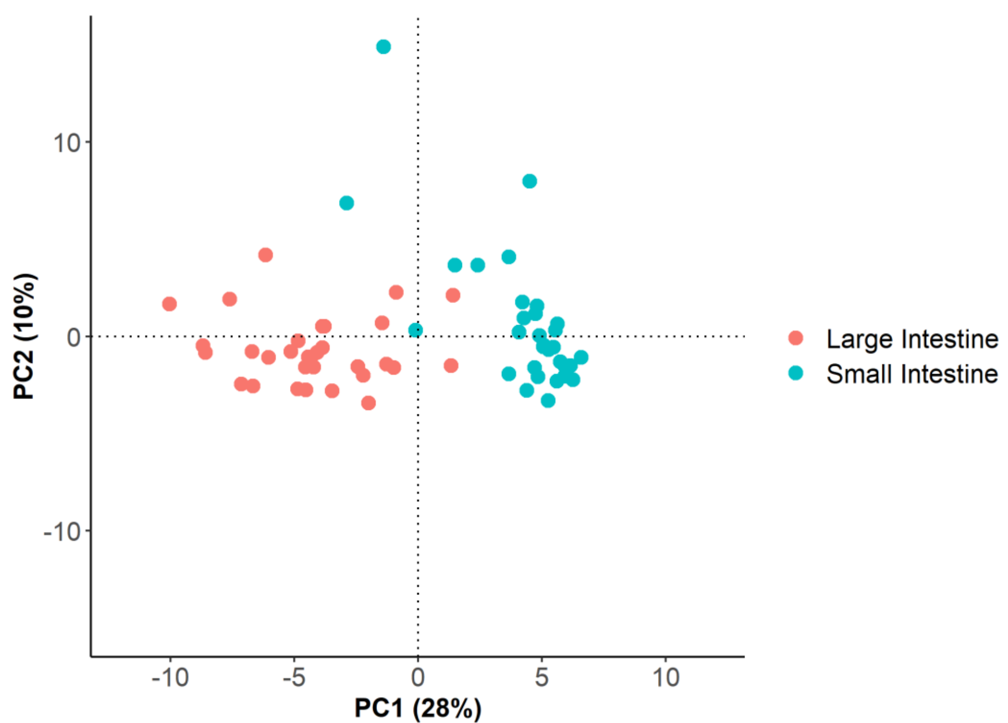

Figure 2. Scatterplot of the first two principal components that segregate the small and large intestine.

GENERAL CONCLUSION

The constant focus on antibiotic resistance has prompted researchers around the world to find alternative solutions whenever possible.

In the livestock sector, prevention further improvement of animal welfare and farm management are the aspects to work on.

This study suggests that the use of olive oil wastewater in the diet of piglets alleviates the negative effects of the weaning phase, such as the percentage of diarrhoea and the number of days in diarrhoea.

In addition, *in vitro* study confirms the antimicrobial capacity versus *Escherichia coli* F4, one of the most responsible for post-weaning diarrhoea in piglets.

The corporation of olive oil wastewater in young piglets increased bacterial diversity. Hight levels of bacterial diversity are associated with functional redundancy, which increases the resilience, resistance, and stability of the gut ecosystem in a stress period.

However, this study confirms the importance of weaning age the formation of the gastrointestinal tract is fundamental to contrast the stress on the delicate phase.

The use of olive oil wastewater in animal nutrition would reduce the amount of waste produced by olive oil production, avoiding environmental pollution problems.