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Green strategies in the finishing pig diet: *in vitro* and *in vivo* studies on the use of an olive mill wastewater phenolic concentrate

Azioni *green* nella dieta del suino pesante: studi *in vitro* e *in vivo* sull'impiego di un concentrato polifenolico da acque di vegetazione

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Abstract

The current growth of the livestock sector has resulted in a higher demand for animal feed, with economic problems for producers. In this context, a recently proposed innovative strategy is the use of alternative natural products, such as the agro-industrial ones, as animal feed supplements, because they are generally nontoxic and already recognized for their high biological value. Furthermore, it is necessary to find a simple and non-invasive approach to address the high yield of such agro-industrial wastes, especially in the main leading producing countries. In this regard, the olive oil production sector, highly developed in the Mediterranean area, produces a substantial amount of by-products (liquid and solid), which imply significant challenges in terms of disposal. Hence, the re-utilization of olive oil wastes can help in their recycling, supporting the transition towards a circular economy.

In addition, by-products from the olive oil industry are considered excellent sources of bioactive molecules, particularly polyphenols, which, for their hydrophilic nature, are usually retained within them up to 90-95%.

Phenolic compounds have been predominantly investigated *in vitro* and *in vivo* for their antioxidant role, but a variety of studies reported other associated beneficial properties in both humans and animals (antimicrobial, anti-inflammatory, anti-cancer, immunomodulatory, and prebiotic functions). These molecules can regulate the oxidative status of monogastric animals and the stability of meat and dry-cured derived products, enhancing their nutritional and qualitative properties. Research into the inclusion of polyphenol-rich products - particularly those from olive oil extraction - in monogastric animal diets is currently ongoing, with emphasis on enhancing animal health and welfare, as well as post-slaughter traits, following the necessities of the modern consumer.

The present doctoral thesis aimed at investigating the effects of an inclusion with a phenolic concentrate from an olive oil bio-waste, olive mill wastewater (OMWW), in the diet of finishing pig.

In Chapter 1, an overview of the utilization of olive co-products as sources of polyphenols in monogastric animal diets (swine, poultry and rabbit) is reported, focusing on their beneficial effects on productive performance and meat quality traits, as well as on antimicrobial, prebiotic, and gene regulatory effects. Notably, polyphenols, thanks to their antioxidant power, may preserve animal oxidative state and the stability of meat and meat-derived products.

In Chapter 2, the *in vitro* effects of a OMWW phenolic product were tested in a continuous porcine jejunal epithelial cell line following *Salmonella typhimurium* infection, through an evaluation of the inflammatory response in terms of gene expression and cytokine production. Polyphenols from OMWW demonstrated to reduce the cellular invasiveness of the intestinal bacterium, simultaneously modulating the expression of many innate immune genes. The phenolic extract had no effect on the cytokine content of pig intestinal cells.

In Chapter 3, the role of dietary supplementation with a polyphenol extract from OMWW was explored, for the first time, on the intestinal ecosystem and morphological characteristics of finishing pigs. Results demonstrated a favorable modulation of gut microbiota diversity and functionality, characterized by an increase in beneficial bacterial genera (*Eubacterium* and *Treponema*) and a reduction in harmful microorganisms (*Fusobacterium*, *Bacteroides helcogenes*, *Corynebacterium urealiticum*). Furthermore, significant intestinal morphological improvements were reported, further supporting the health-promoting effects of polyphenols in swine gut.

In Chapter 4, the same dietary OMWW phenolic extract was investigated on *in vivo* performances, morphology and backfat thickness on heavy finishing pigs, as well as on oxidative

status and quality of meat and a seasoned product (the dry-cured ham). OMWW phenolic compounds did not negatively affect pigs' growth performances, confirming the safety of the extract in animal feed, and showed to partially improve the antioxidant capacity of blood and liver. Interestingly, these molecules also showed to increase water retention in both raw and cured meat, that may positively impact the physico-chemical and sensorial characteristics of the final product. Notably, qualitative analyses demonstrated several modifications in color traits including the yellowness, which is correlated to the acidic composition of fat and oxidative phenomena naturally occurring in meat and dry-cured derived products. By reducing yellowness, polyphenols showed to potentially protect dry-cured ham from lipid oxidation, which is responsible for the development of rancidity and, consequently, worsening of the sensorial characteristics of the final product.

The presented studies, supported by evidences from scientific literature, suggest that the incorporation of olive oil by-products and derived extracts into swine diet is a sustainable practice that can have beneficial effects on monogastric animal health and the technological quality of meat and dry-cured products, and eventually affecting consumer acceptability.

However, the efficacy of dietary enrichment may depend on the phenolic molecules contained and the dosage selection, which emphasizes the necessity for more fine-tuned studies.

Riassunto

La crescita del settore zootecnico comporta un aumento della richiesta dei mangimi destinati all'alimentazione animale, con risvolti di natura economica per gli allevatori. In questo contesto, una strategia innovativa recentemente proposta è l'utilizzo di prodotti naturali alternativi, tra cui quelli agro-industriali, in quanto per lo più privi di tossicità e già conosciuti per il loro elevato valore biologico. Inoltre, risulta quanto mai necessario trovare un approccio semplice e non invasivo allo smaltimento della notevole quantità di tali scarti agroindustriali, soprattutto nei principali Paesi produttori. A questo proposito, va ricordato che il settore della produzione dell'olio d'oliva, altamente sviluppato nell'area del Mediterraneo, produce un'ingente quantità di sottoprodotti (liquidi e solidi) che comporta grandi problemi in termini di smaltimento. Il riutilizzo degli scarti derivanti dalla produzione dell'olio d'oliva può sicuramente agevolare il processo di riciclo, supportando la transizione verso un modello di economia circolare. I sottoprodotti dell'industria elaiotecnica sono inoltre considerati delle fonti eccellenti di molecole bioattive, in particolare di polifenoli che, a motivo della loro natura idrofila, si concentrano in essi in maniera particolare (fino al 90-95%).

I composti fenolici sono stati studiati prevalentemente *in vitro* e *in vivo* per la loro capacità antiossidante, ma diversi studi hanno esplorato altre proprietà benefiche correlate, sia nell'uomo che negli animali (funzioni antimicrobiche, antiinfiammatorie, antitumorali, immunomodulanti, prebiotiche). Tali molecole sono in grado di influenzare lo stato ossidativo degli animali monogastrici e la stabilità della carne e dei prodotti derivati stagionati, migliorandone le caratteristiche nutrizionali e qualitative. Recentemente, numerosi studi sono stati condotti sugli effetti dell'inclusione di prodotti ricchi di polifenoli, in particolare quelli derivanti dall'estrazione dell'olio d'oliva, nella dieta dei monogastrici; l'interesse si è focalizzato sul miglioramento della salute e del benessere animale, nonché sui parametri post-macellazione, così come richiesto dalle necessità del mercato.

La presente tesi di dottorato ha avuto l'obiettivo di studiare gli effetti dell'inclusione di un concentrato polifenolico di un sottoprodotto derivante dall'estrazione dell'olio d'oliva, le acque di vegetazione, all'interno della dieta del suino pesante.

Nel Capitolo 1, viene presentata una panoramica dell'utilizzo dei sottoprodotti dell'oliva come fonti di polifenoli nelle diete degli animali monogastrici (suino, pollo e coniglio), con particolare attenzione agli effetti benefici sulle performance produttive e caratteristiche qualitative della carne, nonché sugli effetti antimicrobici, prebiotici e di regolazione dell'espressione genica. In particolare i polifenoli, grazie al loro potere antiossidante, possono contribuire al mantenimento dello stato ossidativo degli animali e alla stabilità della carne e dei prodotti derivati.

Nel Capitolo 2, gli effetti *in vitro* di un prodotto fenolico da acque di vegetazione sono stati testati su una linea continua di cellule epiteliali intestinali di suino sottoposte ad un'infezione con *Salmonella thyphimurium*, valutando la risposta infiammatoria in termini di espressione genica e produzione di citochine. Il trattamento con i polifenoli delle acque di vegetazione ha comportato una riduzione della capacità invasiva del microrganismo all'interno delle cellule, a cui è risultata correlata una modulazione dell'espressione di alcuni geni coinvolti nella risposta immunitaria innata. L'estratto polifenolico non ha avuto effetto sulla produzione di citochine.

Nel Capitolo 3, il ruolo dell'integrazione alimentare con un estratto di polifenoli da acque di vegetazione è stato indagato, per la prima volta, sull'ecosistema intestinale e sulle caratteristiche morfologiche di suini in finissaggio. I risultati hanno dimostrato un'influenza positiva sulla diversità

e funzionalità del microbiota intestinale del suino, caratterizzata da un aumento di determinati generi (*Eubacterium* e *Treponema*) e dalla riduzione di alcuni batteri considerati nocivi (*Fusobacterium*, *Bacteroides helcogenes*, *Corynebacterium urealiticum*). Inoltre, sono stati riportati dei miglioramenti morfologici a livello della mucosa intestinale, a ulteriore supporto degli effetti favorevoli già osservati sul microbiota.

Nel Capitolo 4, lo stesso estratto di polifenoli da acque di vegetazione, utilizzato come integratore alimentare, è stato studiato sulle performance *in vivo*, caratteristiche morfologiche e sullo spessore del grasso dorsale di suini pesanti, nonché sullo stato ossidativo e sulla qualità della carne e di un prodotto stagionato, il prosciutto. I composti fenolici dalle acque di vegetazione non hanno influenzato negativamente le performance produttive dei suini, confermando la sicurezza di impiego di tale estratto nei mangimi per animali e hanno dimostrato di migliorare in parte la capacità antiossidante nel sangue e nel fegato. Risulta interessante come queste molecole abbiano dimostrato di aumentare la ritenzione idrica sia nella carne che nel prodotto stagionato, con un potenziale impatto positivo sulle caratteristiche fisico-chimiche e sensoriali del prodotto finale. In particolare, l'analisi della qualità di questi prodotti ha riportato una serie di modifiche nei parametri del colore, incluso il colore giallo, che è correlato alla composizione acidica del grasso e ai fenomeni ossidativi che avvengono naturalmente nella carne e nei prodotti derivati. Riducendo la componente gialla del prosciutto crudo stagionato, i polifenoli hanno dimostrato un potenziale effetto protettivo contro l'ossidazione dei lipidi responsabile dell'irrancidimento e, di conseguenza, del deterioramento delle proprietà organolettiche del prodotto finale.

Gli studi presentati, supportati dalle evidenze della letteratura scientifica, suggeriscono che l'inclusione di sottoprodotti dell'olio d'oliva e/o di estratti derivati nella dieta del suino pesante è una pratica sostenibile che può avere effetti benefici sulla salute dell'animale e sulla qualità tecnologica della carne e dei prodotti stagionati, influenzando pertanto l'accettabilità da parte del consumatore finale. Tuttavia, l'efficacia di tale intervento alimentare può dipendere dalla tipologia dei polifenoli presenti e dalla scelta della dose, il che sottolinea la necessità di ulteriori studi a riguardo.

Aim of the study

The general aim of the project is to point out the relevance of the dietary use of a bio-waste from olive oil industry, the olive mill wastewater, on heavy finishing pigs, promoting the application of natural antioxidant sources on livestock feed. The beneficial properties of this product are indeed related to its high content of polyphenols, bioactive molecules which are mainly known for their antioxidant role. Furthermore, the utilization of olive oil industry derivatives can have a positive economic and environmental impact, following the principle of the three Rs (reduce, reuse and recycle).

For this purpose, the objectives of this thesis are:

1. Evaluation of the *in vitro* response of epithelial cells pre-treated with OMWW polyphenols and following an infection with *Salmonella typhimurium*, focusing on the immunomodulatory, anti-inflammatory and antimicrobial capacities of these phenolic molecules;

2. Evaluation of the impact of dietary OMWW polyphenols on animal welfare and intestinal health of finishing pigs, through an examination of gut morphology and analysis of intestinal microbiota composition (richness, diversity and differential abundance);

3. Evaluation of the effects of the dietary OMWW phenolic inclusion on *in vivo* productive performances, quality traits and oxidative status of pork meat and dry-cured ham, following the entire heavy pig supply chain.

CHAPTER 1

GENERAL INTRODUCTION

Dietary supplementation with olive oil co-products rich in polyphenols: a novel nutraceutical approach in monogastric animal nutrition

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Dietary supplementation with olive oil co-products rich in polyphenols: a novel nutraceutical approach in monogastric animal nutrition

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Abstract

In recent years, the increased demand for agri-food products to feed livestock species has stimulated research to identify novel solutions for the valorisation of natural waste, according to the modern concept of a circular economy. Numerous studies have shown the use of plant-derived and agro-industrial co-products that are sources of bioactive molecules for preparing animal feeds. Supplementation with co-products derived from the extraction of olive oil (i.e. olive pomace, olive mill wastewater, olive cake and olive leaf) in diet has been widely considered in recent decades, because these wastes are produced in high quantity and their re-use represents an innovative economic and environmental strategy. Olive oil co-products are characterized by various bioactive molecules such as polyphenols, carbohydrates, proteins, and lipids. Among them, polyphenols are the nutraceuticals most studied, showing to promote health effects in both humans and animals. Olive oil co-products and their phenolic extracts have shown many beneficial and promising effects when added to the diets of monogastric animals, by improving performance parameters and maintaining the oxidative status of meat and derived products. This review provides an update on the use of olive co-products in monogastric animal (swine, poultry and rabbit) diets and their effects on the productive performance, meat quality characteristics and gut health status.

1. Introduction

The global consumption of olive oil has grown over the past three crop years due to its high nutritional value and health benefits, and should reach about 3.055 million tons in the 2022/2023 (1). It is known from the International Olive Council (IOC) that there are more than 800 million olive trees in the word, covering an area of 10 million hectares (2), and that the Mediterranean region accounts for 97% of global olive oil production (1). In Mediterranean countries, there are significant differences in the extraction methods of olive oil in different areas. The three-phase centrifugal extraction method is the most common; other techniques are the two-phase extraction and traditional discontinuous pressing (3). The three-phase decanter has become popular for its ability to decrease labour requirements and simultaneously enhance processing capacity and production of oil. However, it requires a large amount of energy and water during malaxation and generates large quantities of wastewater during the production process. The two-phase decanter separates the liquid part (the oil) from a moist solid part without requiring water, resulting in oil with high antioxidant and aromatic compound levels. However, the two-phase process typically necessitates higher energy consumption and entails a certain degree of loss of oil, because it remains in the pomace (4).

Each production process generates high amounts of co-products, mainly solid and liquid residues including olive cake, olive mill wastewater (OMWW) and olive pomace, which pollute the environment and create disposal problems (5). After two-phase centrifugation, the percentage of wet olive pomace, extra virgin olive oil (EVOO) and OMWW are 78.3%, 18.7% and 3%, respectively. After three-phase centrifugation, the percentages of wet olive pomace, EVOO and OMWW are respectively 37.6%, 17.4% and 45% (4).

In the last decades, innovative strategies including the reutilization of these wastes have become necessary, in order to reduce a negative impact on the ecosystem (6). Interestingly, these co-products can be regarded as excellent economic sources for their content in various beneficial compounds including proteins, lipids, fibres (lignin, cellulose, hemicelluloses, and pectins), polyphenols (phenolic alcohols, phenolic acids, secoiridoids and flavonoids) and other phytochemicals such as tocopherols, triterpenoids and carotenoids (7–9). The content of bioactive molecules differs according to the type of product, and for this reason it is important to consider their individual features and composition, in order to find the most useful applications in different fields (7).

Extra virgin olive oil include a number of polyphenols, among which the phenyl alcohols tyrosol and hydroxytyrosol and the secoiridoid oleuropein, which are abundant and have a considerable antioxidant and antimicrobial activity (10). After extracting olive oil, some olive oil remains in the liquid and solid phases, and these co-products retain about 98% of total phenolic compounds (11). Thus, co-products of olive oil, including OMWW and olive pomace, are considered an excellent source of polyphenols (3.0–50.0 g/kg of dry matter [DM]) (11).

Among the various bioactive molecules contained in olive oil co-products, polyphenols are the most recently studied and recovered molecules, which in several human and animal studies showed multifunctional effects that need to be further investigated (8).

Polyphenols occur naturally in plants either as free aglycones or as esters with polysaccharides or monosaccharides (12). The polyphenols encompass over 8,000 molecules that are characterised by phenolic rings with one or more hydroxyl groups, which are crucial in determining their antioxidant functions (13). Hydroxytyrosol and tyrosol are the major components found in OMWW that could be exploited as high-value compounds (13,14). Oleuropein and hydroxytyrosol represent as much as 10% of the composition in fresh olive leaves (15), while tyrosol and hydroxytyrosol can also be found in leaf extract and olive cake (16). Furthermore, oleuropein is the main polyphenol in olive leaf extract (17), and dried olive pulp also contains antioxidant phenols such as oleuropein and hydroxytyrosol (10). Membrane technologies such as microfiltration, ultrafiltration and nanofiltration have recently been developed to extract phenolic compounds from olive oil co-products. This innovative method could be a valorisation to make the co-products of olive oil a nutritional source of high economic

value. Moreover, the novel recovery processes concerning olive oil co-products, in general, helps also to simplify their disposal and management (18).

Phenolic compounds show important biological effects, including anti-inflammatory, antioxidant, antibacterial, antiproliferative, antifungal and hypoglycaemic activities (19). In particular, based on their antioxidant and antimicrobial power, they have become increasingly important for livestock. Olive oil co-products could provide an advantage for the agri-food and zootechnical industries, and their inclusion in livestock feed may provide benefits without compromising their production traits.

The use of these products for animal feeds could be considered a contribution to the circular economy of Mediterranean countries such as Italy, Spain and Greece, which are the main olive oil producers. Moreover, agro-industrial co-products are usually less expensive compared with the feeds traditionally used (20). Regarding animal nutrition, especially for monogastric animals, there has been extensive research into the use of plant extracts high in polyphenols (21). Plant extracts can be regarded as promising alternatives to synthetic antioxidants, given the characteristics of many natural compounds they contain, especially polyphenols (21). The potent antimicrobial properties of phenolic compounds can also potentially reduce the risk of bacterial infections (22). In this regard, it should be useful be remembered that the European Union (EU) has prohibited the use of antibiotics as growth promoters in livestock since 2006 (EC Regulation No. 1831/2003), owing to the potential detrimental impacts on food safety and animal health (23,24). The use of polyphenols as potential natural feed supplements with a role in the immunity, antimicrobial, antioxidant power and overall production performance of swine and poultry has been confirmed by *in vivo* and *in vitro* studies (21,25). Furthermore, dietary supplementation with polyphenols could enhance food oxidative stability originating from livestock animals (26). Phenolic compounds can also be applied as natural compounds protecting animals from the bad consequences of feed component oxidation, particularly when there is an high unsaturated fatty acid content (25). This review summarises the studies available that have examined in monogastric animals the effects of olive oil co-products and extracts as sources of beneficial bioactive molecules, including polyphenols.

2. Chemical composition of olive oil co-products

2.1 Olive pomace and olive cake

Olive pomace is the primary solid product that remains after extracting olive oil from the fruit; it is composed of oil, skin, pulp and pits and has a relatively high water content. It is a good source of beneficial molecules such as carbohydrates, proteins, lipids and polyphenols (7,27). Olive pomace is rich in fibrous compounds such as cellulose, lignin, pectins and hemicelluloses (xyloglucans, xylans, mannans and glucoroxylans) and also contains small amounts of organic nitrogen as well as minerals (especially potassium) (7,27).

Interestingly, this co-product is composed by oleuropeosides, polyphenols and flavonoids. The extract of olive pomace contains at least 10% of triterpenes and 2% of polyphenols (28).

Olive cake is a semi-liquid paste produced by pressing and compressing the olive pomace to extract any remaining oil. The olive cake consists of stone (18%–32%), pulp, olive skin, kernel, a portion of oil, and approximately 40%–60% of water. The olive cake can be regarded as a source of various phytochemicals such as peptides, flavonoids (quercetin), tocopherols and polyphenols (7). Interestingly, vitamin E is a group of exogenous antioxidants including tocopherols that is lipophilic and can work in a synergically with phenolic acids, potentiating its action (29).

Olive cakes devoid of stones exhibit a significant presence of fibre and lignin (160–557 g/kg DM), while the crude protein (CP) content is relatively low and varies from 44 to 115 g/kg DM (30,31). Some olive cake can be used in the diets of finishing pigs after partial defatting. On dry matter basis, olive cake contains 52–92 g/kg of CP, 7.9 g/kg of Ca, 9 g/kg of total phosphorus, 122 g/kg of ether extract, 8.6 g/kg of total polyphenols (gallic acid equivalents) and 170 g/kg of lignin

(32,33). The semisolid destoned olive cake, called pâté, has an olive oil concentration varying from approximately 8% to 12%, depending on the moisture content. Pâté also has high levels of sugars, structural carbohydrates and a moderate CP content. Fatty acids in pâté mainly comprise oleic acid and polyunsaturated fatty acids (34). The main components of pâté are the following: total polyphenols 120 mg/kg, hydroxytyrosol 54 mg/kg, tyrosol 60 mg/kg, pinoresinol 3 mg/kg, and verbascoside 3.5 mg/kg (34).

2.2 Olive pulp

Olive pulp refers to the fleshy part of the olive fruit that is left over after the oil is extracted. It consists of fragments of olive stones, skin and a small portion of olive oil; it is what remains of the olive cake after drying. Olive pulp is rich in essential fatty acids (73% oleic acid, 13% palmitic acid and 7% linoleic acid) and has a high residual oil (35). Because of this high residual content, dried olive pulp could be a low-cost source of energy. Olive pulp contains fibres, whose contents may change depending on the processing (36). It is also composed by protein, fat, calcium, copper and cobalt, even if it does not have a high nutritional value because of its decreased digestible protein and mineral content and a high lignin concentration (36). Dried olive pulp is considered a relevant reservoir of natural antioxidants, with a high concentration of hydroxytyrosol (228.6 mg/kg DM) and oleuropein (1007 mg/kg DM), and has antibacterial and antifungal effects (10). The proximate chemical composition of dried olive pulp includes 88%–95% of DM, 7%–12% of CP, 28%–35% of crude fibre, 7.9–19 g/kg DM of total phenolic compounds, and 229 mg/kg DM of 3,4-dihydroxyphenylethanol (DHPEA) (10,37,38).

2.3 Olive mill wastewater

Olive mill wastewater is the liquid waste generated during the production of olive oil. It consists of the vegetation water of olives diluted during the extraction of oil and has a high polyphenolic content (up to 53% of olive fruit phenolic compounds) for the elevated water solubility of these molecules (39). This liquid product presents a high content of organic compounds such as proteins, lipids, carbohydrates, tocopherols, carotenoids and polyphenols (7).

The amount of OMWW produced every year in the Mediterranean region is approximately 30 million m^3 (40). Polyphenolic powder can be extracted from OMWW by separating the solids; acidifying the solution; extracting with a solvent; purifying through filtration; and precipitating, drying and storing the isolated polyphenols (40). Different extraction processes have a greater impact on the content of phenolic compounds. The content of total polyphenols is approximately 100 mg/g, with hydroxytyrosol and tyrosol being 0.5 and 0.55 mg/g, respectively (41). In terms of g/kg DM, the content of total polyphenolic is approximately 96.6 g/kg, with hydroxytyrosol and tyrosol representing, respectively, 20.8 and 3.9 g/kg of the composition (15).

2.4 Olive leaf extract

Another interesting co-product of olive fruit is the olive leaf extract, which is a bitter-tasting, dark brown liquid derived from olive leaves; it represents approximately 10% of the material deriving from the olive oil press (42,43). Olive leaves are remnants from the agricultural process of harvesting olives, where the olive trees are beaten to collect the fruits. Pruning yields about 25 kg of olive leaves per tree. The chemical composition of olive leaves consists of a high quantity of extractives (36.52%), lignin (39.6%), cellulose (5.7%) and hemicelluloses (3.8%), as well as crude proteins (ranging from 8.10% to 39.6%) (7).

Fresh olive leaves contain approximately 10% polyphenols (17), specifically oleuropein and hydroxytyrosol. After drying for 2 days at 37°C, olive leaves have up to 25 g/kg total polyphenols

and 22 g/kg oleuropein (43). Deriving from the leaves of the olive tree, olive leaf extract is a dark brown liquid with a bitter taste and is also rich in polyphenols, which have a strong antimicrobial effect (44). Among them, oleuropein is the most abundant, with a concentration ranging from 60 to 90 mg/g in dried olive leaves (45).

3. Antimicrobial properties and effects on gut health

In plants and plant extracts, polyphenols are the main secondary metabolites widely known to have antimicrobial action (46). The antimicrobial effects of co-products from olive oil production (especially olive mill waste water and olive leaf extracts) have been related to the presence of phenolic compounds (47), among which oleuropein and hydroxytyrosol are the two most powerful bioactive molecules contained (48,49). Many polyphenols are able to inhibit the microbial growth and also interfere with oxidative reactions (46). Oleuropein can stimulate the increase of nitric oxide inside the macrophages, protecting them from the endotoxins produced by the gram-negative bacterial species (50). Additionally, extracts can be considered more effective than insolated compounds, because each constituent can be affected by the others contained. In this way, the use of extracts, including the ones from olive oil production, is considered essential for enhancing the synergistic actions of their bioactive compounds (49), with possible applications in animal feed as natural antimicrobial compounds (51). Moreover, a greater antimicrobial activity of olive co-products such as olive leaf extract could be attributed to many phenolic metabolites, including the products of oleuropein hydrolysis (elenolic acid and oleuropein aglycon) rather than the oleuropein glucoside form (49).

The antimicrobial effect of natural extracts can be also related to their the ability to change the intestinal bacterial population (52) and/or modulating the immune response in the gut. In the body, the primary defence system against adverse microorganisms and harmful internal and external substances is the intestinal tract. However, oxidative stress can compromise this function, resulting in intestinal cell damage, apoptosis and reduction in tight junction proteins' expression, that contribute to the function of mucosal barrier (53). Gut health is crucial for animal growth and is closely related to intestinal immune function. In farm animals, oxidative stress is often associated with gut dysbiosis and pathogenic infections, which can lead to reductions in the antioxidant capacity and increased lipid peroxidation, potentially resulting in severe conditions like sepsis (54). In broiler chickens and weaned piglets, oxidative distress may also contribute to microbial infections, causing inflammation and a reduction of feed efficiency related to a gut function impairment (15).

Interestingly, polyphenols from olive oil have been found to maintain the integrity of gut barrier by increasing the expression of genes involved in tight junction stability and modulating the oxidative state, immune and inflammatory response in the intestinal epithelium (55). Polyphenols also act during weaning stress in piglets and calves by improving not only nutrient absorption and digestion, but also intestinal barrier and intestinal microbiota function (56). Furthermore, olive oil co-products rich in phenolic compounds can induce a modification of gut microbiota populations not only by decreasing intestinal pathogens (i.e. *Helicobacter pylori* and *Escherichia coli*) (42), but also exerting a prebiotic action in the gut through an induction of the growth of helpful bacteria such as *Clostridium* (44,57) and *Lactobacillus* (44,58). In every animal species, particularly during the first days of life, is important to have a good microbial composition in the gut, in order to maintain homeostasis and avoid intestinal tract disorders that may occur, especially in the early period (59). In piglets, gut microbiota colonization at birth also derives from the microbiota of the maternal intestinal tract (59). For this reason, the increase of *Lactobacillus* spp. and *Bifidobacterium* spp. in the sow's intestinal tract after dietary administration with olive oil co-products (such as olive pulp) is an important strategy to transfer these effects to the offspring (60). In laying hens, supplementation with olive oil co-products (e.g. with fermented defatted olive pomace) in early life induced an increase of the abundance of Firmicutes, Proteobacteria and Actinobacteria, also stimulating bacterial diversity. These actions can strengthen the innate immune response, help to compete with microbial pathogens and could potentially reduce the antibiotic use (61).

3.1 Olive cake

Dietary supplementation of piglets with an extract of olive cake partially restores intestinal villi height and reduces the depth of crypts by alleviating the lipopolysaccharide (LPS)-induced inflammatory response (53). Furthermore, in the same study the olive cake extract group had a reduced impairment of small intestine morphology, showing that this supplementation can contribute to the morphological and structural integrity of intestine by alleviating LPS-induced damage. Additionally, olive cake extract has been shown to increase the amount of the pro-biotic bacteria *Clostridium* and *Lactobacillus* by lowering the pH, displaying a beneficial effect on pig intestine (53). (Table 1).

3.2 Olive mill waste water

Bonos et al. (57) recently demonstrated that silage supplemented with OMWW significantly reduced the amounts of harmful bacteria such as *Clostridium* spp, *E. coli*, and *Campylobacter jejuni*, suggesting a good correlation between gastrointestinal tract modifications and bird health. Olive mill vegetation water is also able to improve meat microbial quality (20). In poultry, dietary treatment with a phenolic extract from OMWW decreased *Campylobacter* spp. prevalence, by controlling the spread of the bacterium when the production lifespan ended, which could reduce the risk of *Campylobacter* infection in processed poultry meat (5) (Table 1).

3.3 Olive leaves

In one study, olive leaf extract dietary supplementation (100 and 200 mg/kg) was more efficient in decreasing the number of total coliform bacteria and *E. coli* compared to a control diet (44). Additionally, feeding broiler chickens with an olive leaf extract reduced the level of total coliform bacteria. This extract exerted also a beneficial effect on the microflora content of broiler chicken ileum (by increasing the concentration of *Lactobacillus*) and could be used as natural antioxidant alternatives to probiotics (44). Olive leaf extracts have also been shown to successfully inhibit *in vitro* the adhesion of *C. jejuni* and hamper its colonisation (62). Olive leaf extract polyphenols exhibit antimicrobial activity against various pathogenic microorganisms, particularly *E. coli*, and the combined phenolics exert antioxidant and antimicrobial effects comparable to or better than individual phenolics (63). Additionally, in broiler chickens, dietary inclusion of different levels of an olive leaf powder improved the intestinal morphology by enhancing the length and surface of villi and the depth of crypts (64).

Table 1. Effects of olive oil co-products on microbial populations.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
Olive leaf extract	97	0.5, 1.0	Ross 308 male broilers	Total aerobic bacteria →, Total coliform bacteria ↓, *Lactobacilli* bacteria ↑	(44)
OMWPE, DOC	0.0125, 0.0625	160, 330	Ross 308 female broilers	*Campylobacter* spp. ↓	(5)

Olive cake	8.6	120	Finishing pigs	Total aerobic bacteria →, *Enterobacteria →, Bifidobacteria →, Lactobacilli →*	(32)
Olive oil cake extract	-	1.0	Weaned piglets (DLL)	*Clostriudium _sensu_scricto_* ↑, *Lactobacillus* ↑	(53)

DLL, Duroc × Landrace × Large White; DOC, dried olive cake; GA, gallic acid; OMWPE, Olive mill wastewater polyphenolic extract. ↑ increased; ↓ decreased; → no difference.

4. Effects on animal performance

A current issue in livestock industry is the promotion of animal performances without increasing production costs, minimising environmental impacts and maximising welfare (65). In the animal body, different stress conditions including farm practices, changes of environment, transportation, lairage and fasting preceding slaughter can disrupt the balance between the antioxidant defence and the production of free radicals. (66). Reactive oxygen species (ROS) and nitrogen species (RNS), the most common groups of free radicals in biological systems, are usually produced at low or moderate levels in livestock animals being exposed to the stress conditions of the extensive or rangeland production (67). The excess of ROS in the body can be detrimental. Moreover, animals with faster growth rates, and thus high metabolic rates, are more prone to oxidative stress (67), causing negative effects on livestock. Accumulating evidence suggests that oxidative stress can reduce the health status, leading to an impaired immune system and lower productivity and welfare (66).

Stress regulates the functions of the cells by inducing physiological or pathological modifications, and consequently affecting metabolism and the fate of nutrients in the body. Furthermore, oxidative stress could induce DNA, lipid and protein damage, changes that are correlated to various harmful consequences which alter animal production and performances (68). This may contribute to deterioration of the qualitative characteristics of meat products (67). Additionally, in piglets, weaning is a very stressful condition characterised by high production of free radicals, leading to all the oxidative-stress induced negative effects (69). Post-weaning stress is often linked to a decreased growth performance, as well as to an higher susceptibility to microbial infections (70,71).

Animal performance and quality of the derived products are markedly affected in monogastrics, especially chickens (18,28,33,44,72) and pigs (43,73,74). On the farm, the use of antioxidants is required during animal life conditions such as reproduction and growth, in order to maintain the oxidative balance in the biological systems of both cells and tissues (67). Recent research has focused on the addition of natural antioxidants in livestock feed, in order to improve the productivity and health of animals, protecting them from the harmful effects of oxidative stress (75). More specifically, the use of phenolic compounds can reduce the oxidative stress-induced damages in animals due to the antibiotic-like action of these molecules (76). In this way, these molecules can also improve the antioxidant potential of products of animal origin, e.g. meat.

Many *in vitro* and *in vivo* animal studies have demonstrated that polyphenols present in olive oil, olive fruit and extracts (such as OMWW) exert strong biological activities, mostly- but not only exclusively- linked to their antioxidant power (77). For example, pretreatment with hydroxytyrosol protects renal cells from membrane oxidative damage induced by free radicals by improving also the morphology and biochemical functions in the proximal tubular epithelium (78). The protective action of hydroxytyrosol from EVOO has been also demonstrated against the damage induced by

ochratoxins in a Madin–Darby canine kidney cell line (MDCK) and rabbit kidney cell line (RK 13) (79).

Regarding the *in vivo* effects, the use of natural plants and their extract rich in polyphenols in diet has been recently considered as a novel strategy in farm animals, in order to improve their growth and reduce mortality (80). Polyphenols can be considered as good promoters of farm animal growth by stimulating saliva, enzyme digestion, secretion of mucus and bile, and by protecting gut morphology thanks to their anti-inflammatory and anti-oxidant action (80). Moreover, the improvement of chicken growth rate is probably related to a reduction of the passage rate induced by polyphenols during digestion, which increases the digestibility of feed (38). Among the co-products of olive oil extraction, olive leaf extract supplementation in animal feed regulates the digestion and enhances digestive juices stimulating appetite and the consumption of food by the animal. This extract may additionally prevent diseases for its antibacterial and antifungal activity, and improves animal performance (72).

4.1 Olive pomace and olive cake

Herrero-Encinas et al. (28) demonstrated that a bioactive olive pomace extract added to chicken diets (750 mg/kg) improved animal growth given its anti-inflammatory activities and did not induce a negative effect on body weight (BW) and feed conversion ratio (FCR) (Table 2). Moreover, olive cake supplementation at 10% to broiler chicken diets significantly improved the FCR (33), while its inclusion up to 15% in laying hen diets increased feed intake (FI) and improved feed efficiency (81). Branciari et al. (34) confirmed these positive effects in poultry: they found that dietary supplementation with a dehydrated olive cake could increase live weights compared to the controls. Dietary olive cake (10%) with citric acid improved final BW and daily and total body weight gain (BWG) in broiler chickens (82). In rabbit diets, supplementation with olive cake at 30% and bentonite led to improvements of final BW, daily weight gain and FCR parameters (83). Olive cake pulp (up to 25%) added in growing rabbits did not induce harmful effects on their growth performance parameters (84). Furthermore, dietary supplementation with 82.5 and 165 g/kg of pâté olive cake led to greater BW in chickens compared with the control diet (34). A diet based on wet crude olive cake in Iberian pigs resulted in a better growth performance compared with dry olive pulp (85), and the addition of olive cake in finishing pig diets also increased FI and BWG (37). Similarly, Joven et al. (74) reported in pigs an higher consumption of feeds and higher growth rates after a dietary supplementation with 5% or 10% of olive cake (replacing an equivalent proportion of barley in the diet) (Table 2). In contrast, during the pig finishing period, dietary supplementation with 100 mg/kg of partially defatted olive cake had no effect on growth performances, showed only a tendency to increase FCR ($P = 0.059$) and significantly reduced loin depth (32).

4.2. Olive pulp

Dietary supplementation with dried olive pulp can be considered a good strategy for feeding slow-growing broilers, as it has no adverse effects on animal productive performance, carcass weight, yield of breast, and fatty acid composition of breast meat (10). An increase in FI has been observed when supplementing broiler chicken diets with olive pulp (37). In addition, researchers reported an increase in BW and BWG of broilers subjected to heat stress after dietary treatment with olive pulp (86).

4.3. Olive mill wastewater

A natural polyphenolic product from OMWW included in post-weaning pig feed induced higher BW and average daily gain (ADG) (69). Furthermore, this phenolic administration improved clinical performance by decreasing the frequency of post-weaning diarrhoea (69). Branciari et al. (5) demonstrated that in poultry, supplementation with an OMWW polyphenolic extract could increase final BW and carcass weight compared to the control. Sabino et al. (87) did not find differences in feed conversion efficiency after dietary supplementation with OMWW in chicken diets compared to controls, but the observed morphological changes in the jejunum of the OMWW supplemented group suggest that this co-product could have a beneficial effect on the intestinal ecosystem.

4.4 Olive leaves

Researchers have demonstrated that olive leaves can be added to feeds without adversely affecting growth performance (88). The healthful effects of the olive leaf extract have been attributed to the known antioxidant, anti-microbial and anti-inflammatory activity (14) (Table 2). Paiva-Martins et al. (43) investigated the effect of the dietary addition of olive leaf extract on feed digestion, growth performance and meat qualitative characteristics of pigs (Table 2). Olive leaf supplementation improved growth performance, with a better feed-to-gain ratio by comparing with a conventional diet. Sarıca and Ürkmez (44) reported in broiler chickens an increase in BWG and a decrease in the FCR during the 6 weeks after supplementation with a composition of either 100 or 200 mg/kg of olive leaf extract (Table 2). In broiler chickens reared in humid and warm temperature, supplementation of drinking water with olive leaf extract at 15 mL/L (containing 66 mg/L oleuropein) improved BW, BWG, FI and the FCR (18). Furthermore, supplementation of growing rabbits with until 1.5 mL/kg of an olive leaf extract improved several performance parameters (final BW and the FCR) (89). The supplementation of an aqueous extract of olive leaf also increased ADG in growing rabbits (90).

Table 2. Effects of olive co-products supplementation on animal growth performances.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
Olive cake	-	100, 150	Ross 308 broilers	BWG →, FI →, FCR ↓	(81)
Olive pomace extract	20	0.75	Ross 308 broilers	ADG↑, FCR ↓, ADFI→, AID →	(28)
Olive pulp	2.41	30, 60	Ross broilers	BW →, FC →, FCR →, hock burn →, feather cleanliness ↑, foot pad dermatitis ↓	(115)
Olive leaf extract	97	0.5,1.0	Ross 308 broilers	BWG ↑, FCR ↓, FI →	(44)
Olive leaf extract	4.4	5, 10, 15	Arbor acre broilers	ADG ↑, ADFI ↑, FCR ↓	(18)
Olive cake	-	50, 100	Ross broilers	BW →, FI →, FCR →, survival rate ↑	(33)
Pâté olive cake	0.17	82.5, 165	Ross 308 broilers	LW ↑, ADG ↑, FCR ↓	(34)

Olive leaf and grape	7	2	Ross 308 broilers	ADG →, ADFI →, FCR →, EBI →	(103)
Olive pulp	-	25-50, 50-50, 50-80	Cobb 500 broilers	ADG →, ADFI →, FCR ↑	(10)
Olive pulp	7.9	50, 100	Hubbard broilers	BW →, ADG →, ADFI →, FCR →, mortality →	(99)
OMWW extract	100	0.2, 0.5	Hubbard-Sasso broilers	BW →, water consumption →	(41)
Olive cake	-	50, 100, 150	Finishing pig	BW ↑, FI ↑, ADG →, FCR →, ADE ↑	(74)
Olive leaf	25	50, 100	Growing-finishing pigs	FW↓, ADG↓, ADFI↓, F/G↑, ATTD↓	(43)
Olive cake	8.6	120	Finishing pigs	BW →, ADG →, ADFI →, FCR →, LD ↓	(32)
Olive cake	-	100	Finishing Bísaro pigs	BW →, pH →, color →, CW →	(116)
Olive cake	-	50, 100	Pietrain pigs	BW ↑, FCR ↑	(117)

ADFI, average daily feed intake; ADG, average daily gain; ADE, apparent digestible energy; AID, apparent ileal digestibility; ATTD, apparent total tract digestibility; BW, body weight; BWG, body weight gain; EBI, European broiler index; FCR, feed conversion ratio; FI, feed intake; FW, feed weight; GA, gallic acid; LD, loin depth; LW, live weight; OMWW, olive mill wastewater. ↑ increased; ↓ decreased; → no difference.

5. Effects on meat quality

Antioxidants can contribute to animal welfare and productivity by delaying or preventing lipid oxidation through the reduction of free radical activity in meat (43). An excess of reactive oxygen species (such as ROS and RNS) reduce the quality of meat, causing many defects in flavour and taste that compromise the biological and reduce meat shelf life (66). Polyunsaturated fatty acids are highly susceptible to oxidation, and oxidation products can destroy the nutritional, chemical and sensory characteristics of meat, especially tenderness, juiciness, flavour, drip loss and shelf life (17,43,91). Nutritional supplementation is a novel strategy that can improve meat stability by changing the profile of fatty acids or the content of tocopherols in the muscle. Notably, the addition of anti-oxidants in animal feed is considered a useful method to increase meat stability (92). Recently, the use of natural antioxidants including polyphenols has been recommended, in order to restrict lipoperoxidation and maintain qualitative characteristics (flavour, colour, tenderness) and shelf life of animal-derived products, ensuring their healthfulness for the consumers (93). Co-products deriving from olive oil could be employed as potential animal nutrients to produce high-quality meat based on their strong radical scavenger activity from its very high levels of polyphenols, including oleuropein, hydroxytyrosol and verbascoside (94). Hydroxytyrosol is able to scavenge the peroxyl radicals near the surface of the membranes, and oleuropein can interfere with their chain propagation (95).

Increased dietary absorption of polyphenols can also exert a protective effect on the low-molecular-weight antioxidant tocopherol by acting as a barrier against the oxidation of lipids (67).

Oxidative stress can be evaluated with the thiobarbituric acid-reactive substance (TBARS) content, which measures lipoperoxidation due to free radical generation. Branciari et al. (34) reported the TBARS content of meat from animals fed with olive polyphenols. They found that this supplementation improved the oxidative status of the meat. Recent *in vivo* studies have reported that co-products from olive oil rich in polyphenols improve the antioxidant state and welfare of monogastric species including chickens, pigs and rabbits, as well as the quality of their meat (34). Tufarelli et al. (38) also demonstrated an improvement of antioxidant status with lower TBARS in the liver of chickens fed with a supplementation of EVOO. Additionally, a reduction in saturated fatty acids and a monosaturated fatty acids increase improves the meat chemical composition, and these effects can be induced by polyphenols from olive oil co-products (32,74) (Table 3). The analysis of fat content in animal-based products usually consists in the evaluation of nutritional parameters including the ratio between polyunsaturated/saturated fatty acids and of n-6/n-3 fatty acids (96), which are important to ensure healthy human nutrition.

5.1 Olive pomace and olive cake

De Oliveira et al. (97) demonstrated that in chickens olive pomace can induce a modification of the lipid composition in meat by increasing the monounsaturated fatty acids content and decreasing the amount of the saturated ones. The same co-product type (from 5% to 16%) added in rabbit diets significantly increased meat monounsaturated fatty acids, with a correlated reduction in the polyunsaturated ones (94). Moreover, a reduced meat peroxidation has been observed in the olive pomace–supplemented group, in comparison to the control (94).

Dietary supplementation of a chicken diet with a high concentration of pâté led to a reduction of TBARS values in meat (34). Increasing levels of olive cake (up to 15%) led to healthy fatty acid profiles in pig fat by promoting a linear decrease in the proportion of total saturated fatty acids and an increase in the percentage of the total monounsaturated ones (74). The addition of partially defatted olive cake in pig diets did not show a significant effect on carcass quality, microbial counts and subcutaneous fatty acids profile, but induced a lower pH and polyunsaturated fatty acid content and higher monounsaturated fatty acid concentration in the meat (32). Additionally, the inclusion of olive cake silage in the diet (up to 40%) of Apulo-Calabrese pigs promoted a higher proportion of monounsaturated fatty acid, especially oleic acid, and a lower concentration of polyunsaturated acids (98).

5.2 Olive pulp

The results reported by Papadomichelakis et al. (10) confirmed that feeding broiler chickens with dried olive pulp can increase the content of monounsaturated fatty acids in meat. The addition of this olive co-product in broiler diets also induce a significant improvement in meat colour, as indicated by higher meat lightness (L*) and redness (a*) values (99). Moreover, in finishing pigs, meat yellowness decreased and meat oxidation stability tended to be improved after dried olive pulp supplementation (73).

5.3 Olive mill wastewater

Olive mill wastewater supplementation in chicken feed can induce a decrease in the oxidation of lipids and proteins in meat without influencing its colour stability. Moreover, piglets fed with an

OMWW polyphenolic co-product showed lesser damages in proteins and lipids related to oxidative stress, indicated by a decrease in TBARS and protein carbonyl contents (100). The addition of a polyphenolic powder from OMWW to chicken diets reduced oxidative stress–induced damage (41). Gerasopoulos et al. (14) indicated that in piglets, feed containing polyphenolic additives from processed OMWW improved the lipid ratio and the quality of the meat. OMWW supplementation into rabbit diets also decreased *Pseudomonas* spp. growth in the meat (101).

5.4 Olive leaves

Olive leaves contain a large amount of polyphenols, which are strong natural antioxidants potentially able to decrease the excess of free radicals and harmful DNA modifications (41). Olive leaf extract could be used to produce high-quality meat due its very high level of polyphenols, including oleuropein (102). Supplementation of pig diets with 5% or 10% olive leaf extract significantly increased α-tocopherol content in *Longissimus dorsi* muscle and backfat compared with a control diet (43) (Table 3). Even if added at low dosages, olive leaf extract is a beneficial source of biologically-active molecules and could increase tocopherols in meat (43). In broiler chickens, dietary supplementation with olive leaf and a grape co-product (2 g/kg) changed breast colour, by increasing its yellowness (b*) values and colour intensity (103), compared with protected sodium butyrate. The same dietary supplementation seemed to decrease drip loss, a change related to enhanced breast meat quality (103) (Table 3). The combination of oleuropein, magnesium, betaine and vitamin E in pig diet could improve the oxidative state and maintain the stability of lipids in meat (66).

6. Effects on gene transcription

Dietary nutrients may modify gene and protein expression and metabolism directly or indirectly (104,105). Diet is one of the external factors that can influence directly the expression of genes through the biologically-active nutrients contained, which interact with transcriptional factors to positively or negatively affect signal transduction pathways (106–108). Moreover, bioactive molecules in food and feed can have an impact on epigenetics, e.g. methylation of DNA and modifications occurring in histones (106). Transcription factors are found in organs which are metabolically active, like liver, adipose tissue and intestines. Their function consists in acting as molecular sensors through a modification of gene transcriptions as a response to changes in nutrient composition (104). Polyphenols can affect various transcription factors and gene expression (109).

Due to their antioxidant properties, polyphenols can inhibit the negative consequences of excessive ROS production. Two essential regulatory mechanisms inside the cells are the gene expression regulation and the adaptive homeostasis, both of which are redox-induced (110). When the oxidation rate is high, a stress response started inside the cell to control free radicals excess and support redox homeostasis (110). Specifically, stress situations stimulate the translocation of transcription cellular factors (like nuclear factor kappa-light-chain-enhancer of activated B cells [NF-κB]) inside the nucleus; they then bind to specific DNA sites to exert a protective function, but can often exert the opposite effect (110). Oxidative stress induces different cellular processes linked to inflammation, proliferation and apoptosis (111). Polyphenols act by reducing ROS and, consequently, inhibit NF-κB, the most important regulator of the transcription of inflammatory markers (interleukins, tumour-necrosis factor etc). Cappelli et al. (111) showed for the first time the downregulation of the tumour necrosis factor-alpha (*TNFA*), advanced glycosylation end-product specific receptor (*AGER*) and BCL2-associated X apoptosis regulator (*BAX*) genes in rabbit liver after dietary supplementation with OMWW polyphenols. These effects suggest a possible inhibition from polyphenols of the effect of oxidative stress on NF-κB. Dietary OMWW also decreased *BAX* expression in rabbit's ovary system, which highlighted the beneficial action of polyphenols on reproduction linked to inhibition of apoptosis (112).

Additionally, Sabino et al. (87) demonstrated that the incorporation of OMWW into broiler chicken diets modulated, in their jejunum, the expression of innate immune response genes against viral infections (recombinant inhibitory subunit of NF kappa B Epsilon [*IKBE*], Toll-like receptor 3 [*TLR3*], eukaryotic translation initiation factor 2 alpha kinase 2 [*EIF2AK2*], oligoadenylate synthetase like [*OASL*], myxovirus resistance gene [*MX*] and radical S-adenosyl methionine domain containing 2 [*RSAD2*]). The same study supported that an OMWW supplemented diet could regulate sterol biosynthesis and lipid metabolism by downregulating farnesyl diphosphate synthase (*FDPS*), matrix metalloproteinase 1 (*MMP1*) and fatty acid binding protein 3 (*FABP3*) expression in chicken small intestine (87). This nutritional strategy reduced fatty acid transportation as well as body fat accumulation in chickens (87). A few genes involved in lipid metabolism (acetyl CoA carboxylase [*ACC*] and fatty acid synthase [*FAS*]) had an upregulated expression in the serum of laying hens fed with a diet supplemented with olive cake (113). Furthermore, olive oil increased the expression of many genes encoding for heat shock proteins in broiler chickens, improving their tolerance to heat stress (114).

Table 3. Effects of olive co-products supplementation on meat quality.

Co-product	Content of polyphenols (g/kg)	Dosage (g/kg)	Species	Effect	References
OMWW extract, DOC	0.125, 0.0625	160, 330	Ross broilers	LW ↑, CW ↑, dressing percentage →	(5)
Olive leaf and grape	7	2	Ross 308 broilers	CY →, color →, drip loss →	(103)
Olive pulp	-	25-50, 50-50, 50-80	Cobb 500 broilers	SFA ↓, MUFA ↑, MDA →, cooking loss →, shear force →, pH_{24} ↓, a* →, b* → L*↑	(10)
OMWW extract	100	0.2, 0.5	Hubbard-Sasso broilers	CAT ↑, GSH ↑, TAC ↑, protein oxidation ↓, lipid peroxidation ↓	(41)
Olive pulp	7.9	50, 100	Hubbard broilers	Carcass traits →, pH_{24} ↑, FA →, L* ↑, a* ↑, b* ↓, TBARS ↓, SFA ↓, MUFA ↑, PUFA/SFA →, Lipid hydroperoxides ↓	(99)
Pâté olive cake	0.17	82.5, 165	Ross 308 broilers	pH →, drip loss →, cooking loss →, shear force →, TBARS ↓, DPPH ↑	(34)
Olive pulp	-	50	Finishing pigs	CW →, pH →, cooking loss →, b* ↓, shear force →	(73)
Olive leaf	25	50, 100	growing-finishing pigs	α-tocopherol ↑, pH →, color →, cooking loss →, drip loss ↓	(43)
Olive cake	-	50, 100, 150	Finishing pigs	CW ↑, fat depth ↓, SFA ↓, MUFA ↑, PUFA →	(74)
Olive cake	-	200, 400	pigs	MUFA ↑, OA ↑, PUFA ↓, LA ↓	(98)

Olive leaf	67	25, 50	Finishing pigs	Improvement of tocopherol content	(118)
Olive pulp	-	50	Finishing pigs	CW →, pH →, cooking loss →, b* ↓, shear force →	(73)

a*, redness; ADE, apparent digestible energy; b*, yellowness; CAT, catalase; CW, carcass weight; CY, carcass yield; DOC, dried olive cake; DPPH, 2,2-diphenyl-1-picrylhydrazyl; FA, fatty acid; GSH, glutathione; L*, lightness; LA, linoleic acid; LW, live weight; MDA, malondialdehyde; MUFA, monounsaturated fatty acids; OA, oleic acid; OMWW, olive mill wastewater; PUFA, polyunsaturated fatty acids; SFA, saturated fatty acids; TAC, total antioxidant capacity; TBARS, thiobarbituric acid reactive substances. ↑ increased; ↓ decreased; → no difference.

7. Conclusion

The supplementation of co-products from olive oil extraction – olive pomace, olive cake, OMWW, olive pulp and olive leaf – in monogastric animal nutrition is advisable, as these co-products are found to be harmless, sustainable and are sources of several valuable bioactive compounds. In particular, olive co-products retain the majority of EVOO polyphenols, which are the secondary metabolites most studied and recovered, given their multifunctional effects (antioxidant, antimicrobial, and anti-inflammatory) widely demonstrated in both humans and animals. Additionally, the use of these co-products in animal diets represents an innovative and efficient strategy which contributes to the circular economy, ensuring economic and environmental improvements. The suitability of co-products depends on their specific chemical features. Co-products such as OMWW can be added more easily in monogastric diets compared to others (such as olive pomace, olive pulp and olive leaf), which contain a high level of structural carbohydrates and decrease digestibility and palatability in poultry. Furthermore, the beneficial functions of the phenolic compounds contained in olive co-products and the derived extracts are related to many factors including type, dosage, absorption and metabolism. This review suggests that dietary supplementation with olive oil co-products may improve animal health, productive performances and meat quality characteristics, reduce the adverse effect of lipid peroxidation and improve the antioxidant status.

Author Contributions

FF: Writing- original draft. JT: Writing- original draft. KC: Writing-review & editing. MT-M: Writing-review & editing

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Clonflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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CHAPTER 2

Olive Mill Waste-Water Extract Enriched in Hydroxytyrosol and Tyrosol Modulates Host–Pathogen Interaction in IPEC-J2 Cells

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Olive Mill Waste-Water Extract Enriched in Hydroxytyrosol and Tyrosol Modulates Host–Pathogen Interaction in IPEC-J2 Cells

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Keywords: polyphenols; IPEC-J2; cytokine; defensin; immunomodulation; *Salmonella* spp.

Simple Summary

Olive mill waste-water (OMWW) is a liquid waste produced by the olive oil industry that has been recently regarded as a good source of polyphenols. Phenolic molecules are among the most active secondary molecules in the gut for their antioxidant, anti-inflammatory and antimicrobial effects. They may also contribute to positively changing the distribution of gut microbial species, but their effects have not been widely explored in pigs. The intestinal porcine epithelial cell line IPEC-J2 represents a good model for the study of innate immunity and inflammatory response in animal intestinal diseases and has already been used to investigate the effect of phytogenic feed additives on swine intestinal epithelium. This study aimed to evaluate the *in vitro* effects of an OMWW extract enriched in polyphenols on *Salmonella typhimurium* (*S. typhimurium*) infection in IPEC-J2 cells. Polyphenols extracted from OMWW showed the ability to regulate the host–pathogen interaction by decreasing *S. typhimurium* invasiveness and modulating the expression of many innate immune genes.

Abstract

The dietary supplementation of olive oil by-products, including olive mill waste-water (OMWW) in animal diets, is a novel application that allows for their re-utilization and recycling and could potentially decrease the use of antibiotics, antimicrobial resistance risk in livestock species, and the occurrence of intestinal diseases. *Salmonella serovar typhimurium* is one of the most widespread intestinal pathogens in the world, causing enterocolitis in pigs. The aim of this study was to investigate the effect of an OMWW extract enriched in polyphenols (hydroxytyrosol and tyrosol) in the immune response of an intestinal porcine epithelial cell line (IPEC-J2) following *S. typhimurium* infection. Cells were pre-treated with OMWW-extract polyphenols (OMWW-EP, 0.35 and 1.4 µg) for 24 h and then infected with *S. typhimurium* for 1 h. We evaluated bacterial invasiveness and assayed IPEC-J2 gene expression with RT-qPCR and cytokine release with an ELISA test. The obtained results showed that OMWW-EP (1.4 µg) significantly reduced *S. typhimurium* invasiveness; 0.35 µg decreased the IPEC-J2 gene expression of *IL1B*, *MYD88*, *DEFB1* and *DEFB4A*, while 1.4 µg down-regulated *IL1B* and *DEFB4A* and increased *TGFB1.* The cytokine content was unchanged in infected cells. This is the first study demonstrating the *in vitro* immunomodulatory and antimicrobial activity of OMWW extracts enriched in polyphenols, suggesting a protective role of OMWW polyphenols on the pig intestine and their potential application as feed supplements in farm animals such as pigs.

Keywords: polyphenols; IPEC-J2; cytokine; defensin; immunomodulation; *Salmonella* spp.

1. Introduction

The extraction of olive oil produces a series of by-products, including olive mill waste-water (OMWW)—olive vegetation water diluted in the water used during the oil-extraction process. This by-product is characterized by a high organic material load, ranging from 36.07 g/L to 230 g/L, and a content of phenolic compounds that varies from 0.9 to 30.5 g/L [1,2]. The large amount of this by-product (30 million m^3), produced every year in the Mediterranean basin, contributes to environmental pollution due to the high presence of organic compounds, including phenolic ones [3–6]. This by-product comprises about 50% of the total phenolic compounds of the olive fruit [7], with different phenolic types, mainly tyrosol, hydroxytyrosol, verbascoside and oleuropein [8–10], which are highly known for their antioxidant, antimicrobial and anti-inflammatory activities [11–15]. The supplementation of olive by-products, including OMWW, as a source of polyphenols in animal diets potentially represents an innovative strategy for olive oil waste recycling, in line with the current concept of the circular economy [16–22].

In the swine industry, the use of antibiotics can favor the occurrence of antimicrobial resistance in bacteria of the pig intestinal microbiome, therefore increasing the risk of severe intestinal diseases and impairing the pig's growth performance, especially at the weaning stage [23–25]. For this reason, this habit has been limited in various countries. Among pig intestinal diseases, salmonellosis is one of the most common and it represents a severe problem for the swine industry worldwide [26]. *Salmonella enterica* serovar *typhimurium (S. typhimurium)* is the agent of a very widespread enterocolitis form, which can be subclinical, but it can also be associated with a reduction in both productive performance and average daily gain in pigs [27,28].

In order to restrict the use of antibiotics, novel feeding strategies are required to modulate intestinal and immunological functions, as well as to improve the development and health of the swine gastrointestinal tract [23]. Given the correlation between bioactive molecules, such as polyphenols, and the pig intestinal microbiota and immune response to enteric diseases, their use can have a good impact on pig gut health [23]. The health benefits of polyphenols derive from their antioxidant, anti-inflammatory, and/or gene-regulating effects in tissues. Several studies showed that they help decrease the risk of many diseases, including intestinal ones, but the mechanisms correlated are not clear and need further investigation [29–31]. At present, they can be considered among the most active secondary bioactive molecules in the gut, contributing to beneficial changes in the distribution of gut microbial species, reducing pathogenic bacteria, and/or promoting the growth of probiotics [29,32,33]. A number of *in vivo* studies demonstrated that the administration of dietary polyphenols resulted in a reduction of pathogenic species and an increase in probiotic species in the intestinal microbiota of rats, pigs, and calves [32,34–37]. Olive oil by-products rich in polyphenols (e.g., olive leaf extract) were able to interfere with the growth of intestinal bacteria, including *Salmonella* [38,39]. Compared to human and laboratory animals (e.g., rats and mice), responses to polyphenols have been less explored in farm animals, such as pigs [29]. However, it has been recently demonstrated that the supplementation of natural polyphenols in piglets could contribute to alleviating weaning stress and improve intestinal barrier function, thus providing a nutritional strategy to protect intestinal health [40,41]. Other studies examined changes in the pig gut microbiome after the consumption of plant polyphenols, thanks to their ability to reduce oxidative stress and inflammation [42,43] and modulate immune cells and gut microbiota composition [29,44–46]. This action contributes to an improvement in intestinal bacterial function, decreases the release of microbial components into the circulation, and stimulates host immune response [47].

A suitable *in vitro* model to assess the immunomodulatory properties of polyphenols is represented by the porcine jejunal epithelial cell line IPEC-J2. This continuous cell line provides a valuable model to study both innate immunity and inflammatory responses in human and animal intestinal diseases [26,48–52]. Indeed, IPEC-J2 cells are intestinal porcine enterocytes isolated from the jejunum of an unsuckled neonatal pig, which showed the ability to express and produce cytokines, toll-like receptors (TLRs), defensins, and mucins [53]. In particular, these cells spontaneously secrete the pro-inflammatory chemokine IL-8 and possess ideal characteristics for *in vitro* studies on host–

intestinal pathogen interactions [49,50,54]. Indeed, the primary host-cell barrier against pathogens is represented by the mucosal innate immune system, which is characterized by toll-like receptor (TLR) pathways, NF-kB signaling activation (with inflammatory cytokine release), and Type-I Interferon (IFN) responses [49,55]. Moreover, gastrointestinal tract homeostasis can be maintained when the immune response against commensal bacteria is controlled. When this equilibrium is compromised, excessive immune response causes an inflammatory condition [56]. Besides epithelial cells' mechanical function, their role in gut microbiota homeostasis was recently recognized, as they are involved in maintaining the balance between host microbial components and gut immune cells [57]. In addition, IPEC-J2 cells mime the physiological characteristics of intestinal cells and have therefore been employed in several studies on *Salmonella* infections [53,58], providing valuable information on host responses to this bacteria. In fact, invasion with *S. typhimurium* in IPEC-J2 cells was comparable to that occurring in porcine ileal mucosal explants [59]. This cell line has been employed in studies focused on pigs' innate immune response to dietary treatments [60], which can be regarded as a reference for *in vitro* studies of innate immunity in neonatal intra-epithelial cells after dietary stimuli [48,60]. These cells showed high morphological and functional similarities to porcine enterocytes *in vivo*; therefore, they were employed to evaluate the effects of phytogenic feed additives on swine intestinal epithelium [61]. In recent years, various plant-feed additives have demonstrated antioxidant, antimicrobial, and anti-inflammatory actions and other supporting barrier functions in IPEC-J2 cells [62–65].

With this study, we aimed to investigate *in vitro* the IPEC-J2 response to *S. typhimurium* infection after a pre-treatment with OMWW-extract polyphenols (OMWW-EP), to evaluate the influence on bacterial invasion and immune cells' gene expression.

2. Materials and methods

2.1 Olive mill waste-water extract and composition

The OMWW extract enriched in polyphenols (hydroxytyrosol and tyrosol) was provided by Stymon Natural Products P.C., Patras, Greece (www.stymon.com, accessed on 21 December 2023). This product derives from OMWW of the olive (*Olea Europaea* L.) variety Koroneiki and is produced based on a unique patent (Patent number 1,010,150 IOBE (INT.CL.2021.01) A23L 19/00 A23L 33/105, Stymonphen Liquid) using only green technologies. Its total polyphenol content was equal to 15,000 ± 592 mg/kg, according to the Folin–Ciocalteu method [66]; hydroxytyrosol and tyrosol were the main phenolic compounds (8784 mg/kg and 1638 mg/kg, respectively), detected by HPLC-DAD [67]. The stock solution was filtered, vortexed, and diluted in phosphate-buffered saline (PBS, Euroclone, Milan, Italy) to reach 1400 µg/mL; from this, different scalar concentrations of polyphenols (0.35; 0.7; 7; 14; 70; 140 µg) were obtained for the successive analyses by diluting them in complete culture medium.

2.2 Cell cultures

Porcine jejunal epithelial cells (IPEC-J2, IZSLER Cell Bank code BS CL 205) were grown in a mixture (1:1) of Dulbecco's Modified Eagle (DMEM) (Euroclone, Milan, Italy) and Nutrient Mixture F-12 (F12) (Euroclone, Milan, Italy) enriched with 10% Fetal Bovine Serum (FBS, GIBCO™, Thermofisher Scientific, Milan, Italy), 1% L-glutamine solution (Euroclone, Milan, Italy) and 1% penicillin/streptomycin solution (Euroclone, Milan, Italy) and kept in culture at 37 °C under 5% CO_2.

2.2.1. Cell viability

First, to determine the most suitable amount of OMWW extract to be used on IPEC-J2 cells, we tested different scalar phenolic dosages using a 2,3-bis-(2-methoxy-4-nitro-5-sulphophenyl)-2H-tetrazolium-5-carboxanilide (XTT) assay, according to the manufacturer's instructions (XTT Cell Viability Assay, Cell Signaling Technology, Milan, Italy). In brief, IPEC-J2 cells were plated on 96-well plates (100 µL per well, 0.1×10^5) in complete culture medium and incubated for 24 h at 37 °C under 5% CO_2 until confluence. The day after, the seeding cells on the 96-well plates were exposed to different doses of OMWW-EP (0.35, 0.7, 1.4, 14, 70, 140 µg), and untreated cells were employed as a negative control. Two independent experiments were performed, each including four technical replicates (four wells) for each of the seven experimental conditions: untreated cells (control) and cells treated with six different doses of OMWW-EP (0.35, 0.7, 1.4, 14, 70, and 140 µg). An XTT assay was performed at 24 h and at the end of the treatments, and the cell culture medium was removed and replaced with 100 µL of the fresh DMEM/F12 medium supplemented with XTT detected solution (1:50). The plates were then incubated again at 37 °C under 5% CO_2 for 2 h, and the absorbance was measured at 450 nm using a multimode microplate reader (Glomax, Promega, Milan, Italy). This assay was performed two times for each phenolic concentration.

2.2.2. Bacterial invasion

An isolate of *S. typhimurium* strain (ATCC 14028) was used to evaluate bacterial invasion in IPEC-J2 cells. In three independent experiments, IPEC-J2 cells were seeded into a 12-well plate (1 mL per well, 1.5×10^5 cells/mL) and incubated until confluence. Cells were treated with OMWW-EP (0.35 µg and 1.4 µg) for 24 h. *S. typhimurium* was stored at −80 °C until use, then thawed and grown overnight (18–24 h at 37 °C) in Brain Heart Infusion (BHI) (Sigma, Saint Louis, MO, USA). Then, it was sub-cultured in BHI and incubated for 2 h at 37 ± 1 °C to obtain a mid-log phase culture. The strain was pelleted and re-suspended in DMEM/F12 and L-glutamine medium to obtain a concentration of 10^8 CFU/mL and used to infect pig intestinal IPEC-J2 cells pre-treated with 0.35 µg and 1.4 µg of OMWW-EP for 24 h; infected cells without polyphenolic pre-treatment were used as comparison, while uninfected cells were employed as a negative control. For each of the three independent experiments, one plate was used, employing one well for each replicate, resulting in four replicates for each experimental condition: cells without phenolic pre-treatment and infected (ST); cells pre-treated with two OMWW-EP dosages and infected (ST + 0.35 µg POL; ST + 1.4 µg POL). Cells were stimulated with 1 mL/well of bacterial suspension at 10^8 CFU/mL and incubated at 37 °C under 5% CO_2 for 1 h. Then, monolayers were washed five times with DMEM/F12 and L-glutamine medium (1 mL/well) and treated with 1 mL of colistin 300 µg/mL at 37 °C under 5% CO_2 for 2 h to remove all extracellular bacteria. Cells were washed four times with medium and lysed by adding 200 µL/well 1% of Triton X-100 (Merck KgaA, Darmstadt, Germany) in PBS (Euroclone, Milan, Italy) at room temperature for 5 min (min); then, they were blocked by adding 800 µL of PBS to each well. The resulting cell suspension was vortexed, serially diluted in PBS, and seeded on XLD (Sigma, Saint Louis, MO, USA); then, it was incubated for 24–48 h at 37 °C for intracellular bacterial counts.

2.2.3. Modulation of the immune response

Cells from the IPEC-J2 line were seeded into 12-well plates (1 mL per well, 3×10^5 cells/mL) and then incubated at 37 °C under 5% CO_2 until confluence. Two experimental designs were applied: the first one to evaluate the effect of OMWW-EP on IPEC-J2 gene expression and cytokine release, and the second one to investigate cellular pathways modulated by OMWW pre-treatment behind host–pathogen interactions in *S. typhimurium* infection. For the first experiment, cells were treated with OMWW-EP at 0.35 µg, 0.7 µg, 1.4 µg or 7 µg for 24 h, alongside untreated controls. A total of three independent experiments comprising two replicates each (one well for each replicate) were used

for each experimental condition: cells with medium only (control); cells treated with OMWW-EP (0.35 µg POL, 0.7 µg POL, 1.4 µg POL and 7 µg POL). For the second, cells were treated with OMWW-EP at 0.35 µg or 1.4 µg for 24 h, then infected with 1 mL of 10^8 CFU/mL *S. typhimurium* suspension and incubated at 37 °C under 5% CO_2 for 1 h. Moreover, cells infected with *S. typhimurium* without OMWW-EP pre-treatment were used as a control of the infection, and cells with the medium only were used as an untreated and uninfected control. To summarize, four independent experiments, including three replicates (one well for each replicate), were used for each experimental condition: cells with medium only (control); infected cells only (ST); cells pre-treated with polyphenols (0.35 and 1.4 µg); and infected (0.35 µg POL + ST; 1.4 µg POL + ST). After the first incubation, cells were washed five times and again incubated in their medium at 37 °C under 5% CO_2 for 3 h. The resulting IPEC-J2 cell supernatants were stored at −80 °C until our evaluation of the cytokine contents. In parallel, cells were lysed with 400 µL of RLT Buffer (Qiagen, Hilden, Germany) and, after incubation for 10 min (min) at room temperature, collected and stored at −80 °C until RNA extraction and RT-q PCR analysis.

2.3. RNA extraction and Reverse Transcription Quantitative PCR (RT-qPCR)

Total RNA was extracted from the cells described in Section 2.2.3 for both the experimental designs using Rneasy Mini Kit (Qiagen s.r.l., Milan, Italy) in the Qiacube System (Qiagen s.r.l., Milan, Italy), in accordance with the manufacturer's instructions. The quality of extraction was assessed using a Qubit 3.0 Fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). The same amount of RNA for each sample (250 ng) was reverse-transcribed into cDNA using a iScript cDNA Synthesis Kit (Bio-Rad, Milan, Italy). Amplification was performed on a CFX96™ Real-Time System (Bio-Rad, Milan, Italy) using SoFast™ Eva Green Supermix (Bio-Rad, Milan, Italy) following a protocol previously described [48]. Primers of target genes, coding for C-X-C motif chemokine ligand 8 (*CXCL8*), interleukin 1 beta (*IL1B*), *IL18*, nitric oxide synthase 2 (*NOS2*), nuclear factor kappa B subunit 1 (*NFKB1*), RELA proto-oncogene (*NFKB/p65*), toll-like receptor 4 (*TLR4*), toll-like receptor 5 (*TLR5*), myeloid differentiation primary response gene 88 (*MYD88*), transforming growth factor beta 1 (*TGFB1*), beta defensin 1 (*DEFB1*), beta defensin 2 (*DEFB4A*), and reference genes glyceraldehyde 3-phosphate dehydrogenase (*GADPH*) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*) were described in previous studies (Table 1). The relative normalized expression of the selected genes was assessed using the $2^{-\Delta\Delta Ct}$ method [68], comparing different conditions. Samples scored negatively when the Ct was ≥ 39.

Table 1. Primer set sequences of target and reference genes.

Gene	Primer sequences	Amplicon length	Source
IL18	For-5′-CGTGTTTGAGGATATGCCTGATT-3′ Rev-5′-TGGTTACTGCCAGACCTCTAGTGA-3′	106	[48]
IL1B	For- 5′-AATTCGAGTCTGCCCTGTACCC-3′ Rev-5′-TGGTGAAGTCGGTTATATCTTGGC-3′	110	[49]
NOS2	For-5′-CGTTATGCCACCAACAATGG-3′ Rev-5′-AGACCCGGAAGTCGTGCTT-3′	84	[48]
TGFB1	For-5′-CGCGTGCTAATGGTGGAAAG-3′ Rev-5′-CCGACGTGTTGAACAGCATA-3′	87	[48]

CXCL8	For-5′-TTCGATGCCAGTGCATAAATA-3′ Rev-5′-CTGTACAACCTTCTGCACCCA-3′	175	[69]
MYD88	For-5′-GCAGCTGGAACAGACCAACT-3′ Rev-5′-GTGCCAGGCAGGACATCT-3′	62	[69]
NF-kB1	For-5′- CCCATGTAGACAGCACCACCTATGAT-3′ Rev-5′-ACAGAGGCTCAAAGTTCTCCACCA- 3′	131	[69]
NF-kB/p65	For-5′-CGAGAGGAGCACGGATACCA-3′ Rev-5′-GCCCCGTGTAGCCATTGA-3′	61	[69]
DEFB1	For-5′- CTGTTAGCTGCTTAAGGAATAAAGGC-3′ Rev-5′-TGCCACAGGTGCCGATCT-3′	80	[48]
DEFB4A	For-5′-CCAGAGGTCCGACCACTA-3′ Rev-5′-GGTCCCTTCAATCCTGTT-3′	87	[48]
TLR4	For -5′-TGGCAGTTTCTGAGGAGTCATG– 3′ Rev. –5′ –CCGCAGCAGGGACTTCTC – 3′	71	[48]
TLR5	For -5′-TCAAAGATCCTGACCATCACA– 3′ Rev. – 5′ –CCAGCTGTATCAGGGAGCTT – 3′	59	[48]
GAPDH	For-5′-ATGGTGAAGGTCGGAGTGAA-3′ Rev-5′AGTGGAGGTCAATGAAGGGG-3′	61	[48]
HPRT1	For-5′-AACCTTGCTTTCCTTGGTCA-3 Rev-5′-TCAAGGGCATAGCCTACCAC-3′	150	[48]

2.4. Cytokine quantification

The cytokine content was investigated in culture supernatants of IPEC-J2 described in Section 2.2.3, using both experimental designs. Cells were treated for 24 h with OMWW-EP (0.35 and 1.4 µg) without infection. Moreover, after OMWW pre-treatment, cells were infected (1 h) with *S. typhimurium* after a polyphenolic pre-treatment, alongside the corresponding controls. The culture medium was changed, and the cells were incubated for 3 h at 37 °C under 5% CO_2. Then, culture supernatants were collected, centrifuged (at 2500× *g* for 3 min), and kept at -80 °C until use. Levels of GM-CSF, IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-18 were determined using the Porcine Cytokine/Chemokine Magnetic Bead Panel Multiplex assay (Merck Millipore, Darmstadt, Germany) and a Bioplex MAGPIX Multiplex Reader (Bio-Rad, Hercules, CA, USA), following the manufacturer's instructions [48].

2.5. Statistical analyses

A Kolmogorov–Smirnov test was conducted to check for Gaussian distribution in the data sets, concerning the viability assay, gene expression, cells invasion, and protein release. Data showing Gaussian distributions were checked for significant differences by one-way ANOVA or unpaired T-test. Results failing the Kolmogorov–Smirnov test were checked for significant differences by non-

parametric Kruskal–Wallis test, followed by a Dunn's Multiple Comparison post-hoc test. The significance threshold was set at $p < 0.05$ (Prism 5, GraphPad Software, GraphPad Software Inc., San Diego, CA, USA).

3. Results

3.1. Cell viability

Cells from the IPEC-J2 line cells were exposed to scalar doses of OMWW-EP (0.35, 0.7, 1.4, 7, 14, 70, 140 µg), and 24 h later, viability was measured through an XTT assay. The XTT viability test showed that treatment with OMWW-EP at 140 µg and 70 µg induced a statistically significant ($p < 0.0001$) decrease in IPEC-J2 viability after 24 h (Figure 1) OMWW-EP exposition. The other concentrations tested did not show a significant effect. The two doses (0.35, 1.4 µg) not affecting IPEC-J2 viability were therefore selected for the following experiments.

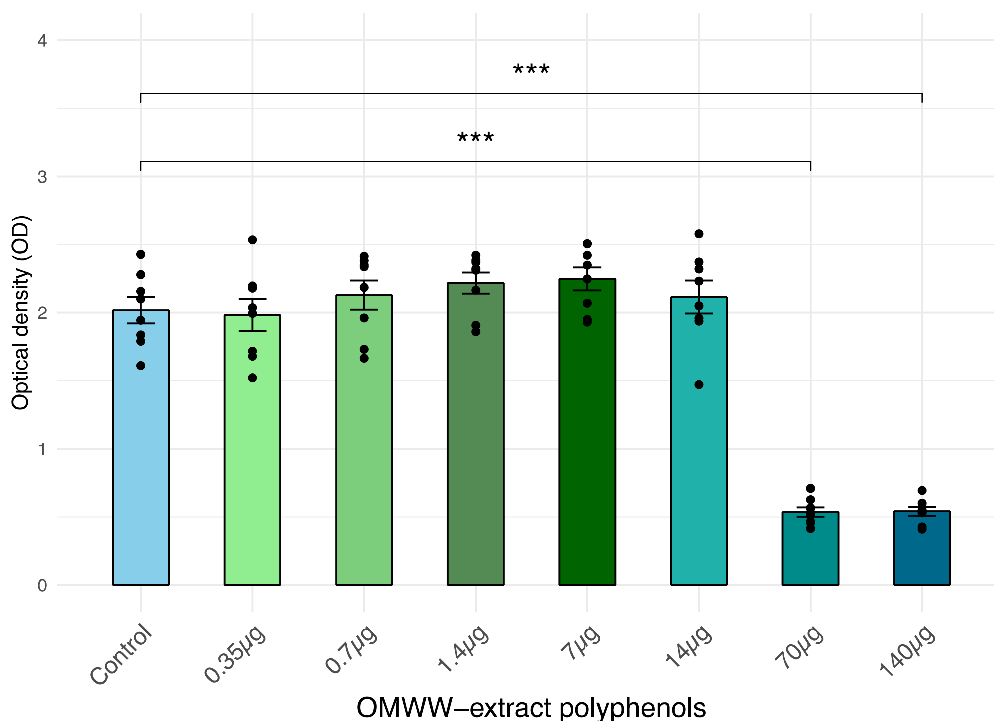

Figure 1. IPEC-J2 viability after a 24 h exposition to the extract of liquid olive mill waste-water (OMWW) polyphenols (OMWW-EP: 0.35 µg; 0.7 µg; 1.4 µg; 7 µg; 14 µg; 70 µg; 140 µg). Cell viability was determined with XTT test. The number of living cells is expressed as optical density (OD) ± standard error (SE) and dots indicate samples included in each group. Statistical difference was calculated for all groups vs. Control (untreated cells): *** $p < 0.001$.

3.2. *Salmonella typhimurium* invasiveness

A significant ($p < 0.05$) decrease in *S. typhimurium* invasiveness into IPEC-J2 cells ($p < 0.05$; $\log_{10}$ CFU/3 × 10^5 cells) after an exposure to OMWW-EP of 1.4 µg for 24 h was demonstrated when compared with controls (untreated infected cells). CFU data were converted into $\log_{10}$ values (Figure 2).

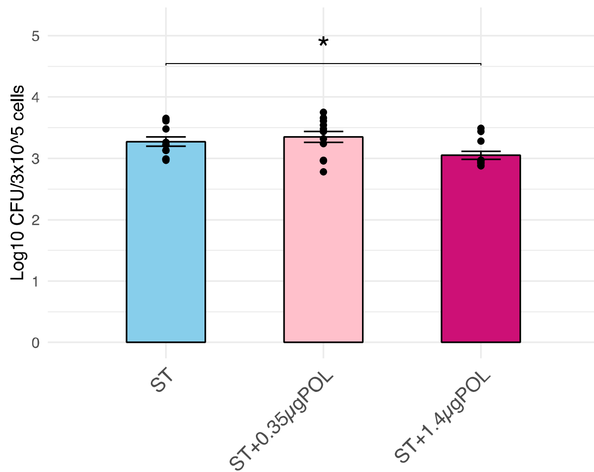

Figure 2. Effects of OMWW-extract polyphenols (OMWW-EP) on *S. typhimurium* penetration into IPEC-J2 cells. Data are expressed as $\log_{10}$ CFU of penetrated, intracellular ST/3 × 10^5 cells. The mean value of five replicates + standard error is presented, and dots indicate samples included in each group. The significant difference between *S. typhimurium* infected cells and pretreated with different concentrations of OMWW-EP (ST + 0.35 µg POL—pink; ST + 1.4 µg POL—fuchsia) and *S. typhimurium* infected cells (ST—blue) is indicated by * (* $p < 0.05$).

3.3. Modulation of immune response

The immunomodulant effect of OMWW-EP at two dosages (0.35 µg and 1.4 µg) was monitored through RT-qPCR and ELISA tests.

3.3.1. OMWW-extract polyphenols' effect on IPEC-J2 gene expression and cytokine release

The effect of OMWW-EP (0.35 µg and 1.4 µg) treatment for 24 h on IPEC-J2 cells was monitored through RT-qPCR. A panel of seven genes was analyzed (Table 1), and the levels of treated cells were compared to untreated control cells. Moreover, complete results for the other polyphenol dosages are reported in the supplementary Figure S1. A significant decrease in *CXCL8* ($p < 0.001$)*, IL18* ($p < 0.05$), and *MYD88* ($p < 0.001$) and a significant increase in *NOS2* ($p < 0.05$) were observed in cells exposed to 0.35 µg of OMWW-EP (Figure 3). The treatment with 1.4 µg of OMWW-EP triggered a significant decrease in *CXCL8* ($p < 0.001$) and *MYD88* ($p < 0.001$). The other genes under study were not significantly modulated (Figure 3).

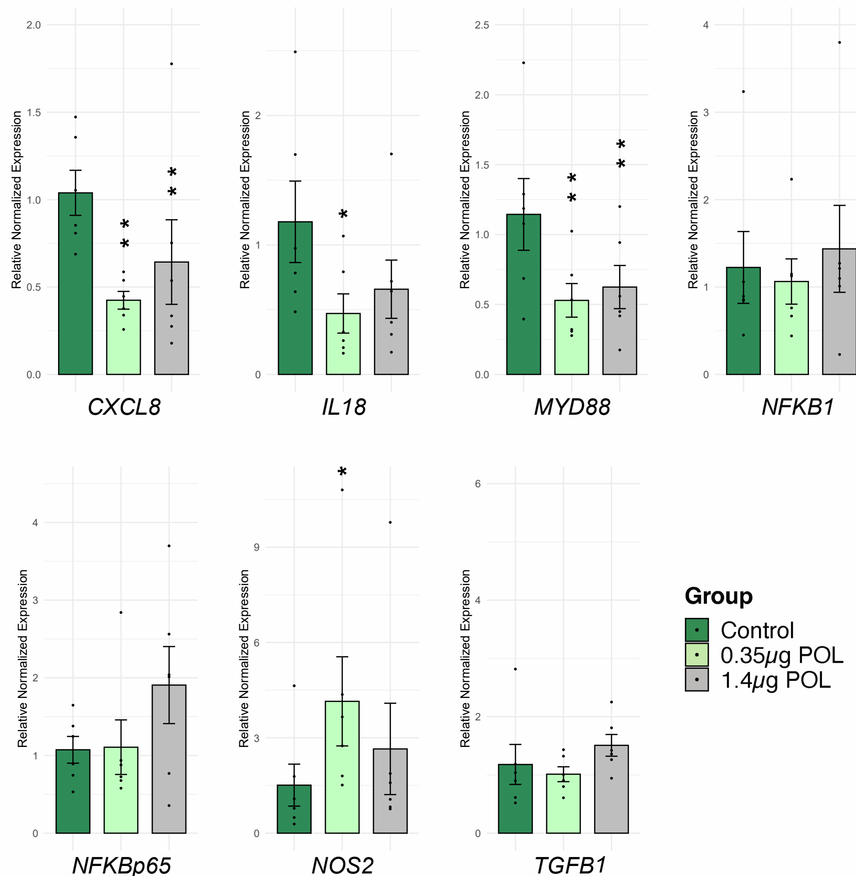

Figure 3. Effects of 24 h OMWW-extract polyphenols on IPEC-J2 gene expression. The RT-qPCR analysis was performed to evaluate *CXCL8, IL18, MYD88, NFKB1, NFKB/p65, TGFB1* and *NOS2* gene expression. Data are presented as bar plots displaying the mean value of normalized expression, standard error as error bars and dots indicate samples included in each group. For each gene and cytokine, differences between treated with polyphenols (0.35 µg POL—light green; 1.4 µg POL—gray) vs. untreated (Control—dark green) cells were evaluated through one-way ANOVA followed by a Dunnett's test or a Kruskal–Wallis test followed by Dunn's multiple comparison test; * $p < 0.05$, ** $p < 0.01$.

3.3.2. OMWW-extract polyphenols' effect on IPEC-J2 cytokine release

In parallel, the impact of scalar doses of OMWW-EP (0.35, 1.4 µg) on cytokine levels in IPEC-J2 culture supernatants was investigated using multiplex ELISA. Eight cytokines were tested: IL-1α, IL-1β, IL-1Ra, IL-6, IL-8, IL-10, IL-18 and GM-CSF (Figure 4). The levels of GM-CSF and IL-β were below the assay detection limit. Exposure to polyphenols did not alter the levels of IL-1α, IL-1Ra, and IL-10 in IPEC-J2 culture supernatants (Figure 4). In agreement with the RT-qPCR data, these compounds decreased the levels of the pro-inflammatory cytokines IL-6, IL-8, and IL-18, although a statistically significant difference was observed only for the latter (Figure 4).

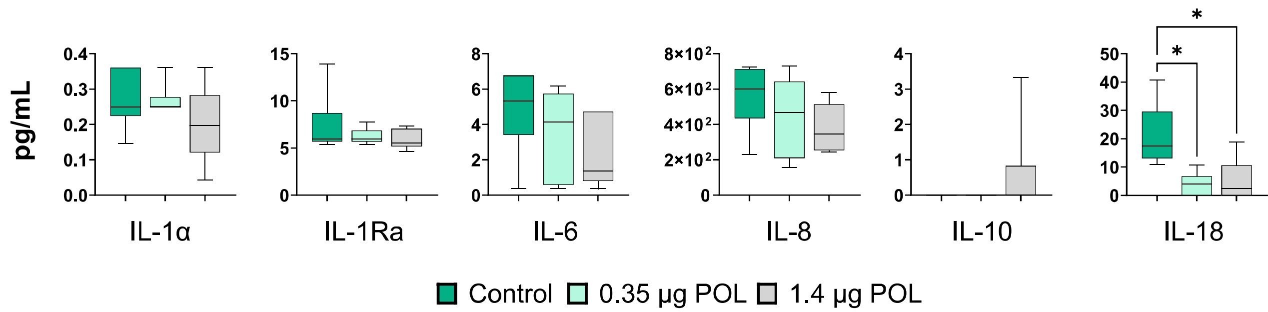

Figure 4. Effects of OMWW-extract polyphenols on IPEC-J2 cytokine release through multiplex ELISA measuring cytokine contents in culture supernatants (IL-1α, IL-1Ra, IL-6, IL-8, IL-10, IL-18). Data are presented as box and whisker plots displaying median and interquartile range (boxes) and minimum and maximum values (whiskers). For each gene and cytokine, differences between treated with polyphenols (0.35 μg POL—light green; 1.4 μg POL—gray) and untreated (Control—dark green) cells were evaluated through one-way ANOVA followed by a Dunnett's test or a Kruskal–Wallis test followed by Dunn's multiple comparison test; * $p < 0.05$.

3.3.3. OMWW-extract polyphenols' and S. typhimurium infection effects on IPEC-J2 gene expression

The effect of OMWW-EP pre-treatment for 24 h in IPEC-J2 responses to *S. typhimurium* infection (for 1 h) was evaluated. First, a panel of 10 genes was analyzed through RT-qPCR (Table 1). We observed a significant increase in *CXCL8* ($p < 0.0001$), *MYD88* ($p < 0.0016$), *DEFB1* ($p < 0.0001$), and *DEFB4A* ($p < 0.0044$) in cells not exposed to OMWW (control) in response to *S. typhimurium infection* (Figure 5).

Then we investigated the impact of OMWW-EP in IPEC-J2 ability to respond to *S. typhimurium* infection. Different results were obtained depending on polyphenol dosages. The pre-treatment of infected cells with 0.35 μg induced a significant decrease in *IL1B* ($p = 0.019$), *MYD88* ($p = 0.062$), *DEFB1* ($p < 0.0001$) and *DEFB4A* ($p = 0.0012$) compared to untreated infected cells, whereas with a pre-treatment of infected cells using 1.4 μg showed a significant decrease in *IL1B* ($p = 0.019$) and *DEFB4A* ($p = 0.023$) and a significant increase in *TGFB1* ($p = 0.008$) (Figure 5). Other genes under study were not significantly modulated (Figure 5).

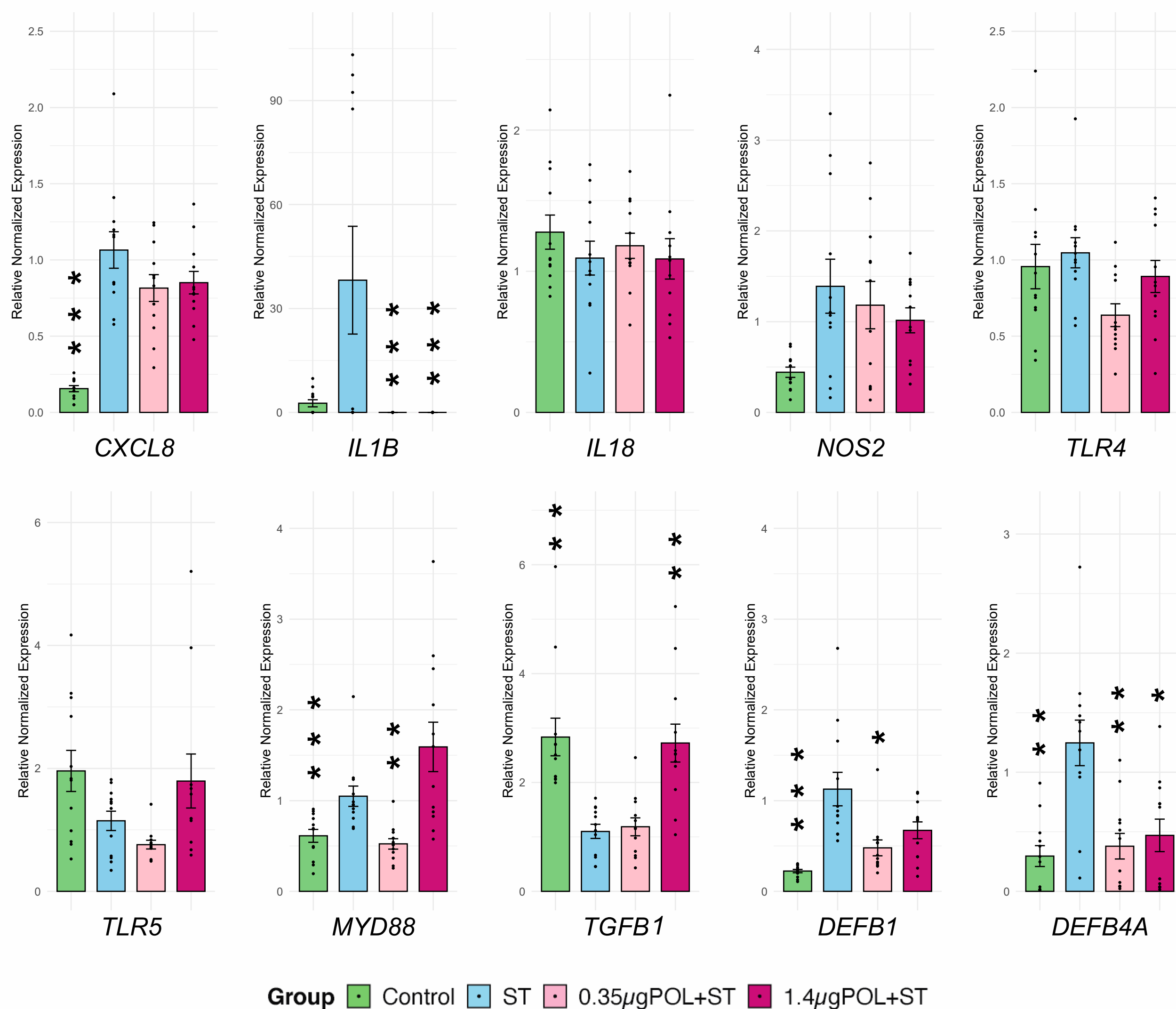

Figure 5. Gene expression of IPEC-J2 cells in response to *S. typhimurium* infection with OMWW-extract polyphenol (OMWW-EP) pre-treatment. The tested conditions for IPEC-J2 cells were: uninfected and untreated cells (Control—dark green), infected with *S. typhimurium* (ST—blue), pre-treated with 0.35 µg OMWW-EP and infected (0.35 µg POL + ST—pink), and pre-treated with 1.4 µg OMWW-EP and infected (1.5 µg POL + ST—fuchsia). Data are reported as mean value and standard error, and dots indicate samples included in each group. Statistical tests were carried out comparing all conditions vs. ST. Significant differences: * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.3.4. OMWW-extract polyphenols' and S. typhimurium infection effects on IPEC-J2 cytokine production

To further investigate the immunomodulatory properties of OMWW-EP, we assayed the cytokine contents in the supernatants of un-infected and untreated IPEC-J2 and *S. typhimurium*-infected cells (ST) pretreated or not with OMWW-EP (0.35 µg or 1.4 µg) (Figure 6). *S. typhimurium* infection triggered an enhanced release of pro-inflammatory cytokines, such as IL-1α ($p = 0.02927$), IL-6 ($p < 0.0001$), and IL-8 ($p = 0.0006$) (Figure 6). OMWW-EP did not affect the cytokine content in *S. typhimurium*-infected IPEC supernatants (Figure 6). Values of GM-CSF and IL-1β were below the reference range values.

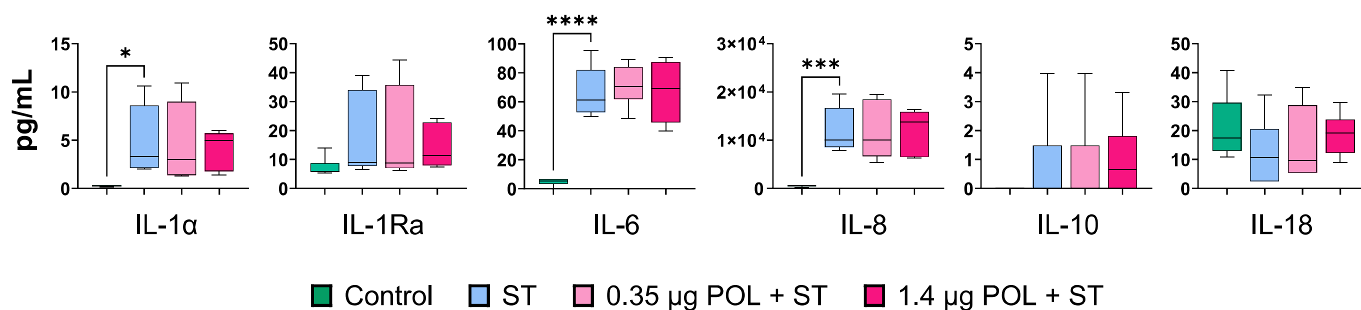

Figure 6. Cytokine release by IPEC-J2 cells in response to *S. typhimurium* infection after OMWW-extract polyphenol (OMWW-EP) pre-treatment. The tested conditions for IPEC-J2 cells were: uninfected and untreated cells (Control—dark green), infected *with S. typhimurium* (ST—blue), pre-treated with 0.35 µg OMWW-EP and infected (0.35 µg POL + ST—pink), and pre-treated with 1.4 µg OMWW-EP and infected (1.5 µg POL + ST—fuchsia). For each cytokine, differences between ST-infected cells and the other conditions were evaluated through one-way ANOVA followed by a Dunnett's test or a Kruskal–Wallis test followed by Dunn's multiple comparison test; * $p < 0.05$, *** $p < 0.001$; **** $p < 0.0001$.

4. Discussion

Gut epithelial cells have a predominant role as the first defense from pathogenic insults [53,57]. Thus, the obtained results regarding the modulation of immune genes in the intestinal epithelium after treatment with polyphenols are a prodromal step to feed supplementation with polyphenols in livestock species. The IPEC-J2 cell line was chosen on the basis of our previous studies [48–50,52]. Indeed, it represents a good model for investigating epithelial immune response in pigs, in order to evaluate the ability of OMWW polyphenols to modulate the in vitro gut immunological response to *S. typhimurium* infection.

Our screening of different amounts of OMWW-extract polyphenols (OMWW-EP) carried out in the first part of the study through the IPEC-J2 viability test allowed us to choose the appropriate dosages for the successive analyses (Figure 1). We then investigated the effect of pre-treatment with OMWW-EP (0.35 and 1.4 µg) on IPEC-J2 cells for 24 h with or without an infective insult and found that exposure to these compounds triggered a decreased expression of *CXCL8, IL18,* and *MYD88* genes; IL-18 release; and an up-regulation of *NOS2* gene expression (Figures 3 and 4). The anti-inflammatory effect of polyphenols is due to complex cellular mechanisms that are still not clear, but most of them have been correlated with the NF-kB pathway [45]. *CXCL8* is a pro-inflammatory chemokine (IL-8)-encoding gene whose expression could be regulated by the TLR4/MyD88/NF-kB pathway. In our study, OMWW-EP seemed to exert anti-inflammatory action by decreasing *MYD88* (a gene with a pivotal role in NF-kB activation) and therefore *CXCL8* gene expression. Indeed, in other studies, dietary supplementation with grape seed cake (another by-product rich in polyphenols) was shown to significantly reduce *MYD88* gene expression in the colon of Dextran Sulfate Sodium (DSS)-treated piglets [70], and Li et al. [71] demonstrated the ability of other natural polyphenols (the flavonoids quercetin and catechin) to restore the increased expression of *MYD88* in LPS-stimulated murine macrophage RAW 264.7 cells.

In addition, our data demonstrated the ability of OMWW-EP to decrease both the expression and secretion of IL-18. IL-18 is a member of the IL-1 family, with an important role in the inflammatory response [72–77]. Its release must be tightly controlled, in order to avoid the development of auto-inflammatory diseases [73].

These data suggest a possible effect of OMWW polyphenols on host–pathogens interaction, which was successively tested *in vitro* using *S. typhimurium* assay. In this way, the ability of a pre-treatment with different dosages of OMWW-EP (0.35 and 1.4 µg) to decrease *S. typhimurium* invasiveness and modulate immune response related-genes in *S. typhimurium*-infected cells was assessed. First of all, our data confirmed *S. typhimurium's* ability to penetrate IPEC-J2 cells (Figure

2), which is known to be related to the up-regulation of the pro-inflammatory molecule *CXCL8* [49], as we found (Figures 5 and 6). In our analysis, we additionally found that *S. typhimurium's* invasion of IPEC-J2 significantly increases the expression of *MYD88* gene (Figure 5) encoding for the MyD88 adaptor protein, which is the mediator of NF-kB activation, essential for the stimulation of pro-inflammatory gene expressions. Not surprisingly, we therefore observed the increased release of other pro-inflammatory cytokines (IL-1α, IL-6, IL-18) after *S. typhimurium* infection, in accordance with a previous study [49] (Figure 6). It is known that mucosal bacteria are able to stimulate the transcription of pro-inflammatory genes through epithelial cell invasion, interacting with many receptors such as TLR or acting on NF-kB [44]. Surface-expressed TLRs are activated by the pathogen-associated molecular patterns (PAMPs), which are microbe structures, exploiting the adaptor molecule MyD88 and stimulating NF-kB translocation into the nucleus [49]. The activation of the nuclear factor NF-kB leads to the increased transcription of pro-inflammatory mediators (such as the cytokines IL-8, IL-1B, IL-6 and TNF) [78], as shown in our experiment (Figure 6).

Interestingly, the pre-treatment with OMWW extract enriched in polyphenols reduced *S. typhimurium* invasiveness. Thus, we tried to highlight molecules influencing this host–pathogen interaction, modulated by the polyphenol treatment. Firstly, the expression of two TLRs (*TLR4* and *TLR5*) was investigated, but no effects on *S. typhimurium* invasion after OMWW-EP pre-treatment were observed.

As for the effects on pro-inflammatory cytokines and related pathways, we observed a down-regulation of *IL1B* in *S. typhimurium*-infected IPEC-J2 cells after OMWW-EP pre-treatment (both dosages). A down-regulation of *MYD88* for the 0.35 μg group was detected as well.

The expression of *MYD88* was also investigated and showed a decrease in *S. typhimurium*-infected cells in the 0.35 μg pre-treatment group, while *MYD88* gene expression was raised in cells infected and not pre-treated with OMWW-EP. The pre-treatment with 0.35 μg OMWW-EP probably prevented the activation of NF-kB and pro-inflammatory mediators through the down-regulation of this adaptor molecule-encoding gene (*MYD88*).

Moreover, inflammasome-induced cell death contributes to host control of *S. typhimurium* infection. Species differences in inflammasomes may contribute to zoonotic immune tolerance. Inflammasomes are molecular platforms that promote the maturation of the proinflammatory cytokines IL-1β and IL-18. During enteric *Salmonella* infection, the activation of caspase-1 and the production of IL-1β and IL-18 provide a protective host response [79]. The inflammasome activation could be mediated by MyD88, but there are other pathways in the activation signaling: various PAMPs, DAMPs, or intracellular changes induce the formation of the NLRP3 inflammasome composed of NLRP3 as a PRR, pro-caspase-1, and adapter proteins such as the apoptosis-associated speck-like protein containing a caspase recruitment domain [80].

At both dosages (0.35 and 1.4 μg), the polyphenolic pre-treatment induced a down-regulation of pro-inflammatory cytokine *IL1B,* which is also involved in the inflammasome reaction, together with IL-18 [73–77]. The combined effect of OMWW-EP on IL-18, which induced gene expression and reduced cytokine release (without *Salmonella* infection), leads the authors to suppose that this is the pathway through which OMWW-EP potentially protects IPEC-J2 cells against *S. typhimurium* infection.

In line with our results, the ability of the polyphenol resveratrol to potentially protect the intestinal barrier against deoxynivalenol (DON)-induced dysfunction and *Escherichia coli* (*E. coli*) translocation in IPEC-J2 cells [64] and *S. typhimurium* infection was demonstrated. Several *in vitro* studies concerning intestinal cells demonstrated that plant extracts rich in polyphenols or isolated molecules can limit induced-inflammation processes [30,81–84]. It was also shown that natural polyphenols can modulate inflammasome activation [77], interfering with the production (both at mRNA and protein levels) of pro-inflammatory mediators [30]. Moreover, it has been demonstrated that IL1-β is reduced by polyphenols such as curcumin and resveratrol [84–86]. Other good sources of polyphenols, i.e., dietary grape seed cake, decreased *IL1B* gene expression and protein concentration in fattening pigs' spleens [87]. Feeding weaned pigs with polyphenol-rich plant products (grape seed, grape marc meal extract, and spent hops) down-regulated various pro-inflammatory cytokines, including IL-1β [44], in the intestine, and the oleuropein glycoside

polyphenol significantly decreased the release of IL1-β in LPS-stimulated human whole-blood cultures [88].

Meanwhile, we did not observe differences between OMWW-EP-treated and untreated IPEC-J2 cells concerning the expression and release of other pro-inflammatory cytokines in response to *S. typhimurium* infection. This probably relates to the fact that these inflammatory molecules are primarily stimulated by TLR4 receptors, whose expression seemed to not be significantly modulated by polyphenols. We also might speculate that OMWW-EP could reduce the levels of pro-inflammatory cytokine release in response to *Salmonella* if a lower infective dose is used.

Not only pro-inflammatory but also anti-inflammatory cytokines such as IL-10 and TGF-β were tested. In particular, TGF-β can dampen the inflammatory effects of cytokines such as IL-1β, IL-12, TNF [89].

In our experiments, we observed that *S. typhimurium* infection determined a decrease in *TGFB1* expression in IPEC-J2 cells. This is not surprising, considering a recent study by Qin et al. [90], who mimed the bacterial infection process with an LPS stimulus in human Caco-2 colon cells. The authors observed a down-regulation of several genes involved in the inflammation response linked to TGF-beta signaling pathways. Interestingly, the pre-treatment with OMWW-EP (1.4 µg) induced the up-regulation of *TGFB1* in infected cells compared to cells that were infected without OMWW-EP pre-treatment. The *TGFB1* gene encodes the TGF-beta superfamily ligands and binds different TGF-beta receptors, regulating gene expression as well as cell growth, proliferation, and differentiation [91]. It is produced by different cell types, including the intestinal cells [92], and is a cytokine involved in the homeostasis of the epithelial barrier, which is normalized by up-regulating the expression of tight junction proteins [93]. The up-regulation of this gene in our experiments may be correlated with a possible inhibitory action of bacterial replication inside the cells, in line with Huang et al. [91]., who demonstrated that, in pigs, the inhibition of *S. typhimurium* intracellular replication can be associated with *TGFB1* increase. Moreover, in a study by Nallathambi et al. [93], a polyphenol-rich grape seed extract was able to enhance *TGFB1* expression in Caco-2 cells, in line with the observed increase in tight junction protein expression.

Finally, the expression of genes coding for antimicrobial peptides (AMPs), released during early response to invading pathogens, was investigated. These molecules show efficacy in disrupting both the Gram-positive and Gram-negative bacterial membranes and are also expressed in epithelial cells of the gastrointestinal tract [26]. Beta-defensins are known AMPs also involved in the maintenance of the homeostasis in the gut microbiota, regulating its composition and thus protecting from microbial pathogens [48,75,94], and it is well known that IPEC-J2 cells express beta-defensin genes [26]. Our results showed that *S. typhimurium* invasion up-regulated *DEFB1* and *DEFB4A* gene expressions in IPEC-J2 cells, as reported by previous studies [26,49]. *DEFB4A* expression was also increased in another porcine ileum epithelial cell line (IPI-2I) after infection with *S. typhimurium* DT104 [26]. Furthermore, it was demonstrated that *E. coli* adhesion increases the expression of *DEFB1* and *DEFB4A* in IPEC-J2 cells [95]. In this study, pre-treatment with OMWW-EP seemed to induce a return to the basal expression of *DEFB1* (at the 0.35 µg dosage) and *DEFB4A* (at both dosages) after *S. typhimurium* infection, counteracting the effect of bacterial invasion and potentially restoring gut homeostasis.

5. Conclusion

Our results confirmed the potential ability of OMWW-EP to modulate host–pathogen interactions in pigs by inducing an alteration of *S. typhimurium* invasiveness. In particular, our data showed a significant reduction of *S. typhimurium's* ability to invade cells pre-treated with 1.4 µg of OMWW-EP compared to untreated IPEC-J2 cells. Furthermore, pre-treatment (independently from the dosage) with OMWW-EP modulated several innate immune-response genes influencing the *S. typhimurium* invasiveness in IPEC-J2, exhibiting potential antimicrobial activity by decreasing intracellular bacterial replication. This is the first study performed in an *in vitro* swine intestinal model that suggests a potential protective role of OMWW polyphenols in the pig intestine, paving the way for *in vivo* studies to confirm these promising results; increasing our knowledge of related molecular mechanisms; and making the possible use of this by-product feasible for livestock animal welfare and health.

Supplementary Materials**:** The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/ani14040564/s1**,** Figure S1: Effect of 24 h OMWW-extract polyphenols on IPEC-J2 gene expression.

Author Contributions: Conceptualization, M.T.-M., E.R. and K.C.; methodology, Flavia Ferlisi, C.G.D.C., Floriana Fruscione; validation, K.C.; formal analysis, E.R., C.G.D.C., Flavia Ferlisi and S.M.; investigation, Flavia Ferlisi, C.G.D.C., G.F., S.Z., Floriana Fruscione and R.G.; resources, E.R.; data curation, E.R. and S.M.; writing—original draft preparation, Flavia Ferlisi and K.C.; writing—review and editing, G.F., S.Z., C.G.D.C., S.M., R.G. M.T.-M. and E.R.; visualization, S.M.; supervision, K.C., E.R. and M.T.-M.; project administration, M.T.-M. All authors have read and agreed to the published version of the manuscript.

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CHAPTER 3

Gastrointestinal health evaluation through a metagenomic and morphologic approach in finishing pigs fed with an olive mill wastewater polyphenol extract

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Gastrointestinal health evaluation through a metagenomic and morphologic approach in finishing pigs fed with an olive mill wastewater polyphenol extract

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Abstract

Pig microbiota influences overall animal health and diet is a possible strategy to target gut microbial composition and improve animal growth, including environmental and economic advantages. Dietary polyphenols may improve gut health because of their antimicrobial and antioxidant properties, although their utilization in dietary formulation for monogastrics is still emerging. The present study investigated the effects of two dosages of polyphenols from an olive mill wastewater (OMWW) extract in pigs' diet on the morphology and intestinal microbiota composition and gastrointestinal pathology. One hundred thirty-five female Landrace × Duroc finishing pigs were randomly allocated into three groups fed with: control diet (C); C diet supplemented with 74 ppm of OMWW polyphenols (P-LOW); C diet with 225 ppm of OMWW polyphenols (P-HIGH). The experiment lasted 85 days and 15 animals per group were slaughtered at final body weight of 148.1 ± 4.6 kg. The intestinal morphological analysis demonstrated a significant increase of villus height:crypt depth (VH:CD) ratio in the ileum of P-LOW and P-HIGH and in the jejunum of P-HIGH compared to C. Metagenomic analysis showed a significant increase for two alpha diversity indexes in the cecum of P-HIGH compared to P-LOW. Differential abundance analysis showed that especially P-HIGH had an increased abundance of beneficial bacteria, in particular *Eubacterium* and *Treponema*, and a decrease of harmful ones, including *Fusobacterium* and *Bacteroides helcogenes* and *Corynebacterium urealiticum*. Results suggest that OMWW polyphenols can have a positive impact on pig gut microbiota and overall intestinal health, although further studies are needed to confirm these data.

Keywords: gut health, metagenomics, olive polyphenols, swine.

HIGHLIGHTS

- Olive mill wastewater (OMWW) extract is particularly concentrated in hydroxytyrosol and tyrosol
- Polyphenols from OMWW affect gut morphology by improving intestinal mucosa characteristics
- OMWW polyphenols can target gut microbiota and positively influence animal health

1. Introduction

The increasing global demand for pig production (FAOSTAT data, 2022) has led to several challenges for the producers related to feed costs and the maintenance of growth performance and animal health status (Vasquez et al. 2022). The modern pig productive sector demands the reduction of antibiotic use to avoid antimicrobial resistance and maintain animal welfare (Lessard et al. 2023; Papakonstantinou et al. 2023). In this sense, phytogenic feed additives (PFAs) have been considered as potential alternatives to antibiotics for their antimicrobial action (Papakonstantinou et al. 2023), and careful attention is currently given to their antioxidant activity (Liu et al. 2021; Papatsiros et al. 2022; Papakonstantinou et al. 2023).

Diet, genetic factors and intestinal microbial composition represent critical aspects affecting feed efficiency (FE) and utilization (Cheng et al. 2023). Pig intestinal microbiota is characterized by high variability of bacterial species playing a crucial role in the regulation of nutritional and metabolic functions and, consequently, swine growth (Le Sciellour et al. 2018; Belloumi et al. 2024).

Metagenomics is a novel approach for investigating the relationship between intestinal bacteria and host characteristics (Mun et al. 2021), indeed the development of high-throughput sequencing helped to obtain distinct profiling of the intestinal microbial communities in pigs (Buzoianu et al. 2012; Buzoianu et al. 2013; Kim et al. 2015) and the correlation between intestinal microbiota and pig growth performances has been recently studied using DNA sequencing techniques (Gardiner et al. 2020).

Dietary inclusion is currently considered one of the most efficient systems to target gut microbiota, especially in growing pigs (Le Sciellour et al. 2018), regulating its composition and, consequently, nutrient digestion, to improve swine growth performance and health (Rebollada-Merino et al. 2019; Vasquez et al. 2022). In the last decades, research has focused on the application of plant-derived feed additives as sources of polyphenols, especially for monogastric animals, including pigs and poultry (Ferlisi et al. 2023). Moreover, the re-utilization of agro-industrial by-products including them in animal diets is a novel strategy that contributes to reducing economic and environmental issues, following the concept of circular economy (Altmann et al. 2018; Altmann et al. 2019). Among these recently used natural feed additives, the secondary by-products deriving from olive oil production are considered an efficient source of polyphenols, as they retain higher concentrations of these bioactive compounds compared to the whole olive fruit (Silvan and Martinez-Rodriguez 2019; Altissimi et al. 2024). In particular, olive mill wastewater (OMWW) is a liquid waste produced by the olive milling industry particularly rich in phenolic types such as hydroxytyrosol, tyrosol, and oleuropein (Davies et al. 2004; Ferlisi et al. 2023; Altissimi et al. 2024). These molecules have demonstrated antioxidant and antimicrobial properties with beneficial effects on growth performances, gut microbiota and overall animal health, potentially helping to decrease antibiotic use in livestock (Van Boeckel et al. 2019; Tian et al. 2023; Belloumi et al. 2024). Many *in vitro* studies demonstrated that natural sources of polyphenols including olive by-products exert a prebiotic action stimulating beneficial intestinal bacteria and, conversely, inhibit the growth of pathogenic species (Paiva-Martins et al. 2009; Verhelst et al. 2014; Fiesel et al. 2014; Silva-Guillen et al. 2020; Xu et al. 2020; Sánchez et al. 2022; Bonos et al. 2022; Almuhayawi et al. 2023). Phenolic compounds have also demonstrated the ability to potentially restore gut health due to their anti-inflammatory function and the modulation of immune response (Selma et al. 2009; Zhang et al. 2020). Finally, it is currently known that microbiota interacts with polyphenols, leading to the formation of powerful bioactive compounds. Previous studies have shown the beneficial effects of dietary olive oil by-products on pig growth performances (Joven et al. 2014; García Casco et al. 2017; Liotta et al. 2019; Papakonstantinou et al. 2023) and intestinal microbiota functions (Liehr et al. 2017; Zhang et al. 2020; Zhang et al. 2021; Sánchez et al. 2022; Belloumi et al. 2024). However, the effects of OMWW on swine gut health and composition have never been tested so far. Thus, this study aimed to determine the effects of a supplementation of OMWW polyphenols in finishing pig diets on gut morphology, pathology, and intestinal microbiota composition.

2. Materials and methods

2.1 Olive mill wastewater extract phenol content

The OMWW extract was provided in powder by Stymon Natural products, P.C., Patras, Greece (www.stymon.com). The OMWW extract was tested for the total phenol content (TPC) with the Folin-Ciocalteu assay as described by Nenadis et al. (Nenadis et al. 2013), using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). Gallic acid was used as the reference standard and results were expressed as gallic acid equivalents (mg GAE/kg). The total polyphenol content of OMWW extract was 36992 ± 58.9 mg/kg. Contents of hydroxytyrosol, tyrosol and oleuropein were 9390 ± 17.4 mg/kg, 1090 ± 3.2 mg/kg and 590 ± 0.4 mg/kg, respectively.

2.2 RP-HPLC-DAD analysis of polyphenols

Hydroxytyrosol, tyrosol, and oleuropein contents of the OMWW were determined by reversed-phase high- performance liquid chromatography (RP-HPLC-DAD) according to Kyriakoudi et al. (Kyriakoudi et al. 2024). The HPLC system consisted of an Agilent 1260 Infinity II Quaternary Pump VL, an Agilent 1260 Infinity II Autosampler and an Agilent 1260 Infinity II Diode Array Detector High Sensitivity. Separation was carried out on a InfinityLab Poroshell 120 EC-C184μm (150 x 4.6 mm i.d.) column (Agilent Technologies, Santa Clara, CA, USA). The column temperature was set at 30 °C. The mobile phase consisted of water (0.1% acetic acid) (A) and acetonitrile (B). The elution protocol was as follow: 0 min, 5% (B), 0–10.0 min, 20% (B); 10.0-15.0 min, 30% (B); 15-18 min, 30% (B); 18.0-20.0 min, 50% (B); 20.0-21.0 min, 100% (B); 21-25 min, 5% (B). The total run time was 25.0 min with flow rate of 1.0 mL/min. The injection volume was 20 μL. Extracts were analyzed after filtration through 0.45 μm PTFE filters (Frisenette, Knebel, Denmark). Monitoring was in the range of 190-600 nm. Chromatographic data were processed using the OpenLab CDS version 3.5 software (2021, Agilent Technologies, Santa Clara, CA, USA). Peak identification was based on retention times and spectral characteristics (absorption maxima) with those of available standards.

2.3 Animal welfare and ethic statement

The experiments were approved by the Bioethics Committee of the University of Perugia (protocol n. 399480). They were performed under the ARRIVE guidelines and animal care procedures followed the European recommendations (Directive 2010/63/EU) for the protection of animals used for scientific purposes. Pigs were raised and slaughtered for conventional meat commerce.

2.4 Animals and diet

The study was conducted on a farm in a hilly area of Umbria, Italy under the conventional farming system for meat production. One hundred thirty-five female Landrace × Duroc finishing pigs (body weight: 102.7 ± 6.8 kg), were randomly divided into three groups (three pens for each group and 15 pigs per pen) fed with the following diets: a) control diet (C) currently used in the farm for the early finishing period (commercial mash-form diet); b) C diet, supplemented with 210 mg/day of polyphenols from OMWW (P-LOW diet); c) C diet, supplemented with 630 mg/day of polyphenols from OMWW (P-HIGH diet). The OMWW polyphenol extract was diluted in water (15 L of water per box), mixed with the feed (2.8 kg/pig), and given to pigs using an automated wet feeding system two times per day. The final concentration of the polyphenols in the complete diet was 74 ppm and 225 ppm for the P-LOW and the P-HIGH diet, respectively. Water was given *ad libitum*, with 15 days of adaptation to the new diets. The composition and nutritional characteristics of the C diet are indicated in Table 1. The trial lasted 85 days and the individual body weight (BW) was recorded at the beginning and at the end of the trial.

Table 1. Ingredients (% as fed basis) and chemical composition (g/100g) of the commercial diet.

	Diet
Raw material	% as fed basis
Grain corn flour	41.8
Grain barley flour	21.7
Wheat middlings	8.1
Proteins	2.5
Wheat flour middlings	9.0
Soybean meal	14.4
Mineral-vitamin supplement	2.5
Analyzed nutrients	g/100 g
Moisture	11.20
CP	15.10
Ether extracts	3.61
Ash	6.83
Crude fiber	4.03
NDF	17.96
ADF	5.79
ADL	1.72
Starch	44.45
Ca	0.68
P	0.53

CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.

2.5 Sample collection

Faeces were collected randomly from 5 pigs per pen at three sampling times: 0 d, 44 d and 85 d. Samples were tested for the presence and consistency of intestinal parasites. At the end of the experiment, 45 pigs (15 pigs per group, 5 pigs per pen) were randomly selected and regularly slaughtered in a local slaughterhouse. Pigs were slaughtered for conventional meat commerce by bleeding after electrical stunning, according to the European Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing. Immediately after, the carcasses were weighed and then stored in a chilling room at 4°C for 24 h. Instead, organs for pathology and morphometry measurements were directly inspected and fixed in formalin. For metabarcoding analysis, 20-30 ml of well-homogenized cecal and jejunal contents were collected and immediately frozen in dry ice and stored at -80°C until further processing.

2.6 Pathology and Morphometry

Macroscopic analysis of the gastrointestinal tract (GI) was performed on the stomach, jejunum, ileum and colon. Due to unpredictable difficulties encountered during the sampling session at the slaughterhouse, 14 animals from C group, 14 from P-LOW group and 11 animals from the P-HIGH group were sampled.

The presence of ulceration in stomach samples was recorded as binomial data (yes/no); additionally, a specific scoring system was used to assess ulceration grade (from 0 to 3) according to Kopinski and McKenzie (Kopinski and McKenzie 2007). Swabs from the pars oesophagea of the stomach were collected from all pigs to investigate the presence of *Helicobacter suis* and *H. pylori* as potential etiological agents of ulcerative lesions. DNA was extracted from the swabs using HiPurA® Multi-Sample DNA Purification (HiMedia, Germany) according to the manufacturer’s protocol. PCR for *H. suis* and *H pylori* was performed using Taq 2× PCR MasterMix (ABM, Canada). Thermal cycling protocols and primers are reported in previous literature (Proietti et al. 2010).

Tissue samples from the stomach, jejunum, ileum and colon were collected and fixed in 10%-buffered formalin. Samples were processed and 3 µm tissue sections were obtained. Histological analysis on these sections assessed leukocyte infiltration, grading severity on a scale from 0 to 3 based on the percentage of affected tissue: 0 indicating no tissue affection, 1 for less than 20%, 2 for 21-60%, and 3 for more than 61%. These criteria also evaluated degeneration, necrosis, and hyperplasia across all segments of the GI tract. The scoring system was established following literature guidelines (Gibson-Corley et al. 2013). Villus height (VH) and crypt depth (CD) measurements in the jejunum, and ileum were obtained by analysing 5 villi at 10× magnification (×100) and averaging the measurements from the apex to the base for VH and from the base to the muscularis mucosae for CD. The VH:CD ratio was calculated for both the jejunum and ileum.

2.7 Metabarcoding analysis

2.7.1 DNA extraction and 16S rRNA gene sequencing

A few samples of jejunum did not contain an adequate amount of digesta and for this reason they were excluded from the analyses (final number of samples kept for analyses: 9 C, 11 P-LOW, and 9 P-HIGH). After thawing, total DNA was extracted using the DNeasy PowerSoil Pro Kit (Qiagen, Maryland, USA) from 350 mg of each cecal and jejunal content sample. After the sample was loaded into the PowerBead Pro tube, the CD1 solution was added. Then, samples were vortexed at a maximum speed for 20 min using a Vortex Adapter for 24 1.5-2.0 ml tubes and DNA was isolated following the manufacturer's protocol. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). Amplicon sequencing of 16S rRNA variable region V3-V4 was performed by preparing libraries through two amplification steps: an initial PCR amplification using locus-specific PCR primers (16S-341F: 5’-CCTACGGGNBGCASCAG-3’ and 16S-805R: 5’-GACTACNVGGGTATCTAATCC-3’) and a subsequent amplification that integrates

relevant flow-cell binding domains and unique indices (NexteraXT Index Kit, FC-131-1001/FC-131-1002). Libraries were sequenced on NovaSeq6000 instrument (Illumina, San Diego, CA) using 250-bp paired-end mode.

2.7.2 Bioinformatic pipeline

Raw sequences were checked for quality using FastQC software, and before and after primer and adapter sequences were checked using Cutadapt. The preprocessed reads were analyzed through the QIIME 2 pipeline (Bolyen et al. 2019), checking for chimaeras and clustering sequences in amplicon sequence variants (ASVs) using the DADA2 algorithm (Callahan et al. 2016). The taxonomy of representative sequences obtained by DADA2 was assigned using the q2-feature-classifier (Bokulich et al. 2013) and the Greengenes2 database (vr. 2022.10) (McDonald et al. 2024). The statistical analyses were performed in R 4.3.0 environment using vegan 2.6.4 (Oksanen et al. 2024) and phyloseq 1.46.0 packages (McMurdie and Holmes 2013). First, ASVs with less than 0.005% of reads among samples (Bokulich et al. 2013) were filtered out and retained sequences were normalized through the rarefaction method setting seed to 1 and choosing the best sample size based on the rarefaction curves produced through the *ggrare* function. The Chao1, Shannon, Simpson and Fisher indices of α-diversity were calculated, and statistical significance was evaluated through student's t-test. The β-diversity was computed using Bray–Curtis distance and weighted generalized Unifrac metrics and statistical significance was calculated by applying the PERMANOVA test with 9999 permutations. Differentially abundant (DA) taxa were retrieved normalizing data and using the negative binomial DESeq2 algorithm after rarefaction (Love et al. 2014). Relative abundance graphs, boxplots for alpha diversity indices, principal coordinates analysis (PCoA) for beta diversity estimation were performed through ggplot2 R package (Wickham 2016).

2.8 Statistical analysis

A simple hierarchical ANOVA model, with the replicates (pen) nested within the diet, was used to analyze pigs' BW. Regarding the morphological and pathological examinations, for binomial (yes/no) factors a Fischer's exact test was conducted to evaluate the association between the factor assessed and the groups. The Kruskal-Wallis test was employed to evaluate differences in various histological parameters across groups. To test differences between groups in the VH:CD ratio in jejunum, and ileum an ANOVA analysis was conducted. In case of significant differences, a Tukey's HSD test was subsequently applied to determine pairwise comparisons between groups. Regarding the morphological and pathological examinations all statistical tests were performed on Rstudio (2023.12.0+369 version). Crosstabulation/chi-square test was used to compare *Helicobacter* detection among groups and to the results of macroscopic and histological analyses of the pig stomach. Statistical significance was set at $p < 0.05$ for any of the assessed parameters.

3. Results

3.1 Growth performance and animals' health

No significant differences among experimental groups were recorded for BW. The average BW recorded at the end of the trial was 148.1 ± 4.6 kg. All fecal samples recorded during the experiment tested negative for intestinal parasites. Furthermore, no diarrheal episodes, except for one pig, were recorded during the whole experimental period.

3.2 Pathology and Morphometry

Gastric ulceration was recorded in all the experimental group C (n = 11/14), P-LOW (n = 7/14), and P-HIGH (n = 5/11), however, the presence of gastric ulceration did not associate significantly with the experimental groups ($p > 0.05$). No difference in *Helicobacter* detection was observed among

the groups. *H. pylori* was not detected in any animal, while *H. suis* was identified in 53.3%, 46.7%, and 63.6% of the animals in the C, P-LOW, and P-HIGH groups, respectively ($p > 0.05$). Nevertheless, *H. suis* was not statistically associated with any of the pathological parameters evaluated (gastric ulceration, leukocyte infiltration, fibrosis, and hyperplasia), supporting its non-etiological role in the gastric lesions observed. The Kruskal-Wallis test revealed no significant differences across the experimental groups for several histological parameters assessed in stomach (grade of gastric ulceration), jejunum, ileum and colon (degeneration, necrosis, hyperplasia, fibrosis, and leukocyte infiltration) ($p > 0.05$).

Regarding morphometry, a summary of data for the VH, CD, and VH:CD ratio is reported in Table 2. A statistically significant difference between groups was identified for the jejunal VH:CD ratio, $F(2,36) = 3.487$, $p < 0.05$ (Figure 1a). The post-hoc comparisons (Tukey HSD test) indicated the following group differences: the mean difference between P-LOW and C, and between Group P-HIGH and P-LOW was not statistically significant ($p > 0.05$); while the mean difference between Group P-HIGH and Group C was 0.671 (95% CI, 0.014 to 1.328), which was statistically significant ($p < 0.05$). Additionally, the VH:CD ratio for the ileum showed a statistically significant difference between groups, $F(2,36) = 9.323$, $p < 0.001$ (Figure 1b). The post-hoc comparisons (Tukey HSD test) indicated the following group differences: the mean difference between P-LOW and C was 0.949 (95% CI, 0.221 to 1.676), which was statistically significant ($p < 0.01$); the mean difference between P-HIGH and C was 1.291 (95% CI, 0.515 to 2.066), which was statistically significant ($p < 0.001$); while the mean difference between P-HIGH and P-LOW was not statistically significant. Features of the jejunal and ileal intestinal mucosa for C, P-LOW and P-HIGH are shown in Figure 2.

Table 2. Summary of villous height (VH), crypt dept (CD), and VH:CD ratio for jejunum and ileum of C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

	VH	CD	VH:CD (median value)
Jejunum			
C	376.30 µm (± 74.80).	152.96 µm (± 38.88)	2.60
P-LOW	417.05 µm (± 52.80)	145.06 µm (± 39.22)	3.25
P-HIGH	424.45 µm (± 54.79)	135.73 µm (± 29.17)	3.40
Ileum			
C	264.73 µm (± 47.19)	146.53 µm (± 25.22)	1.70
P-LOW	330.82 µm (± 69.39)	123.33 µm (± 26.46)	2.67
P-HIGH	310.48 µm (± 41.99)	109.55 µm (± 35.79)	3.26

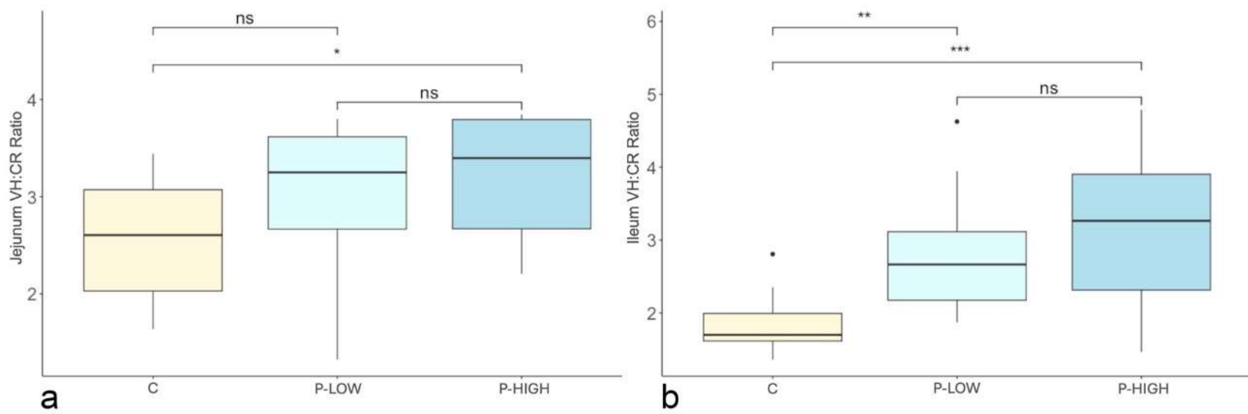

Figure 1. Boxplot summarizing jejunum (a) and ileum (b) Villous Height to Crypt Dept (VH:CD) ratio by diet groups. (a) Group fed with 225 ppm polyphenols (P-HIGH) and group fed with 74 ppm polyphenols (P-LOW) show a higher VH:CD ratio in the jejunum compared to group control (C); differences between Group P-HIGH and Group C are significatively different (*). (b) Group P-HIGH and P-LOW show a higher VH:CD ratio in the ileum compared to group C; differences between P-LOW and C (**) and P-HIGH and C (***) are significatively different. *** $p< 0.001$, ** $p< 0.01$, * $p < 0.05$. *Note*: ns = not significative; VH= Villous height; CD= Crypt Depth.

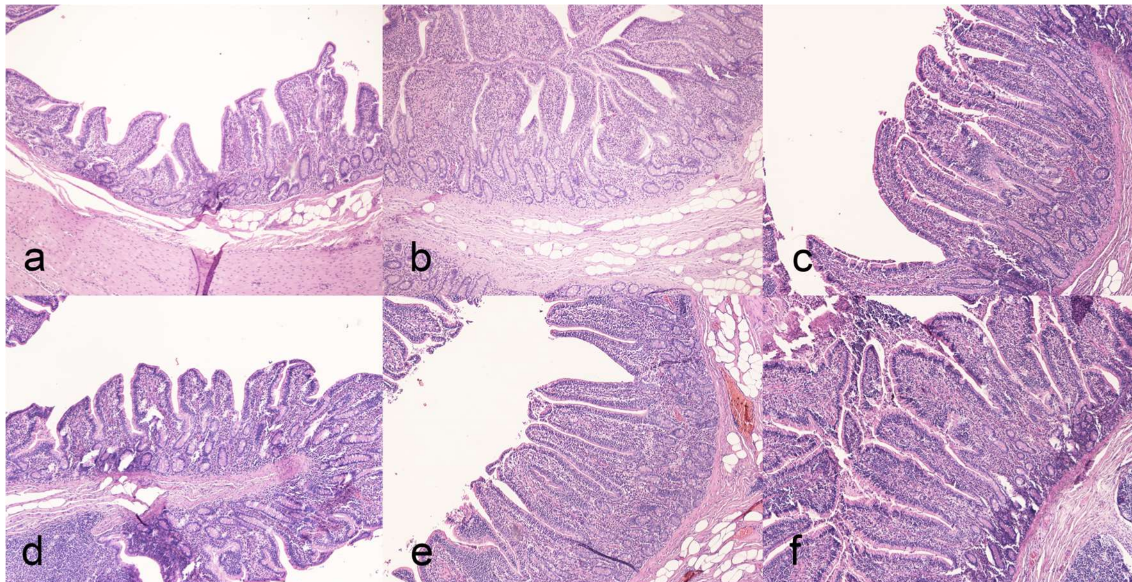

Figure 2. The panel presents histological sections (Haematoxylin and Eosin) of the jejunum (a-c) and ileum (d-f) (x100). The jejunal mucosal architecture of group C (a) shows short, intact villi and crypts. In Group P-LOW (b) and P-HIGH (c), the villi and crypts are notably longer, indicating visible changes. For the ileum, group C (d) reveals shorter villi and deeper crypts compared to P-LOW (e) and P-HIGH (f), where the villi are longer and crypts are shallower, reflecting differences in mucosal architecture across groups.

3.3 Metabarcoding results

For the cecum, metabarcoding library preparation for sequencing was carried out from all 45 subjects (15 C, 15 P-LOW, and 15 P-HIGH) although, unfortunately, the sequencing of 4 samples of the P-HIGH group failed. After each bioinformatic analysis step, over 141 K, on average, high-quality reads per sample were kept (about 75% of raw sequences) (Supplementary Table 1). The histogram of read depth per sample for cleaned reads used for downstream analysis is reported in Supplementary Figure 1A. After filtering, among the 6767 sequences, 879 amplicon sequencing variants (ASV) were retrieved. Rarefaction curves are reported in Supplementary Figure 1B, which allowed the best sample size to be chosen for rarefaction. which allowed to choose the best sample size for rarefaction. The agglomeration step on rarefied data grouped these ASVs into 145 species, 112 genera and 44 families. Figure 3A shows the first 15 most abundant genera and relative families among all samples. The relative abundances calculated did not show differences among the experimental groups at the genus and family levels (Figure 3A).

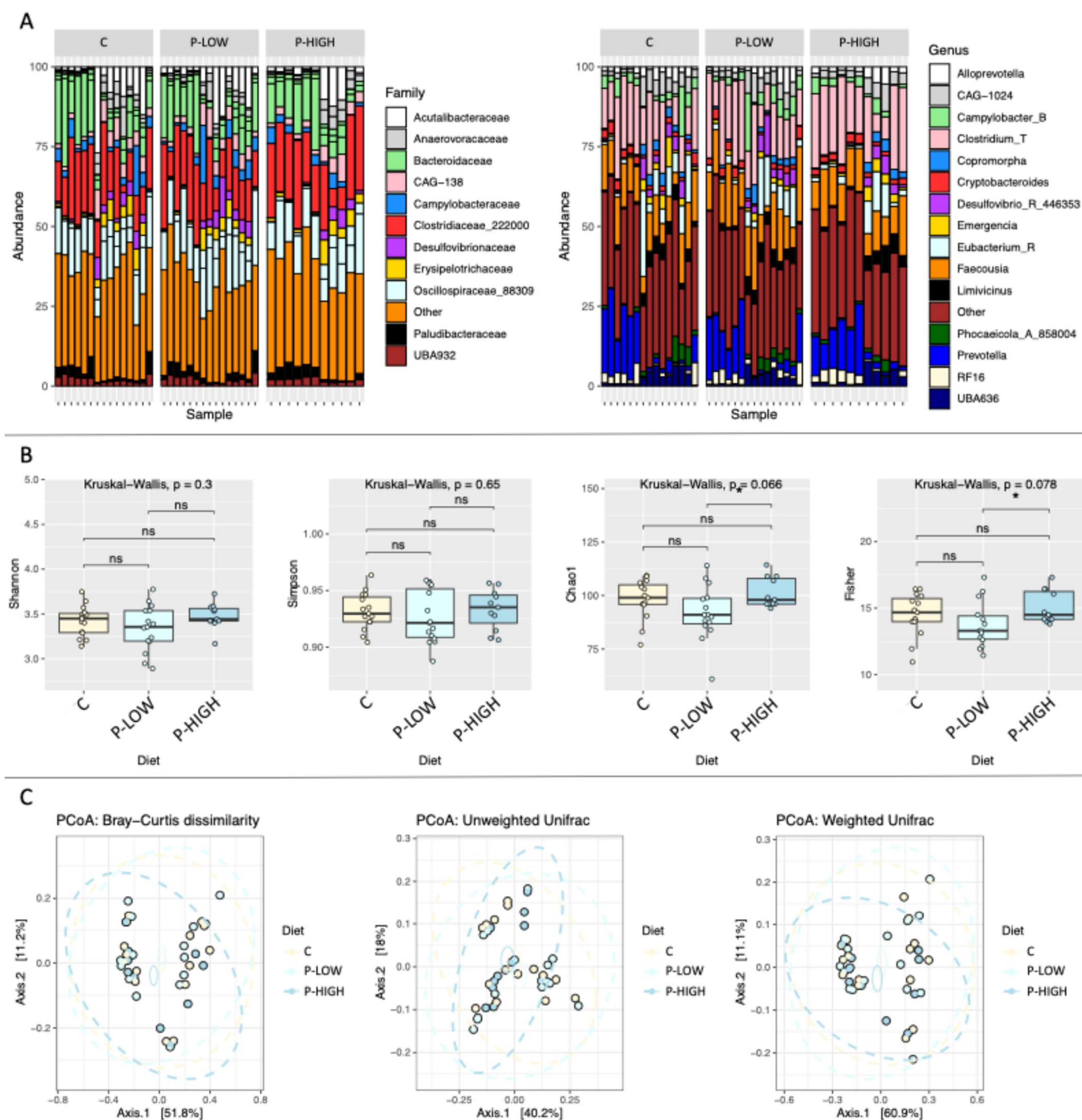

Figure 3. Cecal content characterization and differences between experimental groups (Group Control C, Group fed with 74 ppm of polyphenols P-LOW, Group fed with 225 ppm of polyphenols P-HIGH). A) Relative abundance of the first 15 families and genera; B) Alpha diversity indices (Shannon, Simpson, Chao1 and Fisher), testing relative statistical significance through Kruskal-Wallis test and Wilcox test for pairwise comparison between groups; (C) Principal coordinate analysis graphs for Bray-Curtis dissimilarity index, unweighted Unifrac distance, and weighted Unifrac distance. *Note*: ns= not significative; * p<0.05.

For alpha diversity indexes, no significant differences were found between the three experimental groups in terms of richness (Chao1), except for the P-HIGH group which showed a higher Chao1 index with respect to the P-LOW group (Figure 3B). In the same way, no significant differences were found in terms of species diversity (Shannon, Simpson and Fisher), except for the P-HIGH group which demonstrated a higher Fisher index compared to the P-LOW group (Figure 3B). Concerning beta diversity, the phylogenic tree used to calculate Unifrac distances is shown in Supplementary Figure 1C, while Figure 3C shows that the samples from different diets did not form separated groups for all the indexes, indicating that the general microbial content of the experimental groups was similar. The PERMANOVA test also showed no statistically significant differences (Table 3).

Table 3. Statical significance by PERMANOVA test for the beta diversity calculation among control (C) and treated groups P-LOW (74 ppm) and P-HIGH (225 ppm) of cecal content for the three considered indices.

Beta diversity indices	R^2	F	p-value
Bray-Curtis dissimilarity (rarefaction normalized data)	0.02	0.48	0.91
Unweighted Unifrac distance (rarefaction normalized data)	0.03	0.57	0.87
Weighted Unifrac distance (rarefaction normalized data)	0.01	0.28	0.99

For the jejunum, metabarcoding sequencing was carried out from 27 available samples (9 controls, 11 P-LOW, and 9 P-HIGH). A total of 116K on average high-quality reads per sample were obtained (71% of raw sequences) with a distribution reported in Supplementary Figure 2A. Sequencing statistics are reported in Supplementary Table 2. The initial 3258 sequences, comprising 193 amplicon sequencing variants (ASV), were subjected to the rarefaction step, choosing the best sample size based on the rarefaction curves reported in Supplementary Figure 2B. Three samples for the P-LOW group (2T, 4T and 9T; Supplementary Table 1) were excluded for downstream analysis due to the poor number of sequences. After taxa agglomeration, 58 species, 45 genera and 29 families were identified. Figure 4A shows the first 15 most abundant genera and relative families among all samples. No differences were found for all the alpha diversity indexes between the three experimental groups (Figure 4B) as well as for the beta diversity (Figure 4C). Indeed, the plots indicate that samples from different diets were quite homogeneous, and no statistically significant differences were found (Table 4). The phylogenic tree used to calculate Unifrac distances is shown in Supplementary Figure 2C. Finally, differentially abundant (DA) genera and species are reported in Figure 5 for cecum and Figure 6 for jejunum. Details are reported in Supplementary Tables 3 and 4 for cecum and 5 and 6 for jejunum, while relatives DA families are reported in Supplementary Figure 3A and B for cecal and jejunal substrates, respectively.

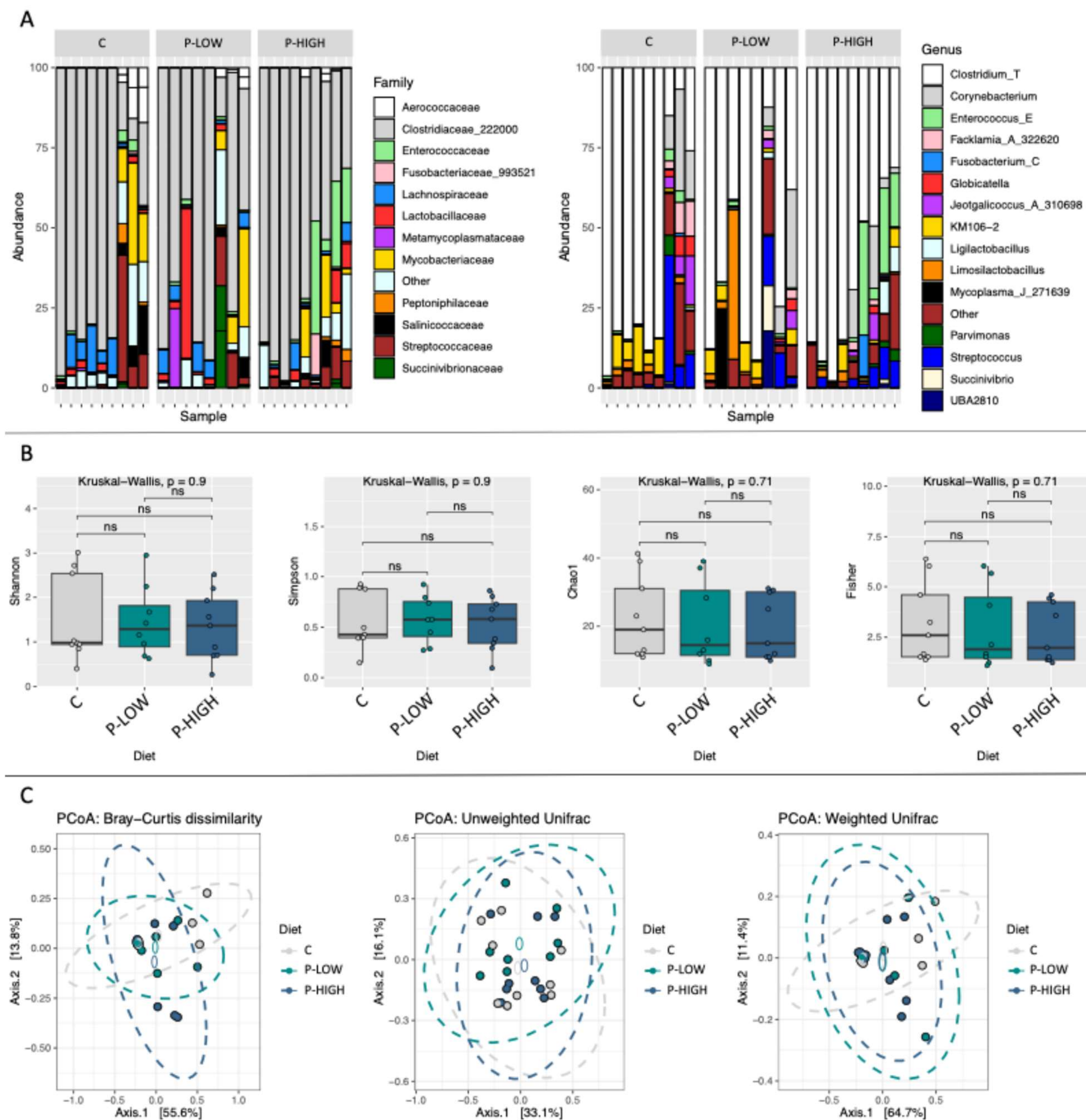

Figure 4. Jejunum content characterization and differences between experimental groups (Group Control C, Group fed with 74 ppm of polyphenols P-LOW, Group fed with 225 ppm of polyphenols P-HIGH). A) Relative abundance of the first 15 families and genera; B) Alpha diversity indices (Shannon, Simpson, Chao1 and Fisher), testing relative statistical significance through Kruskal Wallis test and Wilcox test for pairwise comparison between groups; (C) Principal coordinate analysis graphs for Bray-Curtis dissimilarity index, unweighted Unifrac distance, and weighted Unifrac distance. *Note*: ns= not significative.

Table 4. Statical significance by PERMANOVA test for the beta diversity calculation among control (C) and treated groups P-LOW (74 ppm) and P-HIGH (225 ppm) of jejunal content for the three considered indices.

Beta diversity indices	R^2	F	p-value
Bray-Curtis dissimilarity (rarefaction normalized data)	0.05	0.57	0.80
Unweighted Unifrac distance (rarefaction normalized data)	0.08	1.03	0.40
Weighted Unifrac distance (rarefaction normalized data)	0.02	0.26	0.99

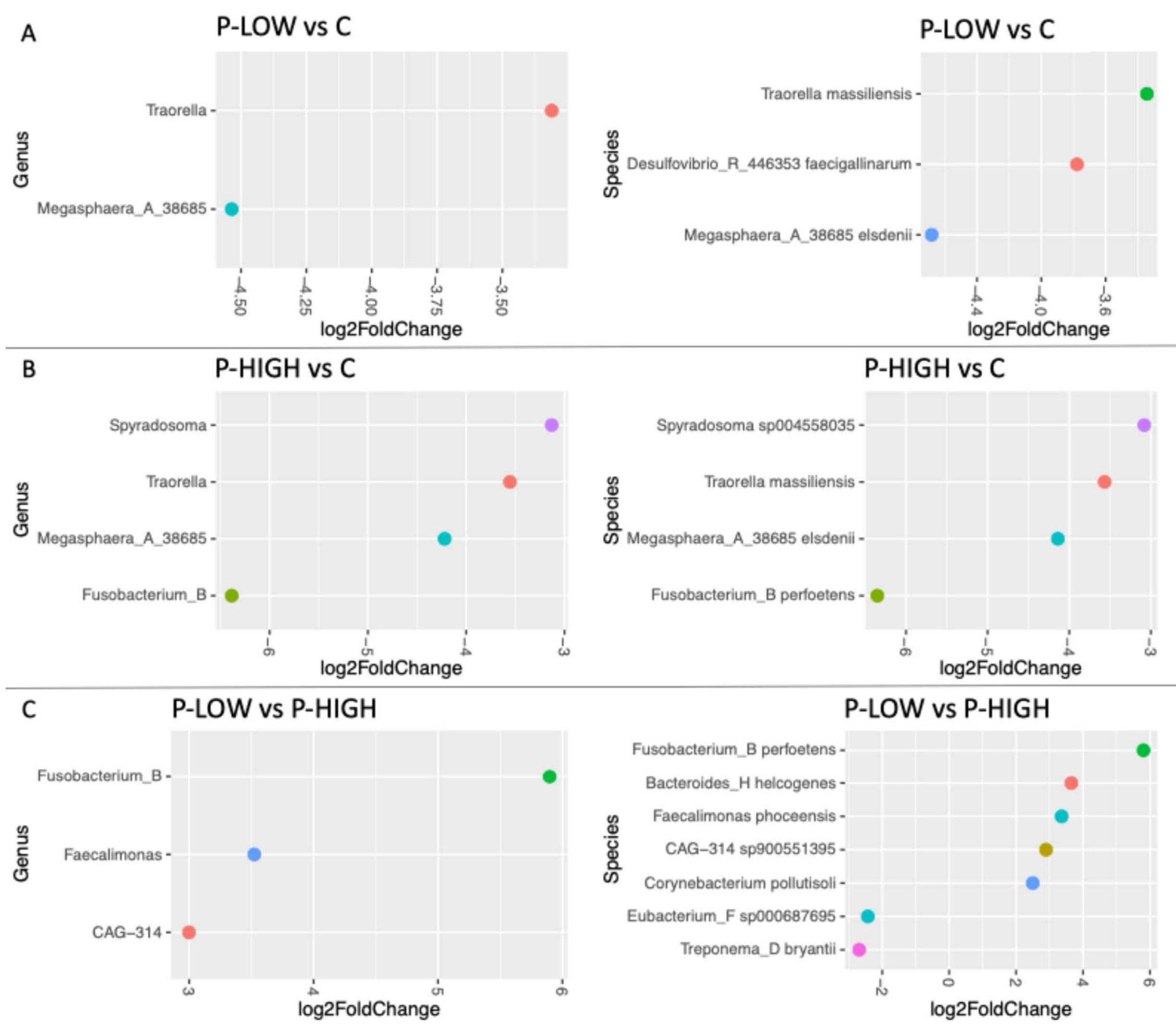

Figure 5. Differentially abundant (DA) genus and species of cecum identified among the various conditions (Control C, olive mill wastewater phenolic diets P-LOW and P-HIGH). For each comparison, genera (left panel) and species (right panel) are shown with a multiple testing corrected p-value ≤ 0.05. Statistically significant features (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) are reported for A) P-LOW vs Control, taxa with a Fold Change greater than 0 are more abundant in the P-LOW condition; B) P-HIGH vs Control, taxa with a Fold Change greater than 0 are more abundant in the P-HIGH condition; and C) P-LOW vs P-HIGH, taxa with a Fold Change greater than 0 are more abundant in the P-LOW condition; colors of the dots indicate the belonging to the same family.

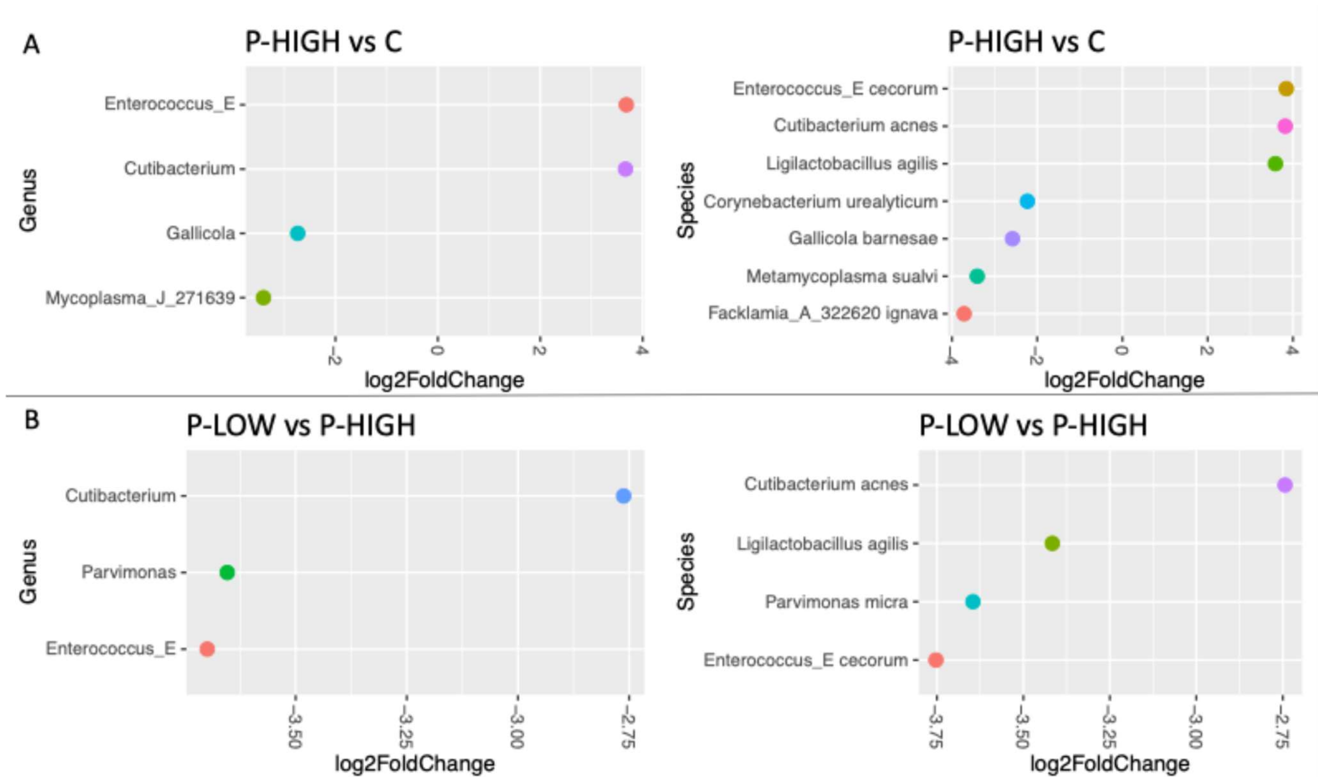

Figure 6. Differentially abundant (DA) genus and species of jejunum identified among the various conditions (Control C, olive mill wastewater phenolic diets P-LOW and P-HIGH). Statistically significant features (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) are reported for A) P-LOW vs Control, taxa with a Fold Change greater than 0 are more abundant in the P-LOW condition; B) P-HIGH vs Control, taxa with a Fold Change greater than 0 are more abundant in the P-HIGH condition; and C) P-LOW vs P-HIGH, taxa with a Fold Change greater than 0 are more abundant in the P-LOW condition; for P-LOW vs C comparison no statistical significance resulted.

4. Discussion

The interest in exploring agro-industrial by-products, including the ones from the olive oil industry, for monogastric diets is increasing, aiming not only to evaluate the impact on animal growth and production efficiency but also to research the potential effects on gut health and microbiota populations (Sánchez et al. 2022; Ferlisi et al. 2023; Belloumi et al. 2024). In this study, we evaluated the effects of dietary supplementation with two dosages (74 ppm and 225 ppm) of a polyphenolic extract from OMWW on gastrointestinal morphology and pathological features and intestinal microbial composition of finishing pigs.

4.1 Pathology and Morphometry

None of the examined parameters of the pathology assessment revealed a significant association and/or a difference in grade distribution with the three experimental groups. However, even if the association of gastric ulcer presence and grade distribution difference in the experimental groups was not significant, a trend for a lower frequency in gastric ulcer development was noticed, within the P-

LOW and P-HIGH groups, compared to group C. Interestingly, there were no significant differences in the semiquantitative scoring of leukocyte infiltration in the intestinal lamina propria, which differs from the findings of a previous study (Varricchio et al. 2019). Similarly to our study, Varricchio et al. reported varying degrees of leukocyte infiltration but found a significantly higher number of infiltrating leukocytes in the group subjected to the supplementation of polyphenol extracted from OMWW.

The impact of the diet on the overall mucosa health was additionally assessed by calculating the VH:CD ratio which, in both the jejunum and ileum, revealed noteworthy differences across different groups. In the jejunum, the P-HIGH group demonstrates a significantly higher VH:CD ratio compared to the C group ($p < 0.05$). Similarly, in the ileum, both P-LOW ($p < 0.01$) and P-HIGH ($p < 0.001$) groups exhibit a significantly higher VH:CD ratios compared to C. This higher VH:CD ratio in both P-LOW and P-HIGH groups could imply enhanced absorptive capacity or a more favourable mucosal structure, which might be beneficial for nutrient absorption or overall gut health. The polyphenol supplementation effects are particularly marked for the ileal tract where the differences with the C group are higher compared to those of the jejunum. Consistent with our results, two studies reported that a dietary supplementation of the polyphenol resveratrol increased the VH:CD ratio in piglets' small intestines (Chen et al. 2021; Xun et al. 2021). Additionally, an olive cake extract could be responsible for the restoration of the VH:CD ratio in the intestine of piglets challenged with lipopolysaccharides (Zhang et al. 2021). The results of the current study suggest the lack of major side effects and OMWW product-associated lesions on the gastrointestinal system of pigs at the examined dosage. These results agree with the current literature, suggesting no major harmful potential and rather beneficial effects on intestinal health for olive oil-processing products.

4.2 Relative abundances and microbial diversity

Concerning microbial composition, our study did not show major differences in the relative abundances of genera and families between the three experimental groups (Figures 3A and 4A), for both cecal and jejunal composition. Also, alpha diversity indexes were comparable among the groups, indicating quite similar levels in terms of biodiversity and richness. The unique significant increases of Chao1 and Fisher indexes were found in P-HIGH compared to the P-LOW group in cecum (Figure 3B). Moreover, no significant differences in beta diversity were found among the groups in both cecum and jejunum, indicating that the two OMWW phenolic diets did not influence the overall microbial composition of the pig gut. Similarly, Belloumi et al. (2024) did not find significative differences in alpha diversity indexes in fecal microbiota of pigs supplemented with an olive cake by-product (Belloumi et al. 2024). It is known that higher microbial diversity is correlated to the maturation of intestinal communities, and contributes to a more stable bacterial ecosystem, preventing stress conditions that could lead to dysbiosis (Luise et al. 2024). Although alfa and beta indexes were similar for the gut content of the enrolled animals, significant variations in the abundances of many bacteria at genera and species levels between the three experimental groups were found, in both cecum and jejunum.

4.3 Differential abundance in cecum

In particular, we found increased levels of potentially beneficial bacteria and a reduction of some possible impairing taxa in P-HIGH compared to P-LOW or control. Concerning taxa that increased after the highest dosage of polyphenols (P-HIGH) compared to P-LOW, we found two species belonging to the *Eubacterium* and *Treponema* genera (Figure 5, $\log_2FC<0$ in P-LOW vs P-HIGH). *Eubacterium* is part of Firmicutes and is one of the most known butyrate-producing bacteria in pig gut (Xu et al. 2021). A study by Bergamaschi et al. (2020) found a positive correlation between the genus *Eubacterium* and FE (Bergamaschi et al. 2020). Furthermore, hydroxytyrosol, the most represented polyphenol in the OMWW extract, is known to increase the abundance of several beneficial bacteria, including *Eubacterium* (Han et al. 2022). Thus, the higher abundance of

Eubacterium F sp000687695 in the P-HIGH group suggests that the beneficial effect of polyphenols on pig cecum may be dose-dependent. In addition, the *Treponema* genus has been associated with the production of short-chain fatty acids, including acetic acid (Belloumi et al. 2024) and, interestingly, with growth and fatness parameters in pigs' small and large intestines (Gardiner et al., 2020) . Quan et al. (2020) demonstrated that many species of the *Treponema* genus were more abundant in the cecum of pigs with high FE and, more in particular, *T. bryantii* (increased in P-HIGH) was cited as one of the bacterial species that may positively influence pig FE (Quan et al. 2020). This microorganism is, indeed, a fiber-degrading bacterium and thus plays a significant role in the digestion of complex carbohydrates in the pig's cecum (Cwyk and Canale-Parola 1979; Stanton and Canale-Parola 1980; Nordhoff et al. 2005). Furthermore, *Treponema* has been associated to beneficial taxa in both small and large swine intestines (Gardiner et al. 2020). Our results are coherent with the scientific literature, which demonstrated that piglets supplemented with an olive cake extract had an increased abundance of this genus (Zhang et al. 2021) and suggest a positive impact on pig productive efficiency. Among taxa that decreased with the highest polyphenol dietary supplementation, the abundance of *Fusobacterium* was lower in terms of genus and species (*Fusobacterium perfoetens*) in the cecum of the P-HIGH pigs, when compared to P-LOW and C. *Fusobacterium* has been associated with human gut inflammation and can interfere with T-cell response and stimulate the expression of many molecular markers implicated in colorectal cancer (Nosho et al. 2016; Wei et al. 2016). Moreover, the increase of this bacterium has been associated with the etiology of diarrhea in piglets (Yang et al. 2017). Our results suggest that the highest OMWW extract dosage used in this study (225 ppm) could decrease the risk of intestinal disorders. Interestingly, P-HIGH pigs also showed lower levels of *Bacteroides helcogenes* compared to the P-LOW. Although it is a rare cause of intestinal damages in intensive livestock animals, *B. helcogenes* is a pathogenic bacterium that was isolated from pig feces and abscesses (Benno and Mitsuoka 1984; Pati et al. 2011). Its reduction following the administration of the P-HIGH diet might positively impact pig gut health.

The present study also showed that the cecum of pigs belonging to the P-HIGH group had a lower abundance of *Faecalimonas*, compared to the P-LOW group. The genus *Faecalimonas* is usually found in the mammalian gut (Pereira et al. 2020) and can produce acetate (Sakamoto et al. 2018; Biagi et al. 2020). Similarly, supplementation with magnolol, a plant polyphenol with health-promoting functions in the pig intestines, has been shown to reduce *Faecalimonas* concentration (Li et al. 2024). Despite these positive actions, we found the unexpected reduction of two controversial bacteria, *Desulfovibrio* and *Megasphaera.* The genus *Desulfovibrio* belongs to the group of sulfate-reducing bacteria, known to produce H_2S (Ran et al. 2019), and, thus, fundamental to removing H_2, and maintaining a healthy gut. Nevertheless, an excess of H_2S has been associated with intestinal damages, with a possible risk of inflammatory bowel disease (Deplancke et al. 2000). Overall, in pig gut microbiota, the diversity of sulfate-reducing bacteria remains unclear and more data are necessary to understand the role of this microbial group (Ran et al. 2019).

Concerning *Megasphaera* and *Megasphaera*_A_38685 *elsdenii*, their abundance was lower in the two treated groups compared to the control. *Megasphera* belongs to the phylum Firmicutes and is known to mainly produce butyrate (Koh et al. 2016), whose synthesis is correlated to intestinal health both in humans and animal species, including pigs (Levine et al. 2013). *Megasphaera* is one of the anaerobic bacteria dominating pig large intestine (Grosu et al. 2019) which plays a central role in fermentation, helps to maintain the intestinal pH and prevents lactic acidosis by regulating lactate levels (Grosu et al. 2020). Furthermore, *Megasphaera elsdenii* is the most isolated anaerobic species that is able to restore the small and large intestine in weaned piglets (Yoshida et al. 2009). Literature reported that *Megasphaera* abundance could be stimulated in pig microbiota with the inclusion of grape seed composed by the polyphenol flavonoid. (Grosu et al. 2019; Grosu et al. 2020). These findings are in contrast with our results, maybe due to the different chemical nature of the olive polyphenols.

4.4 Differential abundance in jejunum

The analysis of jejunum showed an increase of *Enterococcus* abundance at the genus and species (*Enterococcus cecorum*) level in P-HIGH compared to C and P-LOW. *Enterococcus* genus comprehends harmful and also commensal microorganisms that produce lactic acid and have low pathogenic tendencies (Torres et al. 2018; Goel et al. 2022). *E. cecorum* is one of the most studied bacteria in chicken gut microbiota and has been designed as an emerging avian pathogen (EFSA Panel on Animal Health and Welfare (AHAW) et al. 2022), but it was also isolated from healthy intestines of livestock animals including pigs (Jung et al. 2017). This species was additionally found to increase in the feces of diarrheic piglets (Sun et al. 2019). However, in our trial we did not observe diarrhea episodes and, according to the literature, up to now, *Enterococcus cecorum* was not associated with specific swine diseases (Jung et al. 2017). In addition, a grape pomace extract rich in polyphenols (and apigenin in particular) stimulated *in vitro* the increase of *Enterococcus* in human gut microbiota (Sánchez et al. 2016; Wang et al. 2017). Grape pomace has also been shown to increase this bacterium in broilers' intestinal content (Viveros et al. 2011).

Another increased bacterium in the jejunum of P-HIGH pigs was the *Ligilactobacillus agilis*. This microbial species is an important member of the Firmicutes phylum (Wiese et al. 2021). Interestingly, an increase of *Lactobacillus agilis* was observed after dietary supplementation with a fermented herbal feed additive in piglets and tea polyphenols in laying hens (Wang et al. 2024; Xiao et al. 2024). Considering the higher abundance of this helpful microorganism, it is possible to assume that the 225 ppm dosage potentially had a positive impact on pigs' gut health, compared to the P-LOW treatment and the C diet. Among the interesting taxa that decreased in the P-HIGH group, compared to control, there were *Metamycoplasma* and *Corynebacterium. Metamycoplasma* genus includes pathogenic microorganisms associated with severe chronic infections and negative impact on animal health (Chae et al. 2020), although *Mycoplasma sualvi,* the species we found decreased, is generally considered less pathogenic compared to others. Interestingly, members of the *Mycoplasma* genus can cause an increase in oxidative stress in pigs (Helm et al. 2018). Thus, the lower abundance of this bacterium in the P-HIGH group may suggest a protective effect on swine gut, compared to the controls. As already mentioned, our study demonstrated a lower abundance of *Corynebacterium urealiticum* in the jejunum of the P-HIGH group. Corynebacteria are divided into non-pathogenic and pathogenic bacteria, that can cause infectious diseases in farm animals, including pigs (Yibin et al.). In particular, *C. urealiticum* was identified in pigs (Vela 2003; Eiamsam-ang et al. 2023) and can be associated with the urinary tract infections via ascending route (Pathogenesis of bacterial infections in animals 2010).

5. Conclusion

The role of dietary polyphenols in monogastrics' intestinal health is quite unexplored, especially regarding the use of olive by-products as natural sources. Integrating these wastes into livestock diets can help to limit antimicrobial use and excessive costs for the producers, as well as reduce the environmental impact of the olive oil industry. In our study, the supplementation of a polyphenol extract from OMWW in the finishing pig diet seemed to potentially stimulate some beneficial changes in the intestinal environment. This effect may be linked to improvements in intestinal morphological development and the shaping of the microbiota, although further research is needed to confirm the relationship between these bioactive compounds and the maintenance of a healthy and efficient intestinal system in pigs.

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Author contributions

Massimo Trabalza-Marinucci, Katia Cappelli, Flavia Ferlisi, Samanta Mecocci, and Jiayong Tang designed the study. Flavia Ferlisi, Samanta Mecocci, Katia Cappelli, Giuseppe Giglia, Elisa Rampacci, Gabriele Acuti, Jiayong Tang, Anastasia Kyriakoudi, Massimo Trabalza-Marinucci collected the samples and performed the laboratory analyses. Samanta Mecocci e Daniele Pietrucci performed bioinformatic and statistical analyses. Flavia Ferlisi, Samanta Mecocci and Giuseppe Giglia wrote the original draft of the manuscript. Massimo Trabalza- Marinucci, Katia Cappelli, Gabriele Acuti, Elisa Rampacci, Jiayong Tang, Ioannis Mourtzinos, Anastasia Kyriakoudi and Luca Mechelli reviewed and edited the last version. Funding acquisition: Massimo Trabalza-Marinucci, Ioannis Mourtzinos, Luca Mechelli. All authors read and approved the final manuscript.

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

The raw sequencing data that support the findings of this study are openly available in Sequence Read Archive repository (SRA) (Leinonen et al. 2011); BioProject ID PRJNA1185956- sixty-eight BioSamples from SAMN44733558 to SAMN44733598 for cecum content samples and from SAMN44733599 to SAMN44733625 for the jejunum ones).

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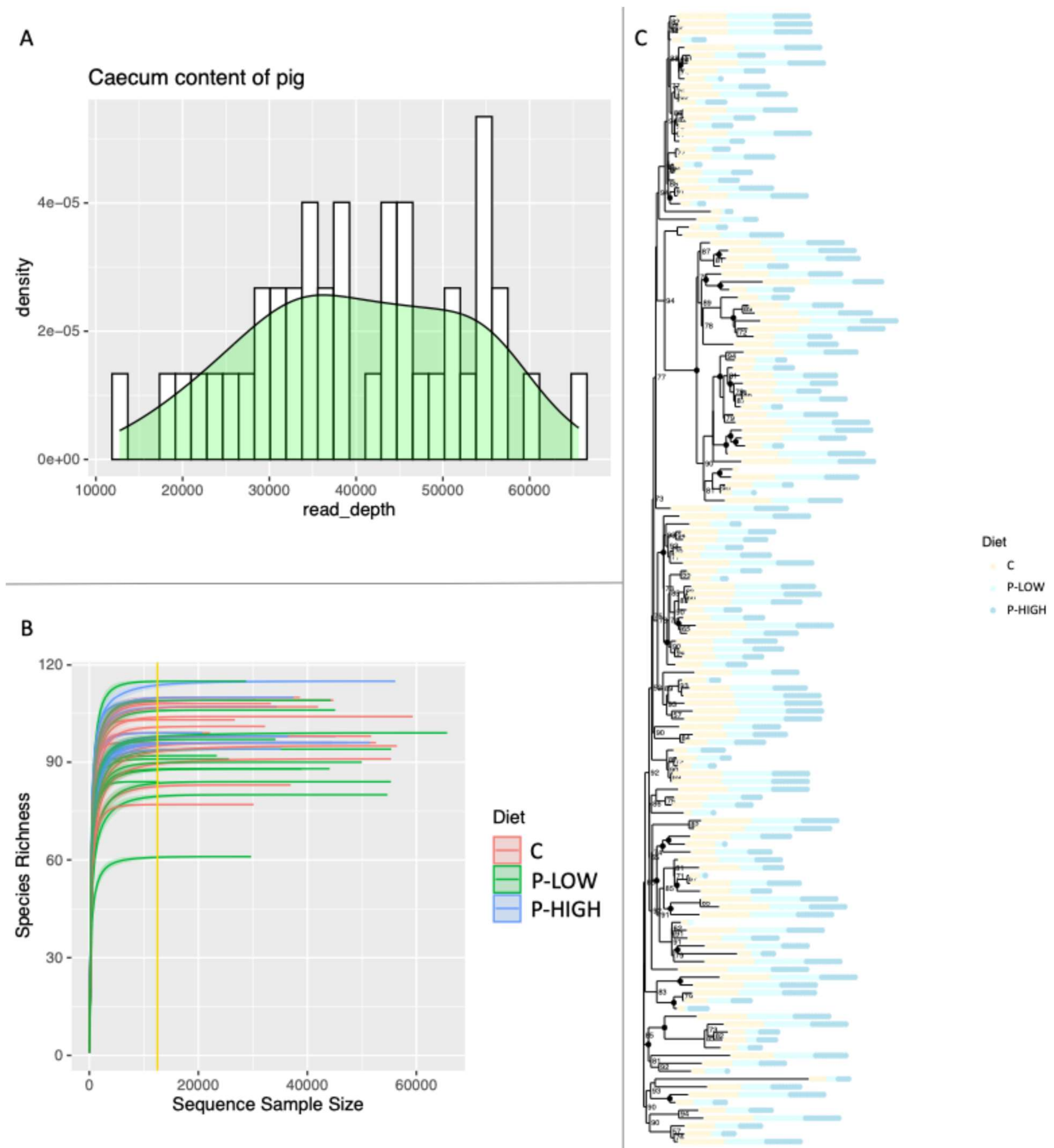
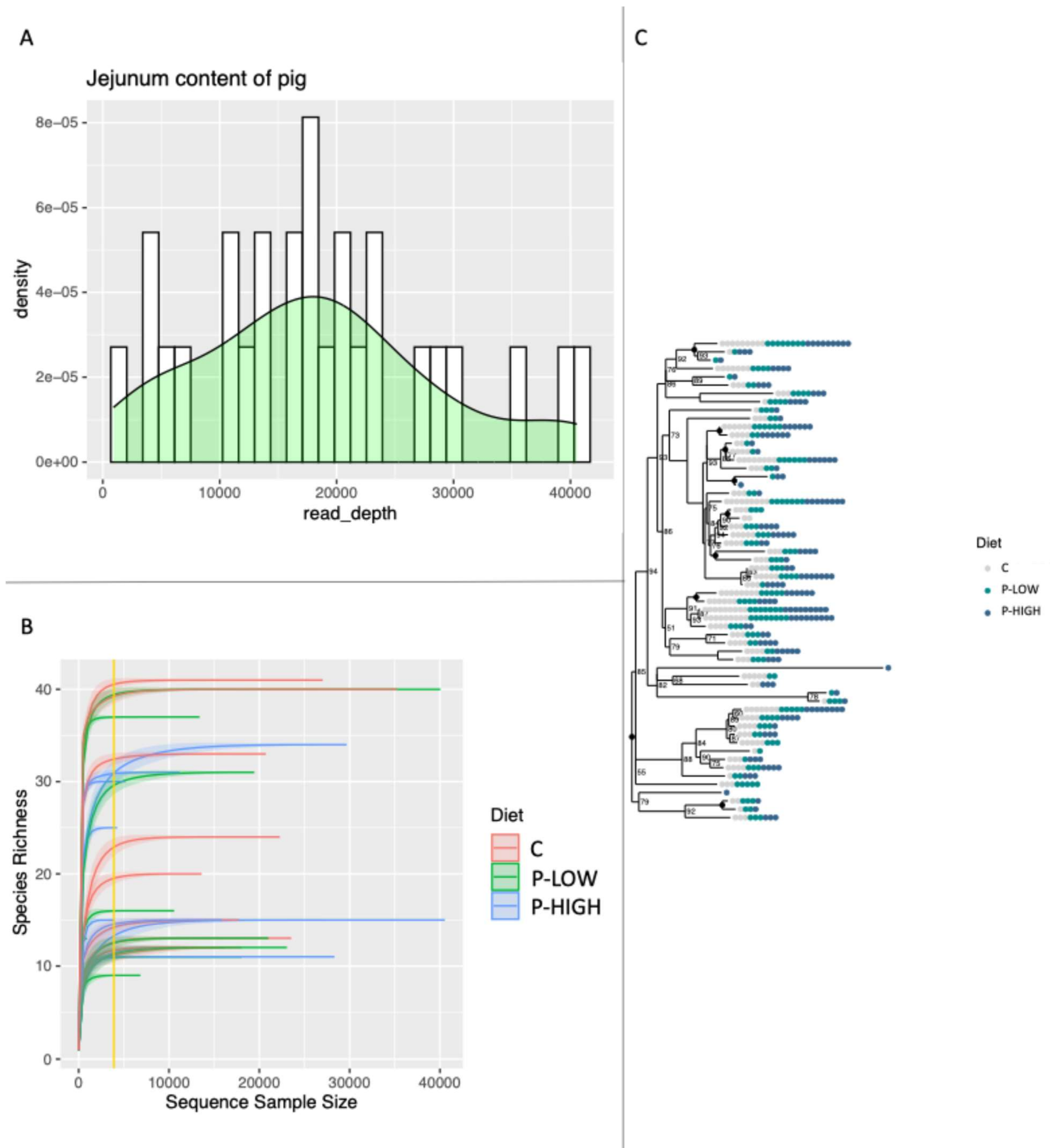
Supplemetary figures and tables

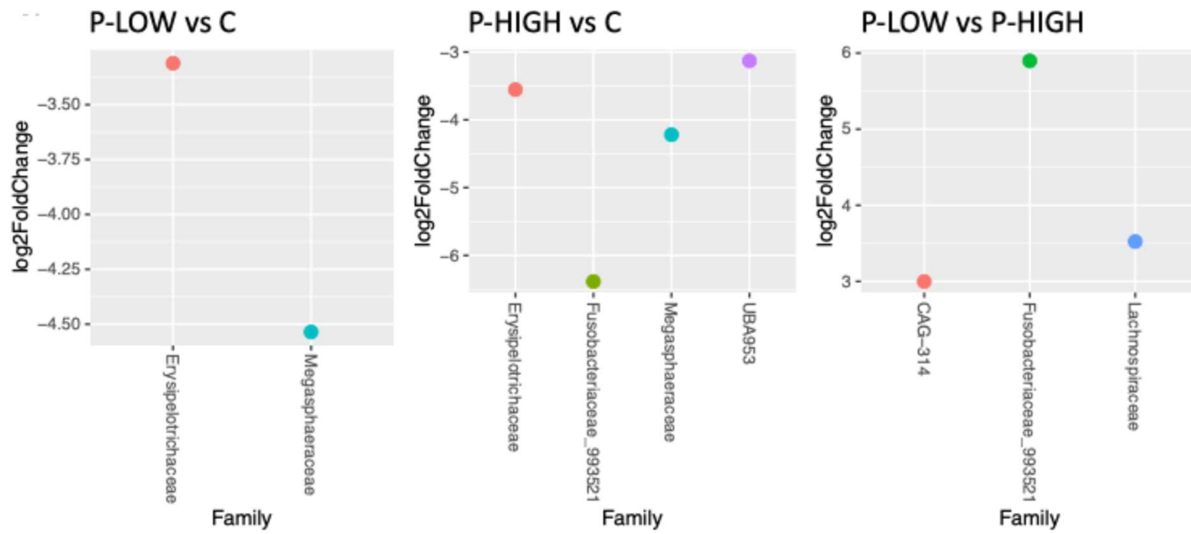


Supplementary Figure 1. Cecum histogram of read depth distribution among samples for cleaned reads used for downstream analysis (A), rarefaction curves which allowed us to choose the best sample size parameter of 12,500 reads for each sample (indicated by the yellow line) (B), and phylogenic tree used to calculate unifrac distances (C); C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

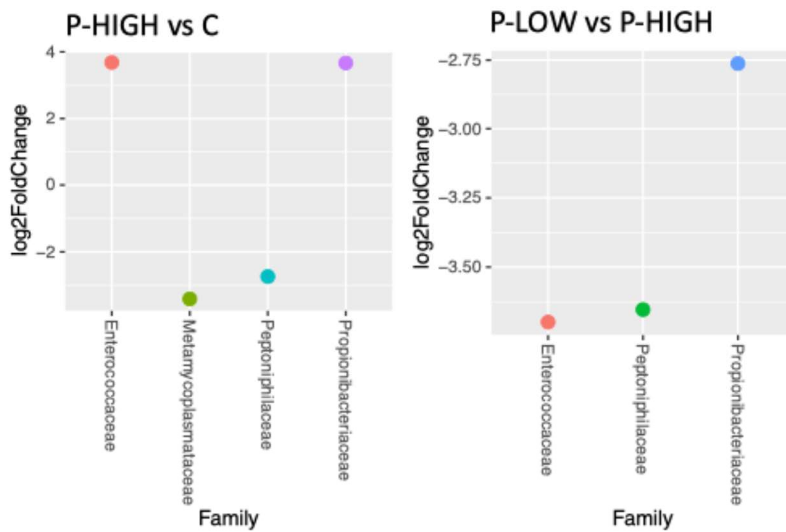


Supplementary Figure 2. Jejunum histogram of read depth distribution among samples for cleaned reads used for downstream analysis (A), rarefaction curves which allowed us to choose the best sample size parameter of 3,900 reads for each sample (indicated by the yellow line) (B), and phylogenic tree used to calculate unifrac distances (C); C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

A) Cecum



B) Jejunum



Supplementary Figure 3. Differentially abundant families (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) for cecum (A) and jejunum (B) substrate comparisons between experimental groups: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Supplementary Table 1. Sequencing statistics of retained reads after each bioinformatic step for cecum content. Diet: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Sample	Diet	input	filtered	% passed filter	denoised	merged	% merged	non-chimeric	% non-chimeric
1C	C	116,036	105,051	90.53	102,964	89,490	77.12	82,968	71.5
2C	C	147,290	134,339	91.21	132,293	116,481	79.08	106,310	72.18
3C	C	186,053	172,018	92.46	169,156	149,346	80.27	137,906	74.12
4C	C	93,991	82,477	87.75	81,047	70,683	75.2	66,232	70.47
5C	C	131,638	120,361	91.43	118,360	105,925	80.47	99,632	75.69
6C	C	232,788	210,063	90.24	207,391	183,178	78.69	171,865	73.83
7C	C	190,274	175,082	92.02	172,983	155,757	81.86	146,118	76.79
8C	C	300,179	278,962	92.93	275,815	246,510	82.12	225,702	75.19
9C	C	215,932	200,156	92.69	197,211	177,671	82.28	168,468	78.02
10C	C	148,165	136,281	91.98	134,004	119,932	80.94	115,054	77.65
11C	C	243,353	224,303	92.17	221,194	200,183	82.26	189,090	77.7
12C	C	219,181	203,134	92.68	200,344	183,041	83.51	173,615	79.21
13C	C	229,443	211,501	92.18	208,813	191,559	83.49	181,400	79.06
14C	C	162,943	121,324	74.46	119,235	106,716	65.49	102,603	62.97
16C	C	248,755	228,520	91.87	224,934	200,592	80.64	187,061	75.2
1T	P-LOW	169,255	155,446	91.84	153,225	136,073	80.4	123,412	72.91
2T	P-LOW	133,669	122,647	91.75	120,943	108,266	81	100,221	74.98
3T	P-LOW	158,383	140,968	89	138,872	118,182	74.62	106,350	67.15
4T	P-LOW	67,756	60,093	88.69	59,073	52,724	77.81	496,60	73.29
5T	P-LOW	125,539	115,433	91.95	113,594	100,877	80.36	95,544	76.11

6T	P-LOW	208,670	192,322	92.17	189,995	169,333	81.15	154,132	73.86
7T	P-LOW	190,617	174,102	91.34	170,852	152,323	79.91	146,074	76.63
8T	P-LOW	228,074	211,311	92.65	208,439	187,477	82.2	173,273	75.97
9T	P-LOW	252,567	229,624	90.92	226,150	205,582	81.4	196,577	77.83
10T	P-LOW	155,401	142,909	91.96	141,043	127,760	82.21	118,907	76.52
11T	P-LOW	190,962	173,742	90.98	170,775	152,512	79.87	144,638	75.74
12T	P-LOW	241,299	219,377	90.92	216,167	194,793	80.73	182,202	75.51
13T	P-LOW	149,612	137,449	91.87	135,036	123,369	82.46	117,474	78.52
14T	P-LOW	292,079	268,678	91.99	265,257	239,965	82.16	228,275	78.16
16T	P-LOW	213,150	194,893	91.43	191,878	172,192	80.78	161,255	75.65
1T+	P-HIGH	196,381	175,850	89.55	173,461	152,976	77.9	143,499	73.07
2T+	P-HIGH	111,243	102,839	92.45	101,266	90,181	81.07	82,734	74.37
3T+	P-HIGH	211,085	192,704	91.29	190,158	168,162	79.67	153,513	72.73
4T+	P-HIGH	238,626	220,251	92.3	217,497	196,422	82.31	182,436	76.45
5T+	P-HIGH	157,955	144,730	91.63	142,594	126,852	80.31	118,403	74.96
6T+	P-HIGH	159,458	145,937	91.52	143,910	128,422	80.54	120,693	75.69
7T+	P-HIGH	213,024	195,492	91.77	192,813	171,578	80.54	162,290	76.18
8T+	P-HIGH	188,496	173,065	91.81	170,396	154,778	82.11	147,559	78.28
9T+	P-HIGH	173,273	158,860	91.68	156,087	140,786	81.25	133,367	76.97
10T+	P-HIGH	185,846	167,804	90.29	165,223	142,519	76.69	134,143	72.18
11T+	P-HIGH	226,223	208,800	92.3	205,221	182,349	80.61	169,280	74.83
Mean		187,918.63	171,436.54	91.04	168,918.76	151,061.39	80.08	141,461.83	74.98

Supplementary Table 2. Sequencing statistics of retained reads after each bioinformatic step for jejunum content. Diet: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Sample	Diet	input	filtered	% passed filter	denoised	merged	% merged	non-chimeric	% non-chimeric
1C	C	201,525	186,405	92.5	185,292	164,940	81.85	137,962	68.46
2C	C	144,122	133,211	92.43	132,283	117,723	81.68	94,829	65.8
3C	C	125,629	116,080	92.4	115,150	102,534	81.62	85,836	68.32
4C	C	152,213	137,305	90.21	136,266	118,191	77.65	102,659	67.44
5C	C	172,485	159,394	92.41	157,661	139,900	81.11	119,666	69.38
6C	C	120,172	111,077	92.43	110,305	97,466	81.11	84,788	70.56
7C	C	203,115	186,111	91.63	182,409	164,390	80.93	158,889	78.23
12C	C	201,583	186,544	92.54	184,269	165,757	82.23	154,477	76.63
14C	C	220,908	205,275	92.92	203,876	184,739	83.63	174,131	78.83
1T	P-LOW	108,270	98,017	90.53	97,378	87,001	80.36	73,690	68.06
2T	P-LOW	78,110	71,163	91.11	70,017	60,054	76.88	52,312	66.97
3T	P-LOW	149,750	138,416	92.43	137,652	123,785	82.66	101,941	68.07
4T	P-LOW	66,047	60,051	90.92	59,558	53,008	80.26	46,022	69.68
5T	P-LOW	158,564	146,489	92.38	145,045	128,294	80.91	109,490	69.05
6T	P-LOW	147,228	134,712	91.5	133,417	116,907	79.41	99,175	67.36
9T	P-LOW	95,780	74,936	78.24	72,872	69,057	72.1	68,038	71.04
10T	P-LOW	201,586	187,884	93.2	186,896	165,438	82.07	157,374	78.07
11T	P-LOW	284,873	263,367	92.45	261,256	232,267	81.53	224,485	78.8
12C	P-LOW	201,583	186,544	92.54	184,269	165,757	82.23	154,477	76.63
14C	P-LOW	220,908	205,275	92.92	203,876	184,739	83.63	174,131	78.83
1T+	P-HIGH	195,376	180,728	92.5	178,971	157,051	80.38	138,408	70.84

3T+	P-HIGH	141,912	130,355	91.86	129,297	113,779	80.18	93,285	65.73
4T+	P-HIGH	117,653	108,104	91.88	107,191	94,797	80.57	80,173	68.14
5T+	P-HIGH	189,476	174,489	92.09	173,275	151,445	79.93	126,906	66.98
6T+	P-HIGH	157,111	146,001	92.93	144,528	129,505	82.43	116,695	74.28
8T+	P-HIGH	191,464	176,444	92.16	174,267	154,188	80.53	148,491	77.56
9T+	P-HIGH	162,486	151,756	93.4	150,819	132,110	81.31	124,893	76.86
10T+	P-HIGH	218,264	203,132	93.07	201,974	177,955	81.53	172,486	79.03
11T+	P-HIGH	129,309	110,386	85.37	107,019	96,573	74.68	93,842	72.57
Mean		160,555.96	147,327.11	91.39	145,886.78	129,587.19	80.35	116,331.22	71.58

Supplementary Table 3. Differentially abundant genus (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) for cecum content: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Comparison	Genus	baseMean	log2FC	pvalue	FDR
P-LOW vs C	Megasphaera_A_38685	21.30	-4.54	9.70E-09	1.09E-06
	Traorella	5.19	-3.31	4.28E-05	2.40E-03
P-HIGH vs C	Fusobacterium_B	96.46	-6.39	1.09E-08	1.22E-06
	Megasphaera_A_38685	21.30	-4.22	5.76E-07	3.22E-05
	Traorella	5.19	-3.55	6.20E-05	2.32E-03
	Spyradosoma	10.53	-3.13	4.66E-04	1.31E-02
P-LOW vs P-HIGH	CAG-314	7.73	3.00	3.84E-04	1.43E-02
	Faecalimonas	13.42	3.52	9.59E-05	5.37E-03
	Fusobacterium_B	96.46	5.90	1.31E-07	1.47E-05

Supplementary Table 4. Differentially abundant species (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) for cecum content: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Comparison	Species	baseMean	log2FC	pvalue	FDR
P-LOW vs C	Megasphaera_A_38685 elsdenii	23.01	-4.68	4.70E-09	6.82E-07
	Desulfovibrio_R_446353 faecigallinarum	17.69	-3.78	3.09E-06	2.24E-04
	Traorella massiliensis	5.42	-3.34	3.69E-05	1.78E-03
P-HIGH vs C	Fusobacterium_B perfoetens	103.23	-6.35	1.54E-08	2.23E-06
	Megasphaera_A_38685 elsdenii	23.01	-4.14	1.04E-06	7.55E-05
	Traorella massiliensis	5.42	-3.57	5.92E-05	2.86E-03
	Spyradosoma sp004558035	10.97	-3.08	5.87E-04	2.13E-02
P-LOW vs P-HIGH	Treponema_D bryantii	8.40	-2.69	5.28E-04	1.87E-02
	Eubacterium_F sp000687695	10.13	-2.43	1.21E-03	2.92E-02
	Corynebacterium pollutisoli	11.60	2.50	1.58E-03	3.27E-02
	CAG-314 sp900551395	8.10	2.90	6.46E-04	1.87E-02
	Faecalimonas phoceensis	13.96	3.36	2.19E-04	1.06E-02
	Bacteroides_H helcogenes	17.46	3.65	9.39E-05	6.81E-03
	Fusobacterium_B perfoetens	103.23	5.81	2.33E-07	3.37E-05

Supplementary Table 5. Differentially abundant genus (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) for jejunum content: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Comparison	Genus	baseMean	log2FC	pvalue	FDR
P-HIGH vs C	Mycoplasma_J_271639	8.48	-3.41	6.37E-04	9.55E-03
	Gallicola	9.83	-2.74	2.51E-03	2.82E-02
	Cutibacterium	7.72	3.66	4.76E-05	1.07E-03
	Enterococcus_E	217.02	3.68	1.59E-05	7.18E-04
P-LOW vs P-HIGH	Enterococcus_E	217.02	-3.70	2.59E-05	1.17E-03
	Parvimonas	21.75	-3.65	6.98E-04	1.57E-02
	Cutibacterium	7.72	-2.76	1.62E-03	2.43E-02

Supplementary Table 6. Differentially abundant species (False Discovery rate – FDR < 0.05; and $\log_2$ Fold Change - $|\log_2 FC| > 1.5$) for jejunum content: C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).

Comparison	Species	baseMean	log2FC	pvalue	FDR
P-HIGH vs C	Facklamia_A_322620 ignava	9.80	-3.72	9.68E-05	1.42E-03
	Metamycoplasma sualvi	8.75	-3.41	7.03E-04	8.15E-03
	Gallicola barnesae	9.34	-2.58	3.99E-03	3.74E-02
	Corynebacterium urealyticum	4.37	-2.23	4.51E-03	3.74E-02
	Ligilactobacillus agilis	17.70	3.59	9.81E-05	1.42E-03
	Cutibacterium acnes	8.13	3.82	2.91E-05	8.45E-04
	Enterococcus_E cecorum	225.71	3.85	5.94E-06	3.45E-04
P-LOW vs P-HIGH	Enterococcus_E cecorum	225.71	-3.75	1.81E-05	1.05E-03
	Parvimonas micra	20.56	-3.65	5.52E-04	1.07E-02
	Ligilactobacillus agilis	17.70	-3.42	3.11E-04	9.02E-03
	Cutibacterium acnes	8.13	-2.74	1.92E-03	2.79E-02

CHAPTER 4

Dietary supplementation with an olive mill wastewater phenolic extract in the finishing pigs: evaluation of the effects on growth performance, oxidative status and quality traits of meat and dry-cured ham

Manuscript in progress

Abstract

Phenolic compounds from olive mill wastewater (OMWW) have demonstrated strong antioxidant capacities, therefore the interest on their application on *in vivo* and post-slaughtering traits of livestock animals, including pigs, is currently increasing. A polyphenol concentrate from OMWW was tested as a dietary supplement in Landrace × Duroc heavy female finishing pigs, which were fed either with a control diet (C group), the C diet supplemented with 74 ppm of OMWW polyphenols (P-LOW group) or the C diet with 225 ppm of OMWW polyphenols (P-HIGH group). Each experimental group was composed of 45 pigs (n= 15 x 3 replicates), for a total of one hundred thirty-five animals. The potential beneficial effects of polyphenols were assessed *in vivo* (growth performance), and *post-mortem* (backfat thickness, pubertal status, and morphological characteristics of several tissues). Furthermore, the present analysis investigated the antioxidant power of these molecules in blood, meat and liver, and their protective role in the quality traits and physico-chemical characteristics of pork meat and dry-cured ham. Texture properties and fatty acid composition were also evaluated in the cured product. The present study demonstrated no significant differences in growth performance traits, tissue morphology and backfat thickness. An increase of antioxidant activity was observed in the liver of P-HIGH pigs through DPPH assay and confirmed by a higher serum paraoxonase and ferric reducing antioxidant power in the same animals. Meat quality analysis showed that cooking loss and redness (a*) values were decreased, while yellowness (b*) was increased in the polyphenol-supplemented animals. Dry-cured ham obtained from the P-HIGH group showed a decrease in yellowness in all the analyzed tissues (*semimembranosus* and *biceps femoris* muscles and fat). No differences were reported in the fatty acid composition of dry-cured ham. Polyphenols seemed to ameliorate water retention capacity of meat and dry-cured ham, potentially influencing the physico-chemical (moisture ad ash content) and sensorial characteristics of the final product. Overall, the observed effects on the technological quality of meat and dry-cured ham suggest that further investigations are needed.

1. Introduction

The Mediterranean region is one of the world's top olive oil-producing areas, with Italy ranking second only to Spain and producing approximately 6 million tons per year (FAOSTAT data, 2022). The olive oil industry generates several residues which are strong pollutants because of their phytotoxicity and high organic content and, for this reason, a correct exploitation is necessary (Dedousi et al. 2023). However, these wastes are particularly rich in bioactive molecules, including polyphenols (Silvan and Martinez-Rodriguez 2019; Altissimi et al. 2024). Polyphenols are secondary metabolites typical of plants and plant extracts, known for their antioxidant activity, but also for their antimicrobial, anti-inflammatory and immunomodulatory functions (Obied et al. 2007; Hume et al. 2011; Servili et al. 2013; Branciari et al. 2016; Veneziani et al. 2017; Mahfuz et al. 2021). Olive mill wastewater (OMWW) is a liquid by-product produced in high quantities from the olive oil industry that contains a wide spectrum of polyphenols, among which hydroxytyrosol, tyrosol, oleuropein, verbascoside and other types including phenolic acids and flavones (Davies et al. 2004; Ferlisi et al. 2024; Altissimi et al. 2024). In particular, hydroxytyrosol is considered, for the presence of several hydroxyl groups, the polyphenol with the highest radical scavenging activity (Baldioli et al. 1996).

Multiple issues are related to animal feeding, such as increasing demand of feeds and increasing costs of raw ingredients for the producers. This context has encouraged the use of unconventional natural feed additives derived from the agro-industrial sector, including the by-products from olive oil industry or derived extracts (Duque-Acevedo et al. 2020; Alimenti et al. 2023). The strategy of including these unconventional matrices in animal diets has proven to be effective in reducing the environmental impact, contributing to the development of a circular economy (Sabino et al. 2018; Vastolo et al. 2019; Sánchez et al. 2022; Dedousi et al. 2023). Recent studies reported that phenolic compounds, as bioactive molecules, can help to improve animal health, oxidative status and productive performance, as well as potentially reducing bacterial infections thanks to the polyphenols' antimicrobial properties (Servili et al. 2013; Lipiński et al. 2017; Mahfuz et al. 2021). Moreover, the supplementation of olive polyphenols in animal diets can contribute to the maintenance of oxidative stability (Dal Bosco et al. 2012; Joven et al. 2014; Branciari et al. 2017) and improvement of lipid composition in meat (Liotta et al. 2019; Rey et al. 2021).

To the authors' knowledge, several studies have been conducted regarding the effects of other olive by-products on pigs' productive performances (Joven et al. 2014; García Casco et al. 2017; Liotta et al. 2019; Papakonstantinou et al. 2023), as well as meat (Paiva-Martins et al. 2009; Liotta et al. 2019; Tsala et al. 2020; Caparra et al. 2023) and dry-cured product quality (Leite, Vasconcelos, Lopez, et al. 2024; Leite, Vasconcelos, Rodrigues, et al. 2024). The objective of this study was to investigate the effects of the dietary inclusion of an OMWW phenolic extract on growth performance, morphological characteristics, backfat thickness, oxidative status and quality of meat and dry-cured ham from heavy pigs intended for cured meat production.

2. Materials and methods

2.1 Animal welfare and ethical statement

The experiments were approved by the Bioethics Committee of the University of Perugia (protocol n. 399480). They were performed under the ARRIVE guidelines and animal care procedures followed the European recommendations (Directive 2010/63/EU) for the protection of animals used for scientific purposes. Pigs were raised and slaughtered for conventional meat commerce.

2.2 Animals and diet

The study was carried out on a farm located in a hilly area of Umbria, Italy. One hundred thirty-five female Landrace × Duroc finishing pigs (body weight: 102.7 ± 6.8 kg), were randomly assigned to three groups (three pens for each group and 15 pigs per pen) fed with the following diets: a) control

diet (C), a commercial mash-form feed used in the finishing period; b) C diet, supplemented with 210 mg/day of polyphenols from OMWW (P-LOW diet); c) C diet, supplemented with 630 mg/day of polyphenols from OMWW (P-HIGH diet). Individual body weight (BW) was recorded at the beginning and at the end of the trial (day 85). The OMWW polyphenol extract was diluted in water (15 L of water per box) and mixed with the feed (2.8 kg/pig), to reach a final phenolic concentration of 74 ppm for the P-LOW and 225 ppm for the P-HIGH diet. Pigs were provided with an automatic wet feeding system and fed two times per day and water was given *ad libitum*. All pigs underwent a period of adaptation of 15 days prior the experiment. Table 1 shows the nutritional characteristics and chemical composition of the control diet.

Table 1. Ingredients (% as fed basis) and chemical composition (g/100g) of the commercial diet.

	Diet
Raw material	% as fed basis
Grain corn flour	41.8
Grain barley flour	21.7
Wheat middlings	8.1
Proteins	2.5
Wheat flour middlings	9.0
Soybean meal	14.4
Mineral-vitamin supplement	2.5
Analyzed nutrients	g/100 g
Moisture	11.20
CP	15.10
Ether extracts	3.61
Ash	6.83
Crude fiber	4.03
NDF	17.96
ADF	5.79
ADL	1.72

Starch	44.45
Ca	0.68
P	0.53

CP: crude protein; NDF: neutral detergent fiber; ADF: acid detergent fiber; ADL: acid detergent lignin.

2.3 Olive mill wastewater extract

The OMWW extract was provided by Stymon Natural products, P.C., Patras, Greece (www.stymon.com) and tested for the total phenol content (TPC) through the Folin-Ciocalteu assay following the protocol of Nenadis et al. (Nenadis et al. 2013) and using a UV-1800 spectrophotometer (Shimadzu, Kyoto, Japan). The extract solution was prepared mixing 0.5 g of OMWW extract with 25 mL of a 80/20 (v/v) methanol:water solvent. Gallic acid was used as the reference standard and results were expressed as gallic acid equivalents (mg GAE/kg). The total phenolic content in OMWW extract was 36992 ± 58.9 mg/kg. Hydroxytyrosol, tyrosol and oleuropein were detected in the extract at the following concentrations: 9390 ± 17.4 mg/kg, 1090 ± 3.2 mg/kg and 590 ± 0.4 mg/kg through reversed-phase high- performance liquid chromatography (RP-HPLC-DAD) analysis.

2.3.1 Reagents and solvents

Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (97%) was from Aldrich Chemical Co. (Steinheim, Germany). DPPH$^{\bullet}$ (1,1-diphenyl-2-picrylhydrazyl radical), ABTS [2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt], and neocuproine were from Sigma Chemical Co. (St. Louis, MO, USA). Potassium persulfate was from Supelco Inc (Bellefonte, PA, USA) whereas copper chloride dehydrate was from Thermo Fisher (Kandel) GmbH (Kandel, Germany). Folin-Ciocalteu's phenol reagent, sodium carbonate, ammonium acetate, sodium sulfate anhydrous, potassium chloride, potassium dihydrogen phosphate, di-sodium hydrogen phosphate, glacial acetic acid as well as HPLC grade acetonitrile were purchased from Chem-Lab (Zedelgen, Belgium).

2.3.2 RP-HPLC-DAD analysis of polyphenols

Hydroxytyrosol, tyrosol, and oleuropein contents were determined in OMWW extract through RP-HPLC-DAD analysis following Kyriakoudi et al. (Kyriakoudi et al. 2024). The HPLC system consisted of an Agilent 1260 Infinity II Quaternary Pump VL, an Agilent 1260 Infinity II Autosampler and an Agilent 1260 Infinity II Diode Array Detector High Sensitivity. Separation was carried out on a InfinityLab Poroshell 120 EC-C184μm (150 x 4.6 mm i.d.) column (Agilent Technologies, Santa Clara, CA, USA). The column temperature was set at 30 °C. The mobile phase consisted of water (0.1% acetic acid) (A) and acetonitrile (B). The elution protocol was as follow: 0 min, 5% (B), 0–10.0 min, 20% (B); 10.0-15.0 min, 30% (B); 15-18 min, 30% (B); 18.0-20.0 min, 50% (B); 20.0-21.0 min, 100% (B); 21-25 min, 5% (B). The total run time was 25.0 min with flow rate of 1.0 mL/min. The injection volume was 20 μL. Extracts were analyzed after filtration through 0.45 μm PTFE filters (Frisenette, Knebel, Denmark). Monitoring was in the range of 190-600 nm. Chromatographic data were processed using the OpenLab CDS version 3.5 software (2021, Agilent Technologies, Santa Clara, CA, USA). Peak identification was based on retention times and spectral characteristics (absorption maxima) with those of available standards.

2.4 Pig performance measurements

Average BW was recorded at the beginning and at the end of the experimental trial. Average daily gain (ADG, g/pig/day) was calculated as the difference between the initial and the final BW divided by the duration of the phase, and feed conversion ratio (FCR) was determined as daily feed intake/daily gain. The pigs were transported in a local authorized slaughterhouse for conventional meat commerce and killed by bleeding after electrical stunning, according to the European Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing. After slaughtering, hot carcass weights were taken, and carcasses were stored in a chilling room for 24 h at 2-4 °C. Finally, the yield at the slaughter (dressing out percentage) was calculated from carcass weight (CW) and body weight (CW/BW ×100).

2.5 Fat deposition

To evaluate the fat deposition, backfat thickness (cm) was assessed at slaughter through real-time ultrasound machine (RKU10, Kaixin ultrasound scanner, Kaixin Mansion, C-01, Economic Development Zone, Xuzhou, Jiangsu, China) equipped with a 4.0-6.5 MHz transducer. As described in Cisneros et al. (1996), the transducer was set at 4.0 MHz and 10cm-deep field of scan, lubricated with ultrasound gel, and then placed on the right flank of the carcass just below the last rib, in a vertical position. Three measurements were recorded, on the upper, downer and central scanning area for each gilt; then, average backfat thickness was calculated. As right and left measurements were highly correlated (Cisneros et al. 1996), we chose one-side assessment with three fat measurements on the single-scan image as representative of the backfat deposition of each gilt.

2.6 Post-mortem analyses

At slaughtering, 45 pigs (15 pigs per group, 5 pigs per pen) were randomly selected. Liver, ovaries and uterus were collected immediately after evisceration as reported in section 2.6.1 while fat and muscle were sampled at the end of the slaughtering line after carcasses were weighted but before chilling. The carcasses were then stored in a chilling room at 4°C for 24.

2.6.1 Morphology and pathology

Macroscopic analysis was performed on the liver and on the ovaries and uterus for the reproductive tract. Tissues from the liver, ovaries, uterus, fat, and muscle were collected and fixed in 10%-buffered formalin for histological analysis. Samples were routinely processed and 3-µm tissue sections were obtained to assess leukocyte infiltration, grading severity on a scale from 0 to 3, based on the percentage of affected tissue: 0 indicating no tissue affected, 1 for less than 20%, 2 for 21-60%, and 3 for more than 61%. These criteria were employed also to evaluate degeneration, necrosis, and hyperplasia across all segments of the evaluated tracts. The scoring system was established following Gibson-Corley et al. (Gibson-Corley et al. 2013). With the same grade scale, muscle atrophy, hypertrophy, degeneration, necrosis, and regeneration were evaluated (0 to 3 based on the percentage of affected tissue). Moreover, for the reproductive tract, the presence of luteal bodies in the ovary was recorded as either yes or no. Ovary leukocyte infiltration, uterus leukocyte infiltration, uterus fibrosis, and uterus hyperplasia were graded on a scale from 0 to 3 each, based on the percentage of affected tissue (0 no tissue affected, 1 for less than 20%, 2 for 21-60%, and 3 for more

than 61%, for each parameter). The presence of adenomyosis in the uterus was also noted as either yes or no. The pubertal status of gilts was also assessed macroscopically, as discussed by Vela et al. (Vela et al. 2022). Vagina, uterus (e.g. body horns) and ovaries were checked for dimensions, aspect and relevant abnormalities. The presence of follicles larger than 6 mm together with corpora lutea and/or corpora albicans were indicative of puberty.

2.6.2 Oxidative status in blood and tissues

Liver and *sternocleidomastoid* muscle tissues were also collected for the evaluation of the antioxidant status and were processed as follows before analysis. Samples were lyophilized using a freeze-dryer, ground with a homogenizer, and kept at -20°C. Extracts were prepared mixing 0.5 g of minced sample with 25 mL of a methanol/water 80/20 (v/v) solution, sonicated at 60 °C for 30 min, and centrifugated at 6000 rpm for 5 min. The supernatants were taken and used for successive analyses.

The DPPH radical scavenging activity of liver and *sternocleidomastoid* muscle samples was determined according to Nenadis et al. (Nenadis et al. 2013) using 25 mL of the prepared extracts with the aid of a UV-1800 spectrophotometer. Radical scavenging activity (%) values (%RSA) were determined by using the formula $\%RSA = [Abs_{515(t=0)} - Abs_{515(t)}] \times 100/Abs_{515(t=0)}$ after correction with appropriate blank. These values were converted to Trolox equivalents via a calibration curve (y=0.6439x+1.6609, R^2=0.996). Radical scavenging activity of liver and muscle extracts (25 mL) against ABTS radical cation was evaluated according to the protocol of Re et al. (1999) (Re et al. 1999) and adjusted according to Nenadis et al. (Nenadis et al. 2013). Inhibition of ABTS radical cation in percent (% Inh) was calculated via the formula $\%\ Inh = [Abs_{734(t=0)} - Abs_{734(t)}] \times 100/Abs_{734(t=0)}$ after correction with an appropriate blank. These values were converted to Trolox equivalents via a calibration curve (y=2.8169x-0.1910, R^2=0.999). The cupric ion reducing antioxidant (CUPRAC) capacity of muscle and liver extracts (25 mL) was measured according to the protocol of Apak et al. (Apak et al. 2004). The absorbance at 450 nm was recorded after standing the solution in the dark 30 min and the results were converted to Trolox equivalents via a calibration curve (y=0.0041x-0.1101, R^2=0.997). In all the assays, measurements were performed in triplicate. Results were finally expressed as µmol Trolox/ g dry sample ± SD in CUPRAC and ABTS assays, and as % of Radical scavenging activity (RSA) in DPPH assay.

At the time of the slaughter, blood samples were collected into empty vacutainer tubes (BD Vacutainer) by jugular vein puncture. All the analyses except retinol and tocopherol were performed at 37°C with a clinical auto-analyzer (ILAB-650; Werfen, Milan, Italy). Concentrations of albumins, globulins, total proteins, cholesterol, total bilirubin, γ-glutamyl transferase (GGT), glutamate oxaloacetate transaminase (GOT) were determined using commercial kits from Instrumentation Laboratory (Werfen, Milan, Italy). Haptoglobin and ceruloplasmin were analyzed using the methods described by Skinner et al. (Skinner et al. 1991) and Sunderman and Nomoto (Sunderman and Nomoto 1970), respectively, adapted to the ILAB 650 conditions. The activity of paraoxonase (PON) was assessed by adapting the method of Ferré et al. (Ferré et al. 2002), as previously described by Bionaz et al. (Bionaz et al. 2007). Ferric reducing antioxidant power (FRAP) was analyzed by adapting a colourimetric method proposed by Benzie and Strain (Benzie and Strain 1996) and at ILAB 650 conditions. Plasma thiol groups were determined using a commercial kit (plasma thiol group test, Diacron, Grosseto, Italy) on ILAB 650 conditions. Plasma retinol and tocopherol were extracted with hexane and analyzed by reverse-phase HPLC using an Allsphere ODS-2 column (3 µm, 150 × 4.6 mm; Grace Davison Discovery Sciences, Deerfield, IL); a UV detector set at 325 nm (for vitamin A), 290 nm (for vitamin E), or 460 nm (for β-carotene); and 80:20 methanol: tetrahydrofurane as the mobile phase (Trevisi et al. 2015).

2.7 Meat and fat quality measurements

2.7.1 Raw meat analysis

From the 45 slaughtered pigs, 6 animals (2 from each replicate) of each group (C, P-LOW and P-HIGH) where chosen for the analysis of meat. All the measurements were performed on *gluteus medius*. The pH was measured at 24 h post-mortem, using a penetrating electrode connected to a portable pH-meter (Mod pH25, Crison, Barcelona, Spain).

The colour measurements were conducted 24 h after slaughtering on raw meat samples following a 1 hour bloom period at the refrigeration temperature (4 ± 1°C) (Honikel 1998). Colour coordinates (Commission International de l'Eclairage, 1976) were determined using a Minolta Chromameter CR400 (Minolta, Osaka, Japan — light source of D65 calibrated against a standard white tile). The results were expressed as lightness (L*), redness (a*), and yellowness (b*).

2.7.2 Cooked meat analysis

Cooking loss was performed on the same muscle type as reported by Honikel (Honikel 1998). For cooking loss determination, meat samples (6.0 × 6.0 × 2.5cm; average weight: 83.32 ± 1.67 g , 84.46 ± 4.15 g and 84.05 ± 2.92 g for CTR, P-LOW and P-HIGH , respectively) were kept in plastic bags, cooked in a water-bath (80 °C for 1 h) and cooled under running tap water for 30 min. Samples were weighed before and after the test, and losses were calculated as 100 × (initial weight -final weight) / initial weight (Honikel 1998). The Warner–Bratzler shear force measurement was also performed according to the Honikel (Honikel 1998) method. Three cylindrical cores (Ø 1.25 cm), which were cut parallel to the gluteal muscle fibres, obtained from cooking loss samples and tested for the shear force value using a Warner–Bratzler (WB) shear device fitted to a texture analyser (TVT6700, Perten Instrument, Segeltorp, Sweden). The peak force was expressed in N/ ½ inches was converted in kg/cm^2.

2.7.3 Fat analysis

Quality measurements were also performed on subcutaneous covering fat overlying the *gluteus medius* at the level of the posterior part of the back (6 samples from C group, 6 samples from P-LOW group, and 6 samples from P-HIGH group). The pH (at 24h) and colour measurements (L*, a*, b*) were carried out following the same protocols for meat analysis but without the bloom period.

2.8 Dry-curing process and sampling

Six animals from each group (the same animals selected for meat analyses) were chosen for ham production. The right hind legs undergo the dry-cured processing chain. The selected legs were precooled and drained in cold storage to maintain a central temperature of 1-1.5 °C. Then, they were dry-cured with sea salt and stored at 1.4 °C for a week. After, a second salting phase occurred at 3.2 °C and 74% of relative humidity. Cured legs were maintained in drying balance stage at 2.9 °C and relative humidity of 79%, until the ham's trimming (after 2 months). During seasoning (T= 17-18 °C, relative humidity 72-28%), hams were subjected to two larding processes on the upper part of the leg, closer to the surface. The entire curing process lasts 12 months. Then, the hams were weighed (average weight: 9.2 kg), deboned and divided in three pieces (front, upper back, lower part) (Figure 1). A 3 cm thick slice was cut from the upper back, containing at least 3 muscle portions (*semimembranosus* - SM, *biceps femoris* - BF and *semitendinosus* - ST) and subcutaneous fat (Figure 2).

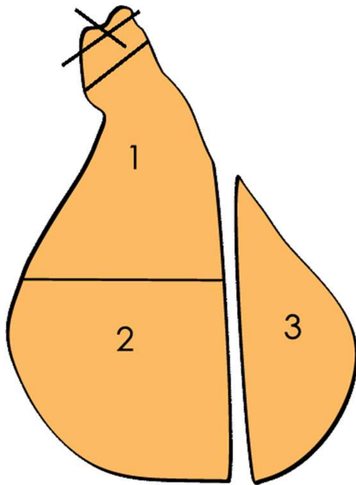


Figure 1. Illustration of the three parts of the dry-cured ham after sectioning: upper back (1), lower part (2), front part (3).

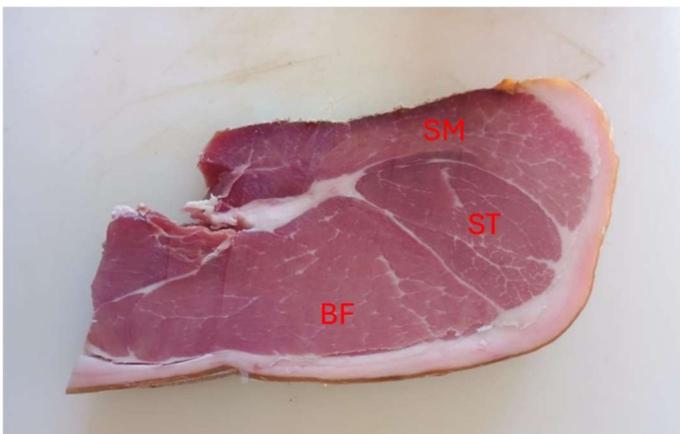


Figure 2. Dry-cured ham muscles*: semimembranosus* (SM), *biceps femoris* (BF) and *semitendinosus* (ST) muscles.

2.8.1 Quality and texture analysis

At sampling, pH and colour (CIE L* a*, b*,) were measured in the SM*,* BF and subcutaneous fat of dry-cured ham, with the same protocol used for raw meat and fat. Three cubes of 1x1x1 cm were removed from each of the SM, BF and subcutaneous fat tissue to perform compression texture profile analysis using a texture analyzer (TVT 6700, Perten Instruments, Milano, Italy) equipped with a 15 kg load cell and a 35 mm diameter cylinder flat-ended probe. Each sample was subjected to a double-cycle compression with the following parameters: test speed 2.0 mm/s; compression rate 30% of the sample high; trigger force 5 g; reaction speed 2.0 mm/s; rest period between the two compressions 5 s. Peak force (hardness expressed in g), cohesiveness, gumminess, springiness (expressed in mm),

chewiness (expressed in mJ), were obtained from the generated force–time curve, as reported in the Perten TexCal software manual (Perten, Milano, Italy, version 4.0.4 67).

2.8.2 Fatty acid composition

The fatty acids content in dry-cured ham (SM and BF) were extracted and transmethylated as follows. Briefly, 10 g of muscle were homogenized with 10 mL of 0.5 M sodium acetate aqueous solution using an Ultraturrax (T25 basic, IKA, Labortechnik, Germany). Then 8 mL of 0.5 M sodium acetate aqueous solution 8 mL of methanol and 4 mL of chloroform were added to 4 g of this mixture and it was mechanically shaken for 3 minutes. After an addiction of 4 mL of chloroform, it was shaken again for 2 minutes and, then, 8 mL of water was added in order to separate the chloroform and methanol phase. After centrifugation, the lower phase was collected in a flask. All solvents are of analytic grade and all chemicals are from Sigma-Aldrich (Bellefonte PA, US). The transmethylation was performed according to the procedure of Branciari et al., 2014. Methyl undecanoate (Sigma-Aldrich, Bellefonte PA, US) was used as internal standard. All chemicals were from Sigma-Aldrich. Methyl esters were analyzed using a gaschromatograph (Agilent Technologies 6890N Network GC System) equipped with a flame ionization detector (FID) and an automatic sampler (Agilent Technologies 7683 Series Injector). A CP-Select CB for FAME fused silica capillary column (100 m x 0.25 mm i.d., film thickness 0.39 µm, J&W, Agilent technologies, Palo Alto, CA, US) was used to separate and analyze the methyl esters. The injector temperature was 270 °C whereas the detector temperature was 300°C. The sample (1 µL) was injected into a split/splitless system (split ratio 1:5). The carrier gas was helium at flow rate of 1.6 mL/min; the oven temperature programme was: the temperature raised from 60°C to 150 °C at a rate of 30°C/min, the temperature was held for 3 minutes and then it raised to 185°C at a rate of 0,5°C; after 1 minute the temperature raised to 220°C at a rate of 1,5 °C/min and was held for 12 minutes. The identification of individual fatty acid methyl esters was done by comparison with a standard mixture containing 37 FAMEs (Supelco, Bellefonte PA, USA) and methyl cis-7,10,13,16,19-docosapentaenoate (Sigma-Aldrich, Bellefonte PA, USA).

2.9 Proximate analysis

Proximate analysis composition (protein, lipid, moisture and ashes content) was assessed in meat (*sternocleidomastoid*) and dry-cured ham (SM and BF) from 6 animals for each group (C, P-LOW, P-HIGH) for chemical composition according to the Association of Analytical Chemists Methods (AOAC methods 2000). The moisture content was obtained by oven-drying meat samples (125 °C for 2h) (method 950.46). The fat content was gravimetrically determined using ether solvent extraction (method 960.30). The nitrogen content was determined using the Kjeldahl method (method 992.15). The protein content was obtained by multiplying the total nitrogen with a coefficient factor of 6.25. The ash content was obtained using a muffle furnace at 600 °C (method 923.03).

2.10 Statistical analyses

Pigs' performance parameters were analysed through a simple hierarchical ANOVA model, with the replicates (pen) nested within the diet and initial body weight used as a covariate. Backfat thickness was compared among groups through ANOVA, while the Chi-Square test was used to compare pubertal rate among groups (P-LOW vs C, P-HIGH vs C, P-LOW vs P-HIGH). Regarding the morphological and pathological examinations, for binomial (yes/no) factors a Fischer's exact test was conducted to evaluate the association between the factor assessed and the groups. The Kruskal-Wallis test was employed to evaluate differences in various histological parameters across groups. For the analysis of the antioxidant assays, a Shapiro-Wilk test was performed to assess the normality

of the data, and a one-way ANOVA was used, followed by a t-test. In the case of non-normally distributed data, Kruskal-Wallis test was performed, followed by a Wilcoxon test for pairwise comparisons.

Results of blood serum parameters, quality traits (raw meat, dry-cured meat and subcutaneous tissues), texture properties (dry-cured ham and adipose tissue), proximate analysis (raw meat and dry-cured ham) and fatty acid composition (dry-cured ham) were analysed through a one-way ANOVA, followed by a Tukey's test. Regarding blood serum analysis, several values (cholesterol, GOT and thiol groups) were log-transformed to normalize the distribution and stabilize the variance of the measurements. A canonical Linear Discriminant Analysis (LDA) was conducted applying the classification function to separate the samples based on the treatment groups. After scaling, blood serum parameters were used as predictor variables, and treatment group was the dependent variable. For all the analyses, R software was employed. Differences between the values were considered significant when $p < 0.05$.

3. Results

3.1 Pig performance

No significant ($p > 0.05$) differences were obtained for BW, ADG, FCR, hot carcass weight (HCW) and dressing out percentage values between the experimental groups (Table 2).

Table 2. Growth performance parameters and carcass characteristics observed in the control group and the OMWW polyphenol-supplemented groups.

	CONTROL	P-LOW	P-HIGH	p-value
Initial BW (kg)	98.48 ± 3.47	103.5 ± 6.35	109.09 ± 4.26	0.47
Final BW (kg)	145.7 ± 16.7	145.2 ± 11.7	153.4 ± 13.6	0.07
ADG (g)	673.97 ± 0.3	536.52 ± 0.1	639.13 ± 0.2	0.15
FCR	4.2 ± 0.3	5.2 ± 0.1	4.4 ± 0.2	0.52
HCW (kg)	120.6 ± 13.7	119.5 ± 9.5	126.3 ± 12.3	0.39
Carcass yield (%)	82.95 ± 0.02	80.79 ± 0.06	83.79 ± 0.04	0.09

BW= body weight; ADG= average daily gain; HCW= hot carcass weight. C= Control; P-LOW= diet supplemented with 74 ppm OMWW polyphenols; P-HIGH= diet supplemented with 225 ppm OMWW polyphenols.

3.2 Fat deposition

Regarding backfat thickness, no differences were found in fat deposition of pigs from the three experimental groups (Table 3).

Table 3. Measurements obtained by ultrasound measurement of the backfat thickness (last rib, cm) in experimental (P-LOW, 74 ppm OMWW polyphenols; P-HIGH 225 ppm OMWW polyphenols) and control pigs.

	Mean	SD	Min	Max
C	2.38	0.90	0.82	3.75
P-LOW	2.36	0.60	1.37	3.19
P-HIGH	1.91	0.64	0.85	2.97
Total	2.23	0.74	0.82	3.75

SD: standard deviation; Min: minimum; Max: maximum. C= Control; P-LOW= diet supplemented with 74 ppm OMWW polyphenols; P-HIGH= diet supplemented with 225 ppm OMWW polyphenols.

3.3 Morphometry and pathology

For the reproductive tract, all binomial (yes/no) factors (presence of luteal bodies, presence of uterine adenomyosis) assessed did not associate with the groups ($p > 0.05$).

No differences across groups ($p > 0.05$) were detected for the parameters evaluated in liver (degeneration, necrosis, hyperplasia, fibrosis and leukocyte infiltration), muscle (atrophy, hypertrophy, degeneration, necrosis, regeneration and leukocyte infiltration), fat (atrophy, necrosis and leukocyte infiltration) and reproductive tract (ovary leukocyte infiltration, uterus leukocyte infiltration, uterus fibrosis, uterus hyperplasia).

3.4 Pubertal status

At slaughter, pre-pubertal and pubertal gilts were characterized by easily distinguishable reproductive tracts, as prepubertal animals showed smaller, smooth ovaries, and shorter (less than 90 cm) uterine horns, while reproductive tracts of pubertal gilts displayed longer (more than 150 cm) uterine horns. In pubertal animals the surface of the ovaries was completely occupied by follicles (greater than 6 mm in diameter) and corpora lutea. There were no differences (C vs P-LOW $p= 0.61$; C vs P-HIGH $p = 0.90$) in the percentage of animals that reached puberty at the end of the observation period, among groups (Table 4).

Table 4. Numbers (n) and percentages (%) of pubertal gilts at the final stage expressed as pubertal/(pubertal+prepubertal) in each treatment group, as identified at inspection after slaughter and isolation of the reproductive tracts.

	C	P-LOW	P-HIGH
Pubertal gilts, n (%)	5 (29.4)	3 (21.4)	3 (27.3)
Prepubertal gilts, n	12	11	8

n: number of animals; C= control; P-LOW= diet supplemented with 74 ppm OMWW polyphenols; P-HIGH= diet supplemented with 225 ppm OMWW polyphenols.

3.5 Blood serum parameters

Several parameters related to oxidative status, i.e FRAP, PON, thiol groups SHp, haptoglobin, and tocopherol were evaluated in pig serum, together with markers of liver function (total proteins, albumins, globulins, cholesterol, ceruloplasmin, GOT, GGT, bilirubin, retinol). Results showed a tendency to a significant difference for PON ($p = 0.06$) and GOT ($p = 0.06$), both showing an increase in the P-HIGH group compared to P-LOW (Table 5). No differences were reported for the other parameters. Results of the canonical discriminant analysis are shown in Figure 3. The obtained results indicate that several parameters distinguished the treated and the control groups. In particular, high values in bilirubin and albumins permit to discriminate the C group, while high values in FRAP and PON permit to discriminate the group P-HIGH.

Table 5. Blood serum parameters of pigs from control and polyphenol-supplemented groups.

Parameter	C	P-LOW	P-HIGH	p-value
Albumins (g/L)	38.1 ± 0.8	35.8 ± 0.8	38.4 ± 1.1	0.07
Total proteins (g/L)	76.2 ± 1.2	77.7 ± 1.2	78.0 ± 1.6	0.53
Globulins (g/L)	37.9 ± 1.3	41.4 ± 1.3	39.0 ± 1.7	0.11
Cholesterol (mmol/L)	2.7 ± 0.1	2.8 ± 0.1	2.7 ± 0.1	0.94
GOT (U/L)	61.1 ± 5.1	49.3 ± 5.1	67.3 ± 7.5	0.06
GGT (U/L)	38.5 ± 2.2	37.9 ± 2.1	38.3 ± 2.9	0.97
Bilirubin (mcmol/L)	1.8 ± 0.7	2.4 ± 0.6	1.2 ± 0.7	0.23
Haptoglobin (g/L)	0.8 ± 0.04	0.8 ± 0.04	0.9 ± 0.06	0.70
Ceruloplasmin(mcmol/L)	16.7 ± 0.7	16.8 ± 0.7	18.2 ± 0.9	0.33
FRAP (mcmol/L)	159 ± 6.0	162 ± 6.0	158 ± 8.0	0.85
Thiol groups (mcmol/L)	253.2 ± 14.3	248.6 ± 14.2	223.6 ±19.0	0.49
Paraoxonase (PON, U/L)	51.7 ± 2.6	49.1 ± 2.6	58.9 ± 3.5	0.06

Retinol (mcg/100 mL)	25.2 ± 1.9	23.7 ± 1.9	25.5± 2.5	0.77
Tocopherol (mg/100 mL)	3.1 ± 0.1	3.4 ± 0.1	3.1 ± 0.1	0.11

C = control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

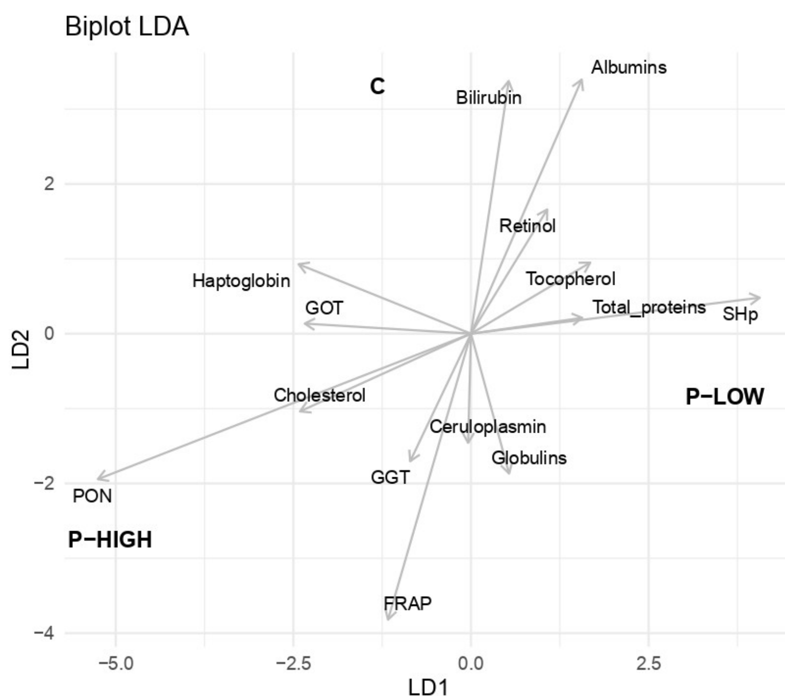


Figure 3. Linear Discriminant Analysis (LDA) diagram representing differentiations of blood serum parameters by the three experimental groups C (control), P-LOW (diet supplemented with 74 ppm OMWW polyphenols), P-HIGH (diet supplemented with 225 ppm OMWW polyphenols).

3.6 Antioxidant activity in muscle and liver

3.6.1 DPPH assay

For the liver, DPPH assay reported a significant ($p = 0.019$) difference in radical scavenging activity (% RSA) between the three experimental groups, with an increase of RSA value in pigs of P-HIGH group compared to control and P-LOW groups (Table 6). For the muscle, no differences ($p > 0.05$) were found between groups (Table 6).

Table 6. DPPH analysis in liver and *sternocleidomastoid* muscle.

	Treatments			
	C	P-LOW	P-HIGH	p-value
Liver	15.9 ± 3.5[a]	17.2 ± 6.1[a]	21.1 ± 3.1[b]	0.02
Muscle	14.2 ± 1.7	15.6 ± 2.6	16.1 ± 2.8	0.10

Data are expressed as means ± standard deviation. [a-c] within a row: means without a common superscript differ ($p \leq 0.05$). C= Control; P-LOW = diet with 74 ppm OMWW polyphenols; P-HIGH = diet with 225 ppm OMWW polyphenols.

3.6.2 CUPRAC assay

In the CUPRAC assay, no differences ($p > 0.05$) were found between experimental groups for both muscle and liver (Table 7).

Table 7. CUPRAC analysis in liver and *sternocleidomastoid* muscle.

	Treatments			
	C	P-LOW	P-HIGH	p-value
Liver	10.4 ± 11.8	21.0 ± 16.9	16.9 ± 19.1	0.15
Muscle	2.3 ± 0.8	2.8 ± 1.1	2.4 ± 0.7	0.52

Data are expressed as means ± standard deviation. C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

3.6.3 ABTS assay

Results of the ABTS assay showed no differences in antioxidant activity in the liver between groups, while a significant ($p = 0.017$) difference between the P-HIGH and P-LOW groups was recorded. (Table 8).

Table 8. ABTS analysis in liver and *sternocleidomastoid* muscle.

	Treatments			
	C	P-LOW	P-HIGH	p-value
Liver	6.3 ± 0.4	6.5 ± 0.4	6.4 ± 0.3	0.27
Muscle	3.9 ± 0.4^{a}	4.0 ± 0.3 ^{a}	4.3 ± 0.4 ^{b}	0.02

Data are expressed as means ± standard deviation. C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols. $^{a-c}$ within a row: means without a common superscript differ ($p \leq 0.05$).

3.7 Meat and fat quality

The pH and colour parameters of meat evaluated on *gluteus medius* are reported in Table 9. No differences ($p > 0.05$) between the experimental groups were found for pH. Regarding the redness a* value of meat, a decrease in the polyphenol-supplemented groups was observed (with a significant difference detected between P-LOW and C), while yellowness b* followed an opposite trend. Cooking loss decreased with the polyphenol supplementation. Finally, no significant differences between the groups ($p > 0.05$) were reported for lightness L* and WB shear force. As for the subcutaneous fat, redness a* value decreased ($p = 0.011$) in P-LOW compared to the C group, while lightness L* increased ($p= 0.015$). No differences were reported for yellowness b* (Table 9).

Table 9. Quality traits of meat and subcutaneous fat of pigs fed control diet or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
Gluteus medius					
pH (24h)	5.47	5.47	5.45	0.031	0.92
L*	50.76	50.96	52.36	0.765	0.28
a*	9.24^{a}	7.59^{b}	8.99^{ab}	0.426	0.02
b*	4.59^{a}	5.36^{ab}	5.77^{b}	0.269	0.01
Cooking loss (%)	34.50^{a}	32.20^{b}	33.72^{ab}	0.535	0.03
WB shear force (kg/cm^{2})	62.90	69.11	60.97	3.956	0.32
Fat					
pH (24h)	5.83	5.80	5.90	0.030	0.07
L*	71.37^{ab}	73.86^{a}	70.67^{b}	0.756	0.02
a*	6.07^{a}	4.37^{b}	5.19^{ab}	0.370	0.01
b*	6.56	5.74	6.03	0.285	0.15

SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

3.8 Dry-cured ham and fat quality

The pH and colour parameters of dry-cured ham evaluated in SM and BF are reported in Table 10. In SM, no differences ($p > 0.05$) between the experimental groups were found for pH, L* and a* values. Regarding b* value, it decreased ($p = 0.01$) in P-HIGH of both SM and BF. In BF, the same experimental group showed decreased values for L* ($p = 0.02$). No significant differences were found for pH and a* value. In the subcutaneous fat, yellowness b* and L* values decreased ($p= 0.02$) in P-HIGH (Table 10), while the pH increased ($p = 0.005$). Fat redness a* decreased in the P-LOW group ($p= 0.02$), when compared to C.

Table 10. Quality traits of dry-cured ham measured on *semimembranosus* and *biceps femoris* muscles and subcutaneous fat from pigs fed control diet or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
Semimembranosus					
pH	6.06	6.02	6.04	0.017	0.20
L*	38.8	36.7	36.2	0.812	0.08
a*	12.3	12.6	11.3	0.397	0.10
b*	8.02^{ab}	8.59^{a}	6.81^{b}	0.368	0.01
Biceps femoris					
pH	6.10	6.07	6.09	0.015	0.41
L*	40.8^{ab}	41.5^{a}	39.3^{b}	0.517	0.02
a*	13.1	13.01	13.3	0.227	0.65
b*	5.47^{a}	5.52^{a}	4.74^{b}	0.138	0.002
Fat					
pH	5.83^{ab}	5.76^{b}	5.90^{a}	0.025	0.005
L*	71.17^{ab}	73.86^{a}	70.67^{b}	0.805	0.03
a*	5.85^{a}	4.37^{b}	5.19^{ab}	0.317	0.02
b*	6.87^{a}	6.02^{ab}	5.74^{b}	0.256	0.02

SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

3.9 Fatty acid profile in dry-cured ham

Results of fatty acid composition in the intra-muscular fat of BF and SM are reported in Tables 11 and 12. No differences were reported between experimental groups regarding total saturated fatty acids (SFAs), total monounsaturated fatty acids (MUFAs), total polyunsaturated fatty acids (PUFAs), MUFA/PUFA ratio and all the fatty acids analysed, with only a tendency observed for margaric acid (C17:0) in SM (p = 0.054) and eicosenoic acid (C20:1n-9) in BF (p= 0.053).

Table 11. Fatty acid profile of intramuscular fat of *biceps femoris* muscle in dry-cured ham from pigs fed control diet or polyphenol-supplemented diets.

Fatty acids	C	P-LOW	P-HIGH	SEM	p-value
Biceps femoris					
C14:0	1.45	1.56	1.43	0.090	0.58
C14:1	0.00	0.02	0.00	0.008	0.14
C15:1	0.01	0.00	0.00	0.004	0.14
C16:0	22.36	22.95	21.55	0.501	0.19
C16:1	3.08	3.41	3.25	0.111	0.18
C17:0	0.18	0.19	0.24	0.023	0.24
C18:0	10.54	10.52	10.33	0.556	0.95
C18:1n-9c	44.72	44.58	0.00	0.303	0.68
C18:1n-7c	4.55	4.40	4.75	0.113	0.15
C18:2n-6c	10.33	9.88	10.75	0.43	0.39
C18:3n-6	0.02	0.00	0.00	0.006	0.14
C18:3n-3	0.70	0.66	0.75	0.041	0.36
C20:0	0.14	0.12	0.13	0.010	0.52
C20:1n-9	0.70	0.60	0.62	0.027	0.053

C20:2	0.50	0.40	0.48	0.040	0.259
C20:3 n-6	0.19	0.23	0.23	0.024	0.5
C20:4 n-6	0.51	0.48	0.55	0.05	0.68
ΣSFA	34.76	35.34	33.67	0.55	0.15
ΣMUFA	53.07	53.00	53.57	0.230	0.21
ΣPUFA	12.26	11.66	12.76	0.473	0.30
PUFA/SFA	0.35	0.33	0.38	0.018	0.23

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

Table 12. Fatty acid profile of intramuscular fat of *semimembranosus* muscle in dry-cured ham from pigs fed control diet or polyphenol-supplemented diets.

Fatty acids	C	P-LOW	P-HIGH	SEM	p-value
Semimembranosus					
C12:0	0.11	0.13	0.11	0.008	0.13
C14:0	1.60	1.64	1.65	0.050	0.78
C16:0	22.69	22.85	22.50	0.139	0.25
C16:1	3.29	3.52	3.58	0.155	0.41
C17:0	0.18	0.15	0.15	0.010	0.054
C18:0	11.38	10.88	10.83	0.299	0.40
C18:1n-9t	0.17	0.17	0.17	0.003	0.20
C18:1n-9c	43.88	44.06	44.24	0.215	0.52

C18:1n-7c	4.27	4.23	4.32	0.182	0.945
C18:2n-6c	10.07	10.02	9.99	0.228	0.97
C18:3n-6	0.04	0.05	0.05	0.008	0.54
C18:3n-3	0.63	0.64	0.64	0.016	0.87
C20:0	0.10	0.09	0.09	0.016	0.84
C20:1n-9	0.66	0.67	0.67	0.007	0.53
C20:2	0.40	0.38	0.39	0.038	0.96
C20:3 n-6	0.12	0.12	0.12	0.009	0.99
C20:4 n-6	0.41	0.40	0.49	0.038	0.28
ΣSFA	36.06	35.74	35.34	0.339	0.36
ΣMUFA	52.27	52.65	52.98	0.37	0.43
ΣPUFA	11.67	11.61	11.68	0.27	0.98
PUFA/SFA	0.32	0.32	0.33	0.009	0.83

SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

3.10 Proximate composition

Proximate composition (protein, lipid, moisture and ashes content) of meat did not change between experimental groups. In dry-cured ham, BF had a higher ($p = 0.005$) moisture and a lower ash content ($p = 0.01$) in the P-HIGH group when compared to C. No differences ($p > 0.05$) were found in SM muscle.

Table 13. Proximate composition analyses of meat (*sternocleidomastoid*) and dry-cured ham (*semimembranosus* and *biceps femoris*) from pigs fed control or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
Meat					
Sternocleidomastoid					
Protein (%)	21.10	21.27	20.94	0.163	0.39
Lipid (%)	1.14	1.13	1.16	0.019	0.65
Moisture (%)	70.52	70.26	70.37	0.202	0.69
Ashes (%)	7.23	7.32	7.52	0.094	0.12
Dry-cured ham					
Semimembranosus					
Protein (%)	33.79	34.55	34.89	0.400	0.18
Lipid (%)	4.17	4.00	3.67	0.144	0.08
Moisture (%)	54.3	54.18	45.36	0.378	0.95
Ashes (%)	7.76	7.26	7.08	0.189	0.06
Biceps femoris					
Protein (%)	31.18	30.55	30.61	0.366	0.35
Lipid (%)	3.21	3.24	3.01	0.117	0.35
Moisture (%)	59.04[b]	60.21[ab]	60.95[a]	0.337	0.006
Ashes (%)	6.56[a]	5.99[ab]	5.43[b]	0.221	0.01

SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

3.11 Texture properties

Regarding the texture parameters evaluated in SM and BF muscles and subcutaneous fat from dry-cured ham, no differences between experimental groups were found in the analysed tissues, except for SM which showed a lower ($p = 0.007$) value of springiness in P-HIGH.

Table 14. Texture properties of dry-cured ham (*semimembranosus* and *biceps femoris*) and subcutaneous fat from pigs fed control diet or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
Semimembranosus					
Peak force A (g)	8957.1	10004.9	7185.2	1188.4	0.27
Springiness	0.66^{a}	0.63^{a}	0.51^{b}	0.029	0.007
Chewiness	3305.08	4006.10	2306.30	505.22	0.09
Gumminess	5088.4	6257.4	4199.6	772.02	0.20
Cohesiveness	0.559	0.624	0.565	0.025	0.16
Biceps femoris					
Peak force A (g)	2150.08	2250.3	2496.8	343.19	0.77
Springiness	0.55	0.53	0.51	0.024	0.59
Chewiness	726.5	656.9	708.5	144.6	0.93
Gumminess	1256.17	1189.5	1333.2	216.25	0.90
Cohesiveness	0.571	0.523	0.522	0.016	0.07
Fat					
Peak force A (g)	2851.56	2492.1	2634.5	508.56	0.88
Springiness	0.53	0.59	0.53	0.025	0.27
Chewiness	669.33	716.10	718.00	151.1	0.97
Gumminess	1366.25	1251.00	1355.4	266.08	0.94
Cohesiveness	0.47	0.51	0.51	0.015	0.20

SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

4. Discussion

It is known that phenolic compounds, including those found in OMWW, possess an antioxidant activity, which involves the donation of a hydrogen atom to alkyl peroxyl radicals originating during lipid peroxidation (Branciari et al. 2017). Olive polyphenols are highly water soluble and, because of that, mainly dispersed in the wastewater (Bertin et al. 2011). The effects of OMWW and phenolic extracts deriving from this matrix have been tested *in vitro* (Caballero-Guerrero et al. 2022; Sar and Akbas 2023; Ferlisi et al. 2024) and *in vivo* (Branciari et al. 2016; Sabino et al. 2018; Papakonstantinou et al. 2023) for their potential application to the diet of livestock animals, mainly pigs and poultry. In our study, two different OMWW phenolic dosages were tested as feed supplements in heavy-weight finishing pigs, to evaluate the impact on growth performance, morphological characteristics, oxidative status and quality of meat and dry-cured ham.

4.1 Growth performance

Growth performance parameters did not significantly differ between the three experimental groups (Table 2). However, although not reaching significance, pigs of P-HIGH group had the highest final BW (+5.3 % compared to C). Several studies reported that olive cake, one of the most used olive by-product, failed to influence growth performance parameters when added to pigs' diet (Hernández-Matamoros et al. 2011; Joven et al. 2014; García Casco et al. 2017; Ferrer et al. 2020). Similarly, no differences were found in the BW of sows after a dietary inclusion of olive pulp (Sánchez et al. 2022) as well as in pigs supplemented with an oleuropein extract (Rey et al. 2021) and partially defatted olive cake (Ferrer et al. 2020). However, a recent study showed that a dietary treatment with OMWW increased BW and ADG in piglets (Papakonstantinou et al. 2023); similarly, Liotta et al. (2019) reported that final BW and ADG of pigs increased when they were supplemented with olive cake (Liotta et al. 2019). Overall, the results obtained suggest that the two dosages of OMWW polyphenols (74 and 225 ppm) could be included in pig commercial diets for heavy pigs without negative effects on productive performances. Phenolic compounds, especially the high dosage, did not impair but could potentially improve pigs' growth rate.

4.2 Backfat thickness and pubertal status

Regarding backfat thickness measured at slaughtering, no differences were found between experimental groups. Two recent studies did not find differences in backfat thickness after a dietary inclusion with olive pulp in gestating sows (Sánchez et al. 2022) nor with olive cake supplementation in Bísaro pigs (Leite et al. 2022), although Palma-Granados et al. (2022) reported a lesser fat deposition after dietary pigs' supplementation with dry olive pulp, compared to wet crude olive cake and control diets (Palma-Granados et al. 2022). In the present study, there were no differences in the onset of puberty between pigs from the three experimental groups (Table 4), although it has been reported that polyphenols, for their chemical structure, could modulate the synthesis of hormones including the follicle-stimulating, steroids (e.g. progesterone) and prostaglandins, which are involved in follicles' growth and ovary size development (Hashem et al. 2020).

4.3 Antioxidant activity in liver and muscle

In the present study, the antioxidant activity of *sternocleidomastoid* muscle and liver was evaluated through DPPH, CUPRAC and ABTS assays. DPPH assay, which measures antioxidant activity by exploiting the electron/ hydrogen transfer ability of the molecules, demonstrated higher radical scavenging activity in the liver of pigs supplemented with 225 ppm of OMWW polyphenols (P-HIGH group), compared to the controls (Table 6). Furthermore, the present analysis showed a difference in RSA value of P-HIGH group compared to P-LOW, suggesting that the increase of the dosage can improve the effect of the OMWW phenolic extract. No significant differences among experimental groups were reported for the muscle samples (Table 6). A recent study of Vasilopoulou et al. (2023) did not find differences in terms of DPPH radical scavenging activity (%) neither in the liver nor in the muscle of broilers following a supplementation with an olive leaf extract (Vasilopoulou et al. 2023). The authors justified these results with the nature of the extract, which is highly made up of oleuropein. This phenolic compound, indeed, when at high doses, is known for exerting pro-oxidant effects (Scicchitano et al. 2023). Additionally, King et al. (2014) did not report significative changes in the antioxidant activity of poultry meat after a dietary supplementation with an olive freeze-dried powder containing 2.5% of hydroxytyrosol (King et al. 2014). Other studies such as the one from Branciari et al. (2017) demonstrated, using the DPPH assay, an increase of antioxidant activity in poultry meat following dietary supplementation of a semi-solid olive cake (patè) (Branciari et al. 2017). Jang et al. (2008) found a higher scavenging activity in breast meat

from broilers fed an herbal extract mix of antioxidants, compared to the control (Jang et al. 2008). These inconsistencies in DPPH results could be related to the different types of olive by-products used, the chemical profile and concentration of phenolic compounds contained (the quantities of each polyphenol may vary depending on climate and cultivar) and the dosages employed. Regarding the CUPRAC assay, which is based on the reduction of copper, no differences among the groups were reported in both muscle and liver (Table 7). Similarly, the ABTS assay did not show any difference between experimental groups in the liver (Table 8). This is supported by Untea et al. (2018), who did not find an enhanced antioxidant activity in the liver of pigs following a dietary supplementation with a plant mixture source of polyphenols (Untea et al. 2018). For the muscle, a significant ($p = 0.02$) difference in ABTS values was observed between P-HIGH and C groups, although the actual difference between the values was relatively small (Table 8). One possible reason could be related to the matrix effect of the muscular tissue. Indeed, to the authors' knowledge, literature does not report any studies in which the ABTS assay has been tested on the muscle of pigs or other monogastric species. Considering our results and literature findings, variations in the outcomes of these three assays could be related to the different antioxidant capacity of compounds (including polyphenols) interacting with the radicals used in the analyses (Echegaray et al. 2020). Moreover, very few antioxidant assays have been performed so far on animal tissues, especially on the liver, suggesting the need for further detailed studies to expand the application of these analyses in monogastric animals.

4.4 Oxidative status in blood

Pig oxidative state was evaluated by analysing several blood serum parameters. A tendency to an increase in PON ($p = 0.06$) was found in the blood of pigs from the P-HIGH group, when compared to P-LOW (Table 5). PON1 is a liver protein influenced by an oxidative environment (Aviram et al., 1998). Indeed, a decrease in this parameter is related to liver inflammation and oxidative stress condition, while in presence of natural antioxidants including polyphenols, its serum activity generally increases (Fuhrman and Aviram 2002; Jaiswal and Rizvi 2014). A recent study showed that the activity of PON1 increased in sows supplemented with a mixture containing natural polyphenols (Rossi et al. 2022). Israr et al. (2021) reported that PON1 increased in broiler chickens after a dietary inclusion of grape seed powder and zinc (Israr et al. 2021). Interestingly, as reported above, an increased antioxidant activity was observed in the liver of the P-HIGH group through DPPH assay, which may confirm the polyphenols' protective effect on the liver. A canonical discriminant analysis was also applied to obtain serum biomarkers that might differentiate the samples into the experimental groups. In particular, the fact that the increase in FRAP and PON has been associated to the P-HIGH group is a potential indication of an increased antioxidant activity in blood (Figure 3).

4.5 Meat and fat quality

Regarding meat quality traits, the pH of *gluteus medius* was measured 24 h post-mortem to evaluate physiological changes and the impact on meat traits. All samples exhibited pH values that were in the expected range for pork (Holmer et al. 2009), and there were no differences between the three experimental groups (Table 9). In raw meat, redness a* values decreased following polyphenol supplementation (Table 9). Similarly, meat redness of pigs feeding a partially-defatted olive cake diet showed a tendency to a decrease (Ferrer et al. 2020). Other studies reported that the antioxidant effects on meat redness becomes more prominent during preservation rather than in raw meat (Garrido et al. 2011; Branciari et al. 2015). Dietary antioxidants are usually known for their protective role on myoglobin, whose oxidation and reduction is related to meat discolouration (Fernández-López et al. 2003; Forte et al. 2017). Notably, oxymyoglobin, which is responsible for meat red colour, is unstable

and can be easily oxidized to metmyoglobin, thus becoming brownish (Masuda et al. 2013). Furthermore, the oxidation of heme protein promotes the formation of a superoxide anion, with detrimental effects on meat quality (Masuda et al. 2013; Wu et al. 2024). Polyphenols could potentially protect oxymyoglobin from oxidation thanks to their antioxidant power. However, their interaction with heme proteins is not always efficient, as it depends on phenolic chemical structure, dosage, pH, or possible interferences with transition metal ions (Wu et al. 2024). Certain polyphenols, including hydroxytyrosol, demonstrated a pro-oxidant action on oxymyoglobin *in vitro* (Masuda et al. 2013), proving that not all the polyphenols have a beneficial effect on heme protein oxidation. Our analysis also showed a higher yellowness b* with increasing dosage of OMWW polyphenols. This change in b* value could be related to the natural pigmentation of the phenolic OMWW extract, that gives a brownish colour to the product. Indeed, pigments in the olive, such as melanin and humic acid-like substances, can be transferred to olive mill wastewater and therefore found in the extracts (Khemakhem et al. 2016). The same effect on yellowness was obtained in fresh pork patties but after the addition of a seaweed extract containing a brown pigment (Moroney et al. 2013).

Among other indicators, cooking loss was evaluated in our study. This parameter is related to the water retention capacity of the muscle and, consequently, meat quality characteristics (i.e. tenderness) (Muzolf-Panek et al. 2016; Zeng et al. 2019). A decrease in cooking loss means a higher water-holding capacity and, consequently, better meat quality and shelf life (He et al. 2023). In our study, a decrease in cooking loss was observed for both polyphenol supplemented groups (although the statistical significance was reached only for P-LOW). Xu et al. (2021) exhibited that certain water-soluble polyphenols (gallic acid, epigallocatechin gallate and tannic acid) enhanced the stability of myofibrillar proteins in a gel and confirmed these outcomes on pork meatballs (Xu et al. 2021). Similarly, hydroxytyrosol and tyrosol, the main polyphenols contained in OMWW extract, are capable of interacting with water and other polar compounds through hydrogen bonds and can potentially reduce the hydrophobic surface of myofibrils in the muscle, maintaining their strength and finally reducing meat cooking loss. The phenolic dietary treatments did not seem to significantly affect meat lightness L* value, as reported by other authors (Liotta et al. 2019; Ferrer et al. 2020; Tsala et al. 2020). Dietary antioxidants are not usually believed to influence meat lightness, although Joven et al. (2014) reported a linear decrease in lightness in response to an addition of olive cake in the diet of finishing pigs (Joven et al. 2014). Similarly to what reported for meat, OMWW dietary polyphenols did not affect pH in fat and there was a decrease of a* value in P-LOW compared to C. Finally, the lightness parameter has shown variations in subcutaneous fat that are not easily explainable with the dietary treatments (Table 9).

4.6 Dry-cured ham and fat quality

In dry-cured ham, the pH of the two muscles (SM and BF) did not change among experimental groups, while yellowness b* decreased in P-HIGH. In BF, lower lightness values were observed in P-HIGH (Table 10). The decrease in lightness may be correlated to the lower yellowness, as they reveal a darker colour in dry-cured ham. Similarly to our results, Serra et al (2018) demonstrated a decrease in yellowness induced by dietary olive pomace in pork sausages (Serra et al. 2018). In SM and BF, redness value was stable among experimental groups, and this is relevant for the acceptability of consumers, which usually prefer a dark red ham (Morales et al. 2013). Parolari et al. (2016) confirmed our findings, reporting that hams at 12 months of seasoning had the highest redness in both muscles (Parolari et al. 2016). The yellowness of dry-cured ham depends on the development of rancidity caused by lipid oxidation (Ruiz et al. 2002). Thus, the decrease in yellowness could be related to a protection of lipid oxidation in dry-cured ham. Interestingly, the same effect is reported

for the subcutaneous fat, where the lowest values were observed for the group with the highest polyphenol dosage. The P-HIGH group also presented a higher pH and a decrease in lightness, with a higher a* value (Tables 9 and 10).

4.7 Fatty acid profile in dry-cured ham

OMWW polyphenols did not seem to influence the acidic composition of SM and BF, in terms of total SFA, MUFA, PUFA and PUFA/SFA ratio and other single fatty acids investigated, despite the decrease in yellowness observed in these muscles after a supplementation with the highest phenolic dosage (P-HIGH). It is believed that the colour and other quality characteristics of subcutaneous fat are more likely to be influenced by the fatty acid composition (Carrapiso and García 2005; Liotta et al. 2019). For instance, saturated fatty acids have a more stable structure that increases fat firmness, potentially influencing the lightness (Carrapiso and García 2005). Thus, the same type of investigation in the subcutaneous fat could be interesting to clarify the effects of dietary polyphenols on the fatty acid profile.

4.8 Proximate composition

In dry-cured ham, particularly in BF muscle, an increase in moisture and a decrease in ash content was reported in the P-HIGH group (Table 13). These results confirm the ability of OMWW polyphenols to retain water, previously demonstrated in raw meat. Indeed, a decrease in ash content suggests a lower amount of salts, as they are correlated in dry-cured products (Ferreira et al. 2013; Marušić Radovčić et al. 2021; Ferreira et al. 2022). Interestingly, BF is differently affected by NaCl and moisture loss compared to SM, which is an external muscle directly exposed to salts (Giovanelli et al. 2016). This suggests that polyphenols can have a more pronounced effect in BF muscle, which is better protected, compared to SM. Moreover, the induction of the increase in water retention capacity by polyphenols is supported by the outcomes of the sensorial analysis, where hams from pigs of the polyphenols- supplemented groups appeared less salty and they were more appreciated than the controls (data not shown).

4.9 Texture properties

The two dietary dosages did not seem to affect the texture properties of dry-cured ham. However, springiness (i.e. elasticity) in SM decreased with an increasing concentration of dietary polyphenols, while there were no significant differences in BF. Polyphenols, for their ability to retain water and increase moisture, should enhance springiness, as other studies showed (Chaves-López et al. 2015; Zhou et al. 2020; Sharma and Yadav 2020). However, in the SM, moisture values did not significantly change with the dietary treatment, most likely because, as previously mentioned, SM is the external muscle directly exposed to salt during curing process. These outcomes may explain the lack of a positive effect of polyphenols on the texture properties of this muscle. Overall, it is known that the differences in rheological characteristics of dry-cured ham can be related to the dietary sources and processing (Zhou et al. 2020).

5. Conclusion

Dietary supplementation with two dosages (74 ppm and 225 ppm) of polyphenols from OMWW in finishing pigs' diet did not negatively affect animal productive performances. Phenolic compounds (particularly the highest dosage) were shown to improve the antioxidant activity in the liver and this action was confirmed by the increase of PON and FRAP levels in blood. No other effects were

observed on liver and meat oxidative status, and the limited data reported in literature make comparisons difficult. An amelioration of water retention capacity by polyphenols was reported in meat and confirmed in dry-cured ham, while their impact on colour parameters, including the decrease in yellowness of dry-cured ham's tissues (lean and fat), needs to be clarified. Finally, it must be said that increasing the number of individuals involved in the experiment may provide further clarifications of the data obtained of this study. The influence of OMWW polyphenols on the oxidation processes typically occurring in dry-cured ham could be an interesting development of research, following the modern consumer demand.

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Closing remarks

Over the past decades, dietary supplementation with olive oil by-products and derived extracts as sources of polyphenols has represented an innovative and sustainable strategy for promoting productive performance and meat quality characteristics of monogastric animals, including pigs. Among other things, phenolic molecules are particularly abundant in olive oil products, giving them a value-added biological property and helping to address the environmental and economic issues related to their disposal (Chapter 1).
The present thesis aimed to explore the value of using polyphenols from an olive mill wastewater extract as dietary supplements throughout the entire heavy pig supply chain.

OMWW polyphenols demonstrated *in vitro* a beneficial role on host-pathogen interaction by reducing *Salmonella typhimurium* invasiveness in pig intestinal epithelial cells, as revealed by the modulation of the innate immune response (pro- and anti-inflammatory gene expressions) and the potential antimicrobial action (decrease of intracellular bacterial replication) (Chapter 2).

Secondly, the inclusion of OMWW polyphenols in finishing pig diets exhibited a potential positive impact on gut health by inducing beneficial changes in the intestinal ecosystem (increase of the abundance of beneficial bacteria and decrease of the harmful ones) and mucosal morphological improvements, although further evidence from the scientific community is needed to confirm the impact of these phenolic types on the maintenance of pig intestinal health (Chapter 3).

Regarding *in vivo* characteristics and post-slaughtering traits, dietary polyphenols did not impair growth performances in heavy finishing pigs and enhanced water retention of meat and a seasoned product (dry-cured ham), with a potential beneficial impact on their qualitative, physico-chemical and sensorial characteristics. Among these effects, polyphenols have shown to decrease yellowness in dry-cured ham, which is an indication of protection against lipid oxidation and, consequently, of the *shelf life* of the product, with a positive impact on consumer acceptability (Chapter 4).

These outcomes may promote the application of natural plant-derived antioxidants, e.g. polyphenols from the olive oil production chain, in the diets of monogastric animals including pigs, and could potentially address the novel preferences of the modern consumer, which requires high-quality, healthy and sustainable products.