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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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*A mio papà,
e a questo percorso,
che spero ci abbia reso ancora più vicini
perché nulla accada per caso.*

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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, histopathology and morphometry of several tissues (liver, ovaries, uterus, fat) and backfat deposition 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, with a favorable impact on 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 lower levels of thiobarbituric acid reactive substances (TBARS) and those of volatile compounds detected in dry-cured ham (aldehydes and alcohols) confirmed the role of polyphenols in preserving the shelf life of the product. The effect of polyphenols in dry-cured ham was also demonstrated on protein oxidation through the reduction in carbonyls.

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, their metabolic fate 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 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. I livelli più bassi di sostanze reattive dell'acido tiobarbiturico e di composti volatili (aldeidi e alcoli) rilevati nel prosciutto stagionato hanno confermato la capacità dei polifenoli di preservare la shelf-life 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, dal loro destino metabolico 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, oxidative status and sensory characteristics 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 world, 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 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 m³ (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 isolated 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 ↓, <i>Lactobacilli</i> bacteria ↑	(44)
OMWPE, DOC	0.0125, 0.0625	160, 330	Ross 308 female broilers	<i>Campylobacter</i> spp. ↓	(5)
Olive cake	8.6	120	Finishing pigs	Total aerobic bacteria →, <i>Enterobacteria</i> →, <i>Bifidobacteria</i> →, <i>Lactobacilli</i> →	(32)
Olive oil cake extract	-	1.0	Weaned piglets (DLL)	<i>Clostridium sensu stricto</i> ↑, <i>Lactobacillus</i> ↑	(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. Sarica 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 peroxy 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- κ B]) 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- κ B, 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- κ B. 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).

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.

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.

Author Contributions

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

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Conflict 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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References

1. Home. *International Olive Council* <https://www.internationaloliveoil.org/> [Accessed July 30, 2023]
2. Abu-Rumman G. Effect of olive mill solid waste on soil physical properties. *International Journal of Soil Science* (2016) 11:94–101. doi: 10.3923/ijss.2016.94.101
3. Fernández-Lobato L, López-Sánchez Y, Blejman G, Jurado F, Moyano-Fuentes J, Vera D. Life cycle assessment of the Spanish virgin olive oil production: A case study for Andalusian region. *Journal of Cleaner Production* (2021) 290:125677. doi: 10.1016/j.jclepro.2020.125677
4. Restuccia D, Prencipe SA, Ruggeri M, Spizzirri UG. Sustainability assessment of different extra virgin olive oil extraction methods through a life cycle thinking approach: challenges and opportunities in the elaiio-technical sector. *Sustainability* (2022) 14:15674. doi: 10.3390/su142315674
5. Branciarri R, Ranucci D, Ortenzi R, Roila R, Trabalza-Marinucci M, Servili M, Papa P, Galarini R, Valiani A. Dietary administration of olive mill wastewater extract reduces campylobacter spp. prevalence in broiler chickens. *Sustainability* (2016) 8:837. doi: 10.3390/su8090837
6. Alimenti C, Lianza M, Antognoni F, Giusti L, Bistoni O, Liotta L, Angeloni C, Lupidi G, Beghelli D. Characterization and biological activities of in vitro digested olive pomace polyphenols evaluated on ex vivo human immune blood cells. *Molecules* (2023) 28:2122. doi: 10.3390/molecules28052122
7. Gullón P, Gullón B, Astray G, Carpena M, Fraga-Corral M, Prieto MA, Simal-Gandara J. Valorization of by-products from olive oil industry and added-value applications for

innovative functional foods. *Food Research International* (2020) 137:109683. doi: 10.1016/j.foodres.2020.109683

8. Rivero-Pino F, Millan-Linares MC, Villanueva-Lazo A, Fernandez-Prior Á, Montserrat-de-la-Paz S. *In vivo* evidences of the health-promoting properties of bioactive compounds obtained from olive by-products and their use as food ingredient. *Critical Reviews in Food Science and Nutrition* (2023)1–13. doi: 10.1080/10408398.2023.2203229
9. Otero P, Garcia-Oliveira P, Carpena M, Barral-Martinez M, Chamorro F, Echave J, Garcia-Perez P, Cao H, Xiao J, Simal-Gandara J, Prieto MA. Applications of by-products from the olive oil processing: Revalorization strategies based on target molecules and green extraction technologies. *Trends in Food Science & Technology* (2021) 116:1084–1104. doi: 10.1016/j.tifs.2021.09.007
10. Papadomichelakis G, Pappas AC, Tsiplakou E, Symeon GK, Sotirakoglou K, Mpekellis V, Fegeros K, Zervas G. Effects of dietary dried olive pulp inclusion on growth performance and meat quality of broiler chickens. *Livestock Science* (2019) 221:115–122.
11. Silvan JM, Martinez-Rodriguez AJ. “Food by-products as natural source of bioactive compounds against *Campylobacter*”. In: Ferranti P, Berry EM, Anderson JR, editors. *Encyclopedia of Food Security and Sustainability*. Oxford: Elsevier (2019). p. 336–350 doi: 10.1016/B978-0-08-100596-5.22139-2
12. Abdel-Moneim A-ME, Shehata AM, Alzahrani SO, Shafi ME, Mesalam NM, Taha AE, Swelum AA, Arif M, Fayyaz M, Abd El-Hack ME. The role of polyphenols in poultry nutrition. *Journal of Animal Physiology and Animal Nutrition* (2020) 104:1851–1866. doi: 10.1111/jpn.13455
13. Varricchio E, Coccia E, Orso G, Lombardi V, Imperatore R, Vito P, Paolucci M. Influence of polyphenols from olive mill wastewater on the gastrointestinal tract, alveolar macrophages and blood leukocytes of pigs. *Italian Journal of Animal Science* (2019) 18:574–586. doi: 10.1080/1828051X.2018.1548911
14. Gerasopoulos K, Stagos D, Krouezas A, Karaveli C, Barda C, Gkika H, Mitsiou D, Petrotos K, Goulas P, Kouretas D. Assessment of fatty acid allocation in plasma and tissues in piglets, using feed supplemented with byproducts from processed olive mill wastewater. *In Vivo* (2016) 30:291–301
15. Savournin C, Baghdikian B, Elias R, Dargouth-Kesraoui F, Boukef K, Balansard G. Rapid high-performance liquid chromatography analysis for the quantitative determination of oleuropein in *Olea europaea* leaves. *J Agric Food Chem* (2001) 49:618–621. doi: 10.1021/jf000596
16. Oke OE, Emeshili UK, Iyasere OS, Abioja MO, Daramola JO, Ladokun AO, Abiona JA, Williams TJ, Rahman SA, Rotimi SO, Balogun SI, Adejuyigbe, AE. Physiological responses and performance of broiler chickens offered olive leaf extract under a hot humid tropical climate. *Journal of Applied Poultry Research* (2017) 26:376–382.
17. Leouifoudi I, Harnafi H, Ziyad A. Olive mill waste extracts: Polyphenols content, antioxidant, and antimicrobial activities. *Adv Pharmacol Sci* (2015) 2015:714138. doi: 10.1155/2015/714138

18. Al-Qodah Z, Al-Zoubi H, Hudaib B, Omar W, Soleimani M, Abu-Romman S, Frontistis Z. Sustainable vs. conventional approach for olive oil wastewater management: a review of the state of the art. *Water* (2022) 14:1695. doi: 10.3390/w14111695
19. Leo M, Muccillo L, Dugo L, Bernini R, Santi L, Sabatino L. Polyphenols extracts from oil production waste products (OPWPS) reduce cell viability and exert anti-inflammatory activity via ppar γ induction in colorectal cancer cells. *Antioxidants (Basel)* (2022) 11:624. doi: 10.3390/antiox11040624
20. Vastolo A, Calabrò S, Cutrignelli MI. A review on the use of agro-industrial co-products in animals' diets. *Italian Journal of Animal Science* (2022) 21:577–594. doi: 10.1080/1828051X.2022.2039562
21. Mahfuz S, Shang Q, Piao X. Phenolic compounds as natural feed additives in poultry and swine diets: a review. *J Anim Sci Biotechnol* (2021) 12:48. doi: 10.1186/s40104-021-00565-3
22. Servili M, Esposito S, Fabiani R, Urbani S, Taticchi A, Mariucci F, Selvaggini R, Montedoro GF. Phenolic compounds in olive oil: antioxidant, health and organoleptic activities according to their chemical structure. *Inflammopharmacology* (2009) 17:76–84. doi: 10.1007/s10787-008-8014-y
23. Fernando U, Biswas D, Allan B, Willson P, Potter AA. Influence of *Campylobacter jejuni* fliA, rpoN and flgK genes on colonization of the chicken gut. *Int J Food Microbiol* (2007) 118:194–200. doi: 10.1016/j.ijfoodmicro.2007.07.038
24. The Community Summary Report on Trends and Sources of Zoonoses and Zoonotic Agents in the European Union in 2007 | EFSA. (2009) <https://www.efsa.europa.eu/en/efsajournal/pub/rn-223> [Accessed April 13, 2023]
25. Lipiński K, Mazur M, Antoszkiewicz Z, Purwin C. Polyphenols in monogastric nutrition – A review. *Annals of Animal Science* (2017) 17:41–58. doi: 10.1515/aoas-2016-0042
26. Starčević K, Krstulović L, Brozić D, Maurić M, Stojević Z, Mikulec Ž, Bajić M, Mašek T. Production performance, meat composition and oxidative susceptibility in broiler chicken fed with different phenolic compounds. *J Sci Food Agric* (2015) 95:1172–1178. doi: 10.1002/jsfa.6805
27. Medouni-Haroune L, Zaidi F, Medouni-Adrar S, Kecha M. Olive pomace: from an olive mill waste to a resource, an overview of the new treatments. *JCR* (2018)1–6. doi: 10.22159/jcr.2018v5i5.28840
28. Herrero-Encinas J, Blanch M, Pastor JJ, Mereu A, Ipharraguerre IR, Menoyo D. Effects of a bioactive olive pomace extract from *Olea europaea* on growth performance, gut function, and intestinal microbiota in broiler chickens. *Poultry Science* (2020) 99:2–10. doi: 10.3382/ps/pez467
29. Shahidi F, Pinaffi-Langley ACC, Fuentes J, Speisky H, de Camargo AC. Vitamin E as an essential micronutrient for human health: Common, novel, and unexplored dietary sources. *Free Radical Biology and Medicine* (2021) 176:312–321. doi: 10.1016/j.freeradbiomed.2021.09.025

30. Molina-Alcaide E, Yáñez-Ruiz DR. Potential use of olive by-products in ruminant feeding: A review. *Animal Feed Science and Technology* (2008) 147:247–264. doi: 10.1016/j.anifeedsci.2007.09.021
31. Blas JC de, Rodriguez CA, Bacha F, Fernandez R, Abad-Guamán R. Nutritive value of co-products derived from olivecake in rabbit feeding. *World Rabbit Science* (2015) 23:255–262. doi: 10.4995/wrs.2015.4036
32. Ferrer P, Calvet S, García-Rebollar P, de Blas C, Jiménez-Belenguer AI, Hernández P, Piquer O, Cerisuelo A. Partially defatted olive cake in finishing pig diets: implications on performance, faecal microbiota, carcass quality, slurry composition and gas emission. *Animal* (2020) 14:426–434. doi: 10.1017/S1751731119002040
33. Al-Harathi MA. The efficacy of using olive cake as a by-product in broiler feeding with or without yeast. *Italian Journal of Animal Science* (2016) 15:512–520. doi: 10.1080/1828051X.2016.1194173
34. Branciarri R, Galarini R, Giusepponi D, Trabalza-Marinucci M, Forte C, Roila R, Miraglia D, Servili M, Acuti G, Valiani A. Oxidative Status and Presence of Bioactive Compounds in Meat from Chickens Fed Polyphenols Extracted from Olive Oil Industry Waste. *Sustainability* (2017) 9:1566. doi: 10.3390/su9091566
35. Ibrahim NS, Sabic EM, Abu-Taleb AM. Effect of inclusion irradiated olive pulp in laying quail diets on biological performance. *Journal of Radiation Research and Applied Sciences* (2018) 11:340–346. doi: 10.1016/j.jrras.2018.06.004
36. Sayehban P, Seidavi A, Dadashbeiki M, Ghorbani A, Araújo W, Albino L. Effects of different dietary levels of two types of olive pulp and exogenous enzyme supplementation on the gastrointestinal tract size, immunology and hematology of broilers. *Rev Bras Cienc Avic* (2015) 17:73–85. doi: 10.1590/1516-635
37. Sayehban P, Seidavi A, Dadashbeiki M, Ghorbani A, de Araújo WAG, Durazzo A, Lucarini M, Gabrielli P, Omri B, Teixeira Albino LF, Souto EB, Santini A. Olive pulp and exogenous enzymes feed supplementation effect on the carcass and offal in broilers: A Preliminary Study. *Agriculture* (2020) 10:359. doi: 10.3390/agriculture10080359
38. Tufarelli V, Laudadio V, Casalino E. An extra-virgin olive oil rich in polyphenolic compounds has antioxidant effects in meat-type broiler chickens. *Environ Sci Pollut Res Int* (2016) 23:6197–6204. doi: 10.1007/s11356-015-5852-1
39. La Scalia G, Micale R, Cannizzaro L, Marra FP. A sustainable phenolic compound extraction system from olive oil mill wastewater. *Journal of Cleaner Production* (2017) 142:3782–3788. doi: 10.1016/j.jclepro.2016.10.086
40. Zahra El Hassani F, El Karkouri A, Errachidi F, Merzouki M, Benlemlih M. The impact of Olive Mill Wastewater spreading on soil and plant in arid and semi-arid areas. *Environmental Nanotechnology, Monitoring & Management* (2023) 20:100798. doi: 10.1016/j.enmm.2023.100798
41. Papadopoulou A, Petrotos K, Stagos D, Gerasopoulos K, Maimaris A, Makris H, Kafantaris I, Makri S, Kerasioti E, Halabalaki M, Brièudes V, Ntasi G, Kokkas S, Tzimas P, Goulas P, Zakharenko AM, Golokhvast KS, Tsatsakis A, Kouretas D. Enhancement of antioxidant

mechanisms and reduction of oxidative stress in chickens after the administration of drinking water enriched with polyphenolic powder from olive mill waste waters. *Oxid Med Cell Longev* (2017) 2017:8273160. doi: 10.1155/2017/8273160

42. Sudjana AN, D’Orazio C, Ryan V, Rasool N, Ng J, Islam N, Riley TV, Hammer KA. Antimicrobial activity of commercial *Olea europaea* (olive) leaf extract. *Int J Antimicrob Agents* (2009) 33:461–463. doi: 10.1016/j.ijantimicag.2008.10.026
43. Paiva-Martins F, Barbosa S, Pinheiro V, Mourão JL, Outor-Monteiro D. The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. *Meat Sci* (2009) 82:438–443. doi: 10.1016/j.meatsci.2009.02.014
44. Şenay Sarıca and Dursen Ürkmez. The use of grape seed-, olive leaf- and pomegranate peel-extracts as alternative natural antimicrobial feed additives in broiler diets, Verlag Eugen Ulmer, Stuttgart. (2016)1612–9199.
45. Benavente-García O, Castillo J, Lorente J, Ortuño A, Del Rio JA. Antioxidant activity of phenolics extracted from *Olea europaea* L. leaves. *Food Chemistry* (2000) 68:457–462. doi: 10.1016/S0308-8146(99)00221-6
46. Sánchez-Gutiérrez M, Bascón-Villegas I, Rodríguez A, Pérez-Rodríguez F, Fernández-Prior Á, Rosal A, Carrasco E. Valorisation of *Olea europaea* L. Olive leaves through the evaluation of their extracts: antioxidant and antimicrobial activity. *Foods* (2021) 10:966. doi: 10.3390/foods10050966
47. Brenes M, García A, De Los Santos B, Medina E, Romero C, De Castro A, Romero F. Olive glutaraldehyde-like compounds against plant pathogenic bacteria and fungi. *Food Chemistry* (2011) 125:1262–1266. doi: 10.1016/j.foodchem.2010.10.055
48. Medina E, Brenes M, Romero C, García A, Castro A de. Main antimicrobial compounds in table olives. *J Agric Food Chem* (2007) 55:9817–9823. doi: 10.1021/jf0719757
49. Pereira A, Ferreira I, Marcelino F, Valentão P, Andrade P, Seabra R, Estevinho L, Bento A, Pereira J. Phenolic compounds and antimicrobial activity of olive (*Olea europaea* L. Cv. Cobrançosa) Leaves. *Molecules* (2007) 12:1153–1162. doi: 10.3390/12051153
50. Özkaya FD, Özkaya MT. Oleuropein using as an additive for feed and products used for humans. *J Food Process Technol* (2011) 02: doi: 10.4172/2157-7110.1000113
51. Yakhlef W, Arhab R, Romero C, Brenes M, de Castro A, Medina E. Phenolic composition and antimicrobial activity of Algerian olive products and by-products. *LWT* (2018) 93:323–328. doi: 10.1016/j.lwt.2018.03.044
52. Selma MV, Espín JC, Tomás-Barberán FA. Interaction between phenolics and gut microbiota: role in human health. *J Agric Food Chem* (2009) 57:6485–6501. doi: 10.1021/jf902107d
53. Zhang Y, Deng Z-X, He M-L, Pastor JJ, Tedo G, Liu J-X, Wang H-F. Olive oil cake extract stabilizes the physiological condition of lipopolysaccharide-challenged piglets by reducing oxidative stress and inflammatory responses and modulating the ileal microbiome. *Food Funct* (2021) 12:10171–10183. doi: 10.1039/D0FO03012K

54. Scott MB, Styring AK, McCullagh JSO. Polyphenols: Bioavailability, Microbiome interactions and cellular effects on health in humans and animals. *Pathogens* (2022) 11:770. doi: 10.3390/pathogens11070770
55. Shehata AA, Yalçın S, Latorre JD, Basiouni S, Attia YA, Abd El-Wahab A, Visscher C, El-Seedi HR, Huber C, Hafez HM, Eisenreich W, Tellez-Isaias G. Probiotics, prebiotics, and phytochemical substances for optimizing gut health in poultry. *Microorganisms* (2022) 10:395. doi: 10.3390/microorganisms10020395
56. Jiao X, Wang Y, Lin Y, Lang Y, Li E, Zhang X, Zhang Q, Feng Y, Meng X, Li B. Blueberry polyphenols extract as a potential prebiotic with anti-obesity effects on C57BL/6 J mice by modulating the gut microbiota. *The Journal of Nutritional Biochemistry* (2019) 64:88–100. doi: 10.1016/j.jnutbio.2018.07.008
57. Bonos E, Skoufos I, Petrotos K, Giavasis I, Mitsagga C, Fotou K, Vasilopoulou K, Giannenas I, Gouva E, Tsinas A, D'Alessandro AG, Cardinali A, Tzora A. Innovative use of olive, winery and cheese waste by-products as functional ingredients in broiler nutrition. *Vet Sci* (2022) 9:290. doi: 10.3390/vetsci9060290
58. Almuhayawi M, Alruhaili M, Gattan H, Alharbi M, Nagshabandi M, Almehayawi M, Al Jaouni SK, Selim S, Alqahtani F, El-Saadony M, Alagawany M. Evaluation of antimicrobial effect of olive leaves powder and its role in improving the broiler productivity, carcass traits, blood metabolites, and caecal microbiota. *Poultry Science* (2023)103054. doi: 10.1016/j.psj.2023.103054
59. Kubasova T, Davidova-Gerzova L, Merlot E, Medvecký M, Polansky O, Gardan-Salmon D, Quesnel H, Rychlik I. Housing systems influence gut microbiota composition of sows but not of their piglets. *PLOS ONE* (2017) 12:e0170051. doi: 10.1371/journal.pone.0170051
60. Sánchez CJ, Barrero-Domínguez B, Martínez-Miró S, Madrid J, Baños A, Aguinaga MA, López S, Hernández F. Use of olive pulp for gestating Iberian sow feeding: influence on performance, health status indicators, and fecal microbiota. *Animals* (2022) 12:3178. doi: 10.3390/ani12223178
61. Rebollada-Merino A, Bárcena C, Ugarte-Ruiz M, Porrás N, Mayoral-Alegre FJ, Tomé-Sánchez I, Domínguez L, Rodríguez-Bertos A. Effects on intestinal mucosal morphology, productive parameters and microbiota composition after supplementation with fermented defatted alperujo (FDA) in laying hens. *Antibiotics* (2019) 8:215. doi: 10.3390/antibiotics8040215
62. Šikić Pogačar M, Klančnik A, Bucar F, Langerholc T, Smole Možina S. Anti-adhesion activity of thyme (*Thymus vulgaris* L.) extract, thyme post-distillation waste, and olive (*Olea europaea* L.) leaf extract against *Campylobacter jejuni* on polystyrene and intestine epithelial cells. *J Sci Food Agric* (2016) 96:2723–2730. doi: 10.1002/jsfa.7391
63. Lee O-H, Lee B-Y. Antioxidant and antimicrobial activities of individual and combined phenolics in *Olea europaea* leaf extract. *Bioresour Technol* (2010) 101:3751–3754. doi: 10.1016/j.biortech.2009.12.052
64. Sabetan Shiraze AA, Hassanabadi A, Agah MJ, Nasiri Moghadam H. Effect of dietary inclusion of olive leaf powder on performance, small intestine morphology and nutrient

- digestibility in broiler chickens. *Animal Production* (2017) 19:371–387. doi: 10.22059/jap.2017.62053
65. Hume DA, Whitelaw CBA, Archibald AL. The future of animal production: improving productivity and sustainability. *The Journal of Agricultural Science* (2011) 149:9–16. doi: 10.1017/S0021859610001188
 66. Rey AI, De Cara A, Segura JF, Martí P, Hechavarría T, Calvo L. Dietary oleuropein extract supplementation and its combination with α -tocopheryl acetate and selenium modifies the free fatty acid profile of pork and improves its stability. *J Sci Food Agric* (2021) 101:2337–2344. doi: 10.1002/jsfa.10855
 67. Ponnampalam EN, Kiani A, Santhiravel S, Holman BWB, Lauridsen C, Dunshea FR. The Importance of dietary antioxidants on oxidative stress, meat and milk production, and their preservative aspects in farm animals: antioxidant action, animal health, and product quality—Invited review. *Animals* (2022) 12:3279. doi: 10.3390/ani12233279
 68. Xing T, Gao F, Tume RK, Zhou G, Xu X. Stress effects on meat quality: A mechanistic perspective. *Compr Rev Food Sci Food Saf* (2019) 18:380–401. doi: 10.1111/1541-4337.12417
 69. Papakonstantinou GI, Meletis E, Petrotos K, Kostoulas P, Tsekouras N, Kantere MC, Voulgarakis N, Gougoulis D, Filippopoulos L, Christodoulopoulos G, Athanasiou L, Papatsiros VG. Effects of a natural polyphenolic product from olive mill wastewater on oxidative stress and post-weaning diarrhea in piglets. *Agriculture* (2023) 13:1356. doi: 10.3390/agriculture13071356
 70. Kluess J, Schoenhusen U, Souffrant WB, Jones PH, Miller BG. Impact of diet composition on ileal digestibility and small intestinal morphology in early-weaned pigs fitted with a T-cannula. *Animal* (2010) 4:586–594. doi: 10.1017/S1751731109991455
 71. Heo JM, Opapeju FO, Pluske JR, Kim JC, Hampson DJ, Nyachoti CM. Gastrointestinal health and function in weaned pigs: a review of feeding strategies to control post-weaning diarrhoea without using in-feed antimicrobial compounds. *Journal of Animal Physiology and Animal Nutrition* (2013) 97:207–237. doi: 10.1111/j.1439-0396.2012.01284.x
 72. Agah M, Mirakzahi MT, Saleh H. Effects of olive leaf extract (*Olea europea* L.) On growth performance, blood metabolites and antioxidant activities in broiler chickens under heat stress. *Journal of Animal and Plant Sciences* (2019) 29: 657-66.
 73. Tsala A, Mpekeli V, Karvelis G, Tsikakis P, Goliomytis M, Simitzis P. Effects of dried olive pulp dietary supplementation on quality characteristics and antioxidant capacity of pig meat. *Foods* (2020) 9:81. doi: 10.3390/foods9010081
 74. Joven M, Pintos E, Latorre MA, Suárez-Belloch J, Guada JA, Fondevila M. Effect of replacing barley by increasing levels of olive cake in the diet of finishing pigs: Growth performances, digestibility, carcass, meat and fat quality. *Animal Feed Science and Technology* (2014) 197:185–193. doi: 10.1016/j.anifeedsci.2014.08.007
 75. Skaperda Z-V, Veskoukis A, Kouretas D. Farm animal welfare, productivity and meat quality: Interrelation with redox status regulation and antioxidant supplementation as a nutritional intervention (Review). *World Academy of Sciences Journal* (2019) doi: 10.3892/wasj.2019.19

76. Scicutella F, Mannelli F, Daghighio M, Viti C, Buccioni A. Polyphenols and organic acids as alternatives to antimicrobials in poultry rearing: A review. *Antibiotics* (2021) 10:1010. doi: 10.3390/antibiotics10081010
77. Cardinali A, Pati S, Minervini F, D'Antuono I, Linsalata V, Lattanzio V. Verbascoside, isoverbascoside, and their derivatives recovered from olive mill wastewater as possible food antioxidants. *J Agric Food Chem* (2012) 60:1822–1829. doi: 10.1021/jf204001p
78. Loru D, Incani A, Deiana M, Corona G, Atzeri A, Melis MP, Rosa A, Dessì MA. Protective effect of hydroxytyrosol and tyrosol against oxidative stress in kidney cells. *Toxicol Ind Health* (2009) 25:301–310. doi: 10.1177/0748233709103028
79. Crupi R, Palma E, Siracusa R, Fusco R, Gugliandolo E, Cordaro M, Impellizzeri D, De Caro C, Calzetta L, Cuzzocrea S, Di Paola R. Protective Effect of hydroxytyrosol against oxidative stress induced by the ochratoxin in kidney cells: in vitro and in vivo study. *Front Vet Sci* (2020) 7:136. doi: 10.3389/fvets.2020.00136
80. Valenzuela-Grijalva NV, Pinelli-Saavedra A, Muhlia-Almazan A, Domínguez-Díaz D, González-Ríos H. Dietary inclusion effects of phytochemicals as growth promoters in animal production. *J Anim Sci Technol* (2017) 59:8. doi: 10.1186/s40781-017-0133-9
81. Al-Harhi MA, Attia YA, El-Shafey AS, Elgandy MF. Impact of phytase on improving the utilisation of pelleted broiler diets containing olive by-products. *Italian Journal of Animal Science* (2020) 19:310–318. doi: 10.1080/1828051X.2020.1740896
82. Azazi I, Gadelrab S, Elkomy H, Ahmed A. Growth performance and feed utilization of growing rabbits fed diets containing olive cake meal supplemented with or without citric acid. *Egyptian Journal of Rabbit Science* (2018) 28:241–262. doi: 10.21608/ejrs.2018.44114
83. Salama WA, Basyony M, Suliman M, Matari R, Hassanein H. Effect of feeding olive cake supplemented with or without bentonite on performance of growing rabbits. *Egyptian Journal of Rabbit Science* (2016) 26:211–230. doi: 10.21608/ejrs.2016.42076
84. Bakr E. O. A., M. M. Shetaewi and A. El. M. I. El-Desoky. Effect of olive cake pulp as a partial or a complete substitute of wheat bran in growing rabbit's diet on growth performance, carcass traits and blood constituents under north sinai conditions. *J Animall and Polutry Prod, Mansoura Univ* (2019) 10:83–88. doi: <http://dx.doi.org/10.21608/jappmu.2019.40530>
85. García-Casco JM, Muñoz M, Martínez-Torres JM, García-López A, Fernández-Barroso MA, González-Sánchez E. Alternative feeding in iberian pigs during growth period: incorporation of olive cake in a dry or wet (silage) form. *Agriculturae Conspectus Scientificus* (2017) 82:147–150.
86. Dedousi A, Kritsa M-Z, Sossidou EN. Thermal comfort, growth performance and welfare of olive pulp fed broilers during hot season. *Sustainability* (2023) 15:10932. doi: 10.3390/su151410932
87. Sabino M, Cappelli K, Capomaccio S, Pascucci L, Biasato I, Verini-Supplizi A, Valiani A, Trabalza-Marinucci M. Dietary supplementation with olive mill wastewaters induces modifications on chicken jejunum epithelial cell transcriptome and modulates jejunum morphology. *BMC Genomics* (2018) 19:576. doi: 10.1186/s12864-018-4962-9

88. Yeniçeri M, Filik AG, Filik G. The effect of some selected fruit wastes for poultry feed on growth performance of broilers. *Palandöken Journal of Animal Sciences Technology and Economics* (2022) 1:33–41.
89. Younan GE, Mohamed MS, Morsy WA. Effect of dietary supplementation of olive leaf extract on productive performance, blood parameters and carcass traits of growing rabbits. *Egyptian Journal of Nutrition and Feeds* (2019) 22:173–182. doi: 10.21608/ejnf.2019.103477
90. Héla Y, Aymen H, Jihen J, Khalil A, Mohamed K, Jamel R, Atef M. Use of olive leaf aqueous extract (*Olea europaea*) in growing rabbits' diets. *Journal of New Sciences* (2019) 68:4209–4213.
91. Hedji C, Houndonougbo F, Tougan UP, Marcel H, Fiogbe E. Technological, sensorial and nutritional meat quality traits from pig fed with conventional and unconventional diets. *Food and Nutrition Sciences* (2015) 06:1514–1521. doi: 10.4236/fns.2015.616156
92. Maraschiello C, Sárraga C, García Regueiro JA. Glutathione peroxidase activity, TBARS, and alpha-tocopherol in meat from chickens fed different diets. *J Agric Food Chem* (1999) 47:867–872. doi: 10.1021/jf980824o
93. North MK, Dalle Zotte A, Hoffman LC. The use of dietary flavonoids in meat production: A review. *Animal Feed Science and Technology* (2019) 257:114291. doi: 10.1016/j.anifeedsci.2019.114291
94. Dal Bosco A, Mourvaki E, Cardinali R, Servili M, Sebastiani B, Ruggeri S, Mattioli S, Taticchi A, Esposito S, Castellini C. Effect of dietary supplementation with olive pomaces on the performance and meat quality of growing rabbits. *Meat Sci* (2012) 92:783–788. doi: 10.1016/j.meatsci.2012.07.001
95. Saija A, Trombetta D, Tomaino A, Lo Cascio R, Princi P, Uccella N, Bonina F, Castelli F. 'In vitro' evaluation of the antioxidant activity and biomembrane interaction of the plant phenols oleuropein and hydroxytyrosol. *International Journal of Pharmaceutics* (1998) 166:123–133. doi: 10.1016/S0378-5173(98)00018-0
96. World Health Organization. Diet, nutrition and the prevention of chronic diseases : report of a joint WHO/FAO expert consultation, Geneva, 28 January - 1 February 2002. World Health Organization (2003). 149 p. <https://apps.who.int/iris/handle/10665/42665> [Accessed June 23, 2023]
97. de Oliveira CO, Roll AAP, Medeiros Gonçalves FM, Lopes DCN, Xavier EG. Olive pomace for the feeding of commercial poultry: effects on performance, meat and eggs quality, haematological parameters, microbiota and immunity. *World's Poultry Science Journal* (2021) 77:363–376. doi: 10.1080/00439339.2021.1894409
98. Caparra P, Chies L, Scerra M, Foti F, Bognanno M, Cilione C, De Caria P, Claps S, Cifuni GF. Effect of dietary ensiled olive cake supplementation on performance and meat quality of apulo-calabrese pigs. *Animals* (2023) 13:2022. doi: 10.3390/ani13122022
99. Tufarelli V, Passantino L, Zupa R, Crupi P, Laudadio V. Suitability of dried olive pulp in slow-growing broilers: performance, meat quality, oxidation products, and intestinal mucosa features. *Poultry Science* (2022) 101:102230. doi: 10.1016/j.psj.2022.102230

100. Gerasopoulos K, Stagos D, Petrotos K, Kokkas S, Kantas D, Goulas P, Kouretas D. Feed supplemented with polyphenolic byproduct from olive mill wastewater processing improves the redox status in blood and tissues of piglets. *Food and Chemical Toxicology* (2015) 86:319–327. doi: 10.1016/j.fct.2015.11.007
101. Branciarri R, Galarini R, Trabalza-Marinucci M, Miraglia D, Roila R, Acuti G, Giusepponi D, Dal Bosco A, Ranucci D. Effects of olive mill vegetation water phenol metabolites transferred to muscle through animal diet on rabbit meat microbial quality. *Sustainability* (2021) 13:4522. doi: 10.3390/su13084522
102. Xie P, Deng Y, Huang L, Zhang C. Effect of olive leaf (*Olea europaea* L.) extract addition to broiler diets on the growth performance, breast meat quality, antioxidant capacity and caecal bacterial populations. *Italian Journal of Animal Science* (2022) 21:1246–1258. doi: 10.1080/1828051X.2022.2105265
103. de-Cara A, Saldaña B, Vázquez P, Rey AI. Dietary protected sodium butyrate and/or olive leaf and grape-based by-product supplementation modifies productive performance, antioxidant status and meat quality in broilers. *Antioxidants* (2023) 12:201. doi: 10.3390/antiox12010201
104. Hashemi SR, Davoodi H, Arabiyan E. Nutrigenomics: A new approach to feed formulation. *The Journal of Qazvin University of Medical Sciences* (2020) 24:80–93. doi: 10.32598/JQUMS.23.5.8
105. Fekete SG, Brown DL. Veterinary aspects and perspectives of nutrigenomics: a critical review. *Acta Vet Hung* (2007) 55:229–239. doi: 10.1556/AVet.55.2007.2.9
106. Sabino M, Capomaccio S, Cappelli K, Verini-Supplizi A, Bomba L, Ajmone-Marsan P, Cobellis G, Olivieri O, Pieramati C, Trabalza-Marinucci M. Oregano dietary supplementation modifies the liver transcriptome profile in broilers: RNASeq analysis. *Res Vet Sci* (2018) 117:85–91. doi: 10.1016/j.rvsc.2017.11.009
107. Dauncey M, Katsumata M, White P. “Nutrition, hormone receptor expression and gene interactions: implications for development and disease” (2004). p. 103–124 doi: 10.1079/9780851998114.0103
108. Kaput J, Rodriguez RL. Nutritional genomics: the next frontier in the postgenomic era. *Physiological Genomics* (2004) 16:166–177. doi: 10.1152/physiolgenomics.00107.2003
109. Surai PF. Integrated antioxidant defence network in animals. *EC Nutrition* (2023) 18:18–20.
110. Surai PF, Kochish II, Fisinin VI, Kidd MT. Antioxidant defence systems and oxidative stress in poultry biology: An update. *Antioxidants (Basel)* (2019) 8:235. doi: 10.3390/antiox8070235
111. Cappelli K, Ferlisi F, Mecocci S, Maranesi M, Trabalza-Marinucci M, Zerani M, Dal Bosco A, Acuti G. Dietary supplementation of olive mill waste water polyphenols in rabbits: Evaluation of the potential effects on hepatic apoptosis, inflammation and metabolism through RT-qPCR approach. *Animals* (2021) 11:2932. doi: 10.3390/ani11102932
112. Maranesi M, Dall’Aglia C, Acuti G, Cappelli K, Trabalza Marinucci M, Galarini R, Suvieri C, Zerani M. Effects of dietary polyphenols from olive mill waste waters on inflammatory and apoptotic effectors in rabbit ovary. *Animals* (2021) 11:1727. doi: 10.3390/ani11061727

113. Safwat GM, Kandiel MA, Abozaid OAR, Arafa MM, Mohamed SO. Biochemical effect of olive cake as feed additive on antioxidants and molecular expression of FAS, ANS, ACC in laying hens. *Adv Anim Vet Sci* (2022) 10: doi: 10.17582/journal.aavs/2022/10.4.731.738
114. Tari AR, Sadeghi AA, Mousavi SN. Dietary vegetable oils inclusion on the performance, hormonal levels and hsp 70 gene expression in broilers under heat stress. *Acta Scientiarum Animal Sciences* (2020) 42:e45517–e45517. doi: 10.4025/actascianimsci.v42i1.45517
115. Dedousi A, Kotzamanidis C, Kritsa M-Z, Tsourekis A, Andreadelli A, Patsios SI, Sossidou E. Growth performance, gut health, welfare and qualitative behavior characteristics of broilers fed diets supplemented with dried common (*Olea europaea*) olive pulp. *Sustainability* (2023) 15:501. doi: 10.3390/su15010501
116. Leite A, Vasconcelos L, Ferreira I, Domínguez R, Pateiro M, Rodrigues S, Pereira E, Campagnol PCB, Pérez-Alvarez J, Lorenzo JM, Teixeira A. Did the addition of olive cakes obtained by different methods of oil extraction in the finishing diet of bísaro pigs affect the volatile compounds and sensory characteristics of dry-cured loin and “Cachaço”? *Foods* (2023) 12:2499. doi: 10.3390/foods12132499
117. Liotta L, Chiofalo V, Lo Presti V, Chiofalo B. In vivo performances, carcass traits, and meat quality of pigs fed olive cake processing waste. *Animals* (2019) 9:1155. doi: 10.3390/ani9121155
118. Paiva-Martins F, Ribeirinha T, Silva A, Gonçalves R, Pinheiro V, Mourão JL, Outor-Monteiro D. Effects of the dietary incorporation of olive leaves on growth performance, digestibility, blood parameters and meat quality of growing pigs. *J Sci Food Agric* (2014) 94:3023–3029. doi: 10.1002/jsfa.6650

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 phytochemical 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 m³), 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- κ B 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 phytogetic 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 $15,000 \pm 592$ 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 μ g/mL; from this, different scalar concentrations of polyphenols (0.35; 0.7; 7; 14; 70; 140 μ g) 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% CO₂.

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 μ L per well, 0.1×10^5) in complete culture medium and incubated for 24 h at 37 °C under 5% CO₂ 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 μ g), 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 μ g). 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 μ L of the fresh DMEM/F12 medium supplemented with XTT detected solution (1:50). The plates were then incubated again at 37 °C under 5% CO₂ 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 μ g and 1.4 μ g) 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 μ g and 1.4 μ g 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 μ g POL; ST + 1.4 μ g POL). Cells were stimulated with 1 mL/well of bacterial suspension at 10^8 CFU/mL and incubated at 37 °C under 5% CO₂ 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 μ g/mL at 37 °C under 5% CO₂ for 2 h to remove all extracellular bacteria. Cells were washed four times with medium and lysed by adding 200 μ L/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 μ L 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% CO₂ 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 μ g, 0.7 μ g, 1.4 μ g or 7 μ g 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 μ g POL, 0.7 μ g POL, 1.4 μ g POL and 7 μ g POL). For the second, cells were treated with OMWW-EP at 0.35 μ g or 1.4 μ g for 24 h, then infected with 1 mL of 10^8 CFU/mL *S. typhimurium* suspension and incubated at 37 °C under 5% CO₂ 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% CO₂ 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 $2^{-\Delta\Delta\text{Ct}}$ method [68], comparing different conditions. Samples scored negatively when the Ct was ≥ 39 .

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% CO₂. Then, culture supernatants were collected, centrifuged (at 2500 \times g for 3 min), and kept at -80 °C until use. Levels of GM-CSF, IL-1 α , IL-1 β , 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).

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

Gene	Primer sequences	Amplicon length	Source
<i>IL18</i>	For-5'-CGTGTTTGAGGATATGCCTGATT-3' Rev-5'-TGGTACTGCCAGACCTCTAGTGA-3'	106	[48]
<i>IL1B</i>	For-5'-AATTCGAGTCTGCCCTGTACCC-3' Rev-5'-TGGTGAAGTCCGTTATATCTTGGC-3'	110	[49]
<i>NOS2</i>	For-5'-CGTTATGCCACCAACAATGG-3' Rev-5'-AGACCCGGAAGTCGTGCTT-3'	84	[48]
<i>TGFB1</i>	For-5'-CGCGTGCTAATGGTGGAAAG-3' Rev-5'-CCGACGTGTTGAACAGCATA-3'	87	[48]
<i>CXCL8</i>	For-5'-TTCGATGCCAGTGCATAAATA-3' Rev-5'-CTGTACAACCTTCTGCACCCA-3'	175	[69]
<i>MYD88</i>	For-5'-GCAGCTGGAACAGACCAACT-3' Rev-5'-GTGCCAGGCAGGACATCT-3'	62	[69]
<i>NF-kB1</i>	For-5'- CCCATGTAGACAGCACCACCTATGAT-3' Rev-5'-ACAGAGGCTCAAAGTTCTCCACCA-3'	131	[69]
<i>NF-kB/p65</i>	For-5'-CGAGAGGAGCACGGATACCA-3' Rev-5'-GCCCGTGTAGCCATTGA-3'	61	[69]
<i>DEFB1</i>	For-5'- CTGTTAGCTGCTTAAGGAATAAAGGC-3' Rev-5'-TGCCACAGGTGCCGATCT-3'	80	[48]
<i>DEFB4A</i>	For-5'-CCAGAGGTCCGACCACTA-3' Rev-5'-GGTCCCTTCAATCCTGTT-3'	87	[48]
<i>TLR4</i>	For -5'-TGGCAGTTTCTGAGGAGTCATG- 3' Rev. -5' -CCGCAGCAGGGACTTCTC - 3'	71	[48]
<i>TLR5</i>	For -5'-TCAAAGATCCTGACCATCACA- 3' Rev. - 5' -CCAGCTGTATCAGGGAGCTT - 3'	59	[48]
<i>GAPDH</i>	For-5'-ATGGTGAAGGTCGGAGTGAA-3' Rev-5' AGTGGAGGTCAATGAAGGGG-3'	61	[48]
<i>HPRT1</i>	For-5'-AACCTTGCTTTCCTTGGTCA-3 Rev-5'-TCAAGGGCATAGCCTACCAC-3'	150	[48]

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.

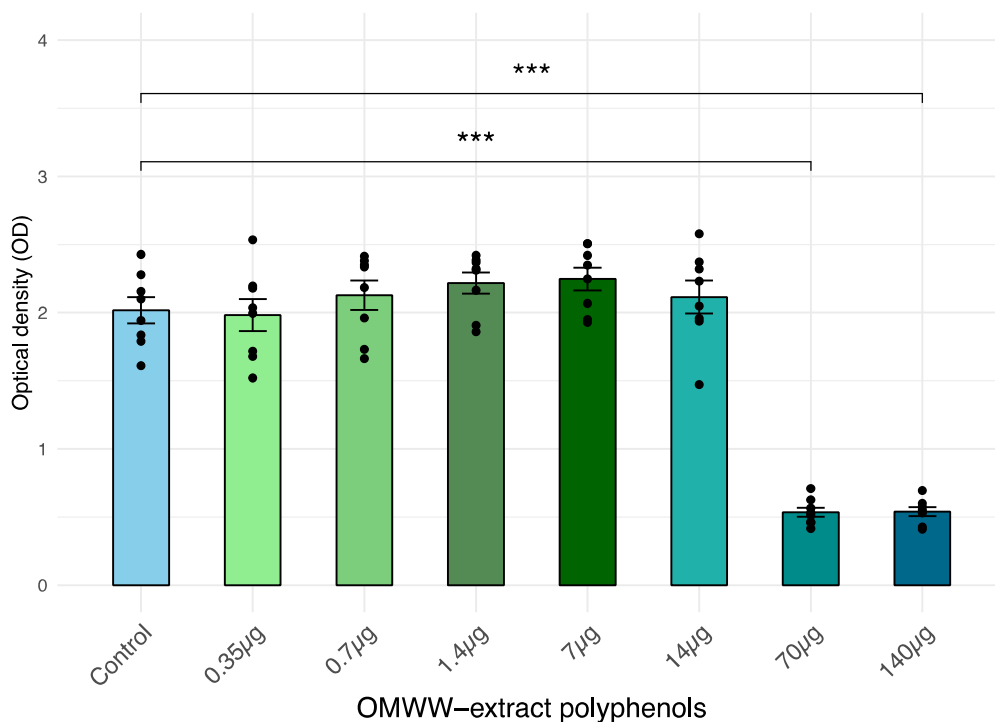


Figure 1. IPEC-J2 viability after a 24 h exposition to the extract of liquid olive mill waste-water (OMWW) polyphenols (OMWW-EP: 0.35 μg ; 0.7 μg ; 1.4 μg ; 7 μg ; 14 μg ; 70 μg ; 140 μg). Cell viability was determined with XTT test. The number of living cells is expressed as optical density (OD) \pm 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).

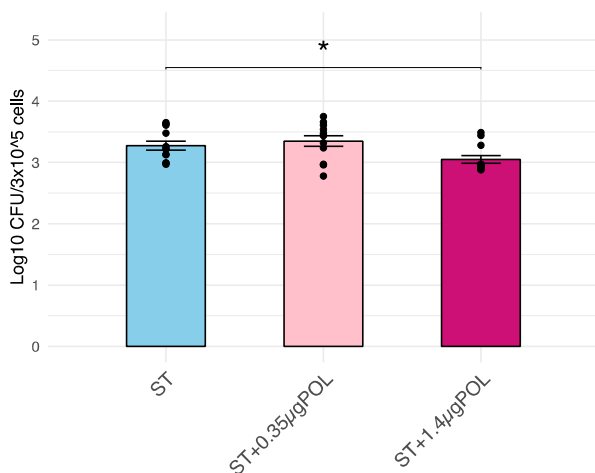


Figure 2. Effects of OMWW-extract polyphenols (OMWW-EP) on *S. typhimurium* penetration into IPEC-J2 cells. Data are expressed as \log_{10} CFU of penetrated, intracellular ST/ 3×10^5 cells. The mean value of five replicates + standard error is presented, and dots indicate samples included in each group.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

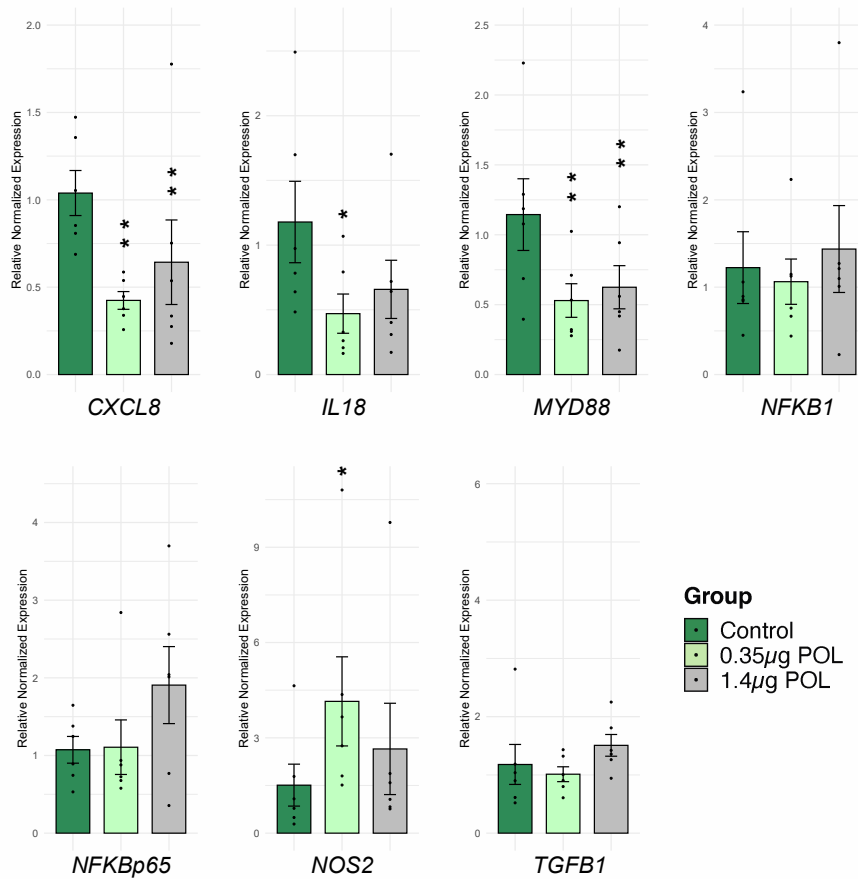


Figure 3. Effects of 24 h OMWW-extract polyphenols on IPEC-J2 gene expression. The RT-qPCR analysis was performed to evaluate *CXCL8*, *IL18*, *MYD88*, *NFKB1*, *NFKB/p65*, *TGFB1* and *NOS2* gene expression. Data are presented as bar plots displaying the mean value of normalized expression, standard 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-1 α , IL-1 β , 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-1 α , 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).

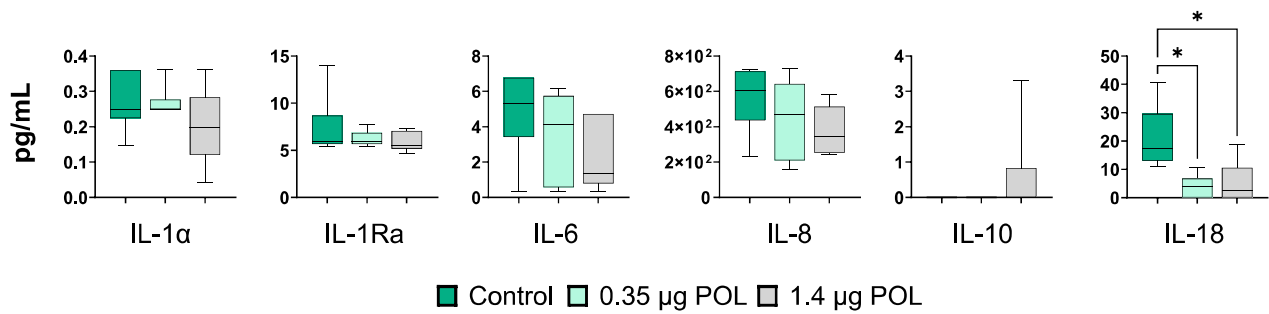


Figure 4. Effects of OMWW-extract polyphenols on IPEC-J2 cytokine release through multiplex ELISA measuring cytokine contents in culture supernatants (IL-1 α , IL-1Ra, IL-6, IL-8, IL-10, IL-18). Data are presented as box and whisker plots displaying median and interquartile range (boxes) and minimum and maximum values (whiskers). For each gene and cytokine, differences between treated with polyphenols (0.35 μ g POL—light green; 1.4 μ g 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 μ g 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 μ g 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).

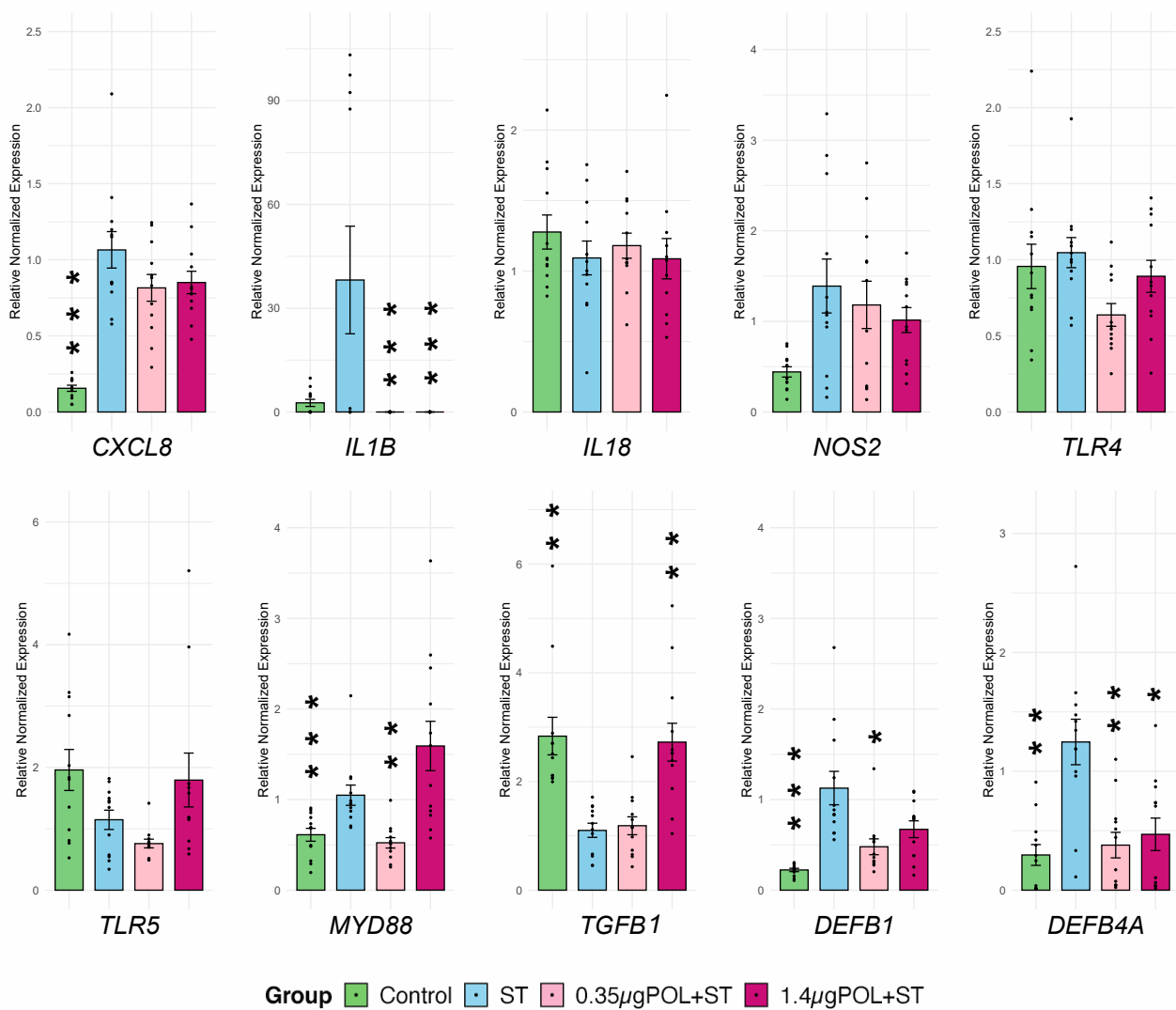


Figure 5. Gene expression of IPEC-J2 cells in response to *S. typhimurium* infection with OMWW-extract polyphenol (OMWW-EP) pre-treatment. The tested conditions for IPEC-J2 cells were: uninfected and untreated cells (Control—dark green), infected with *S. typhimurium* (ST—blue), pre-treated with 0.35 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-1 α ($p = 0.02927$), 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-1 β were below the reference range values.

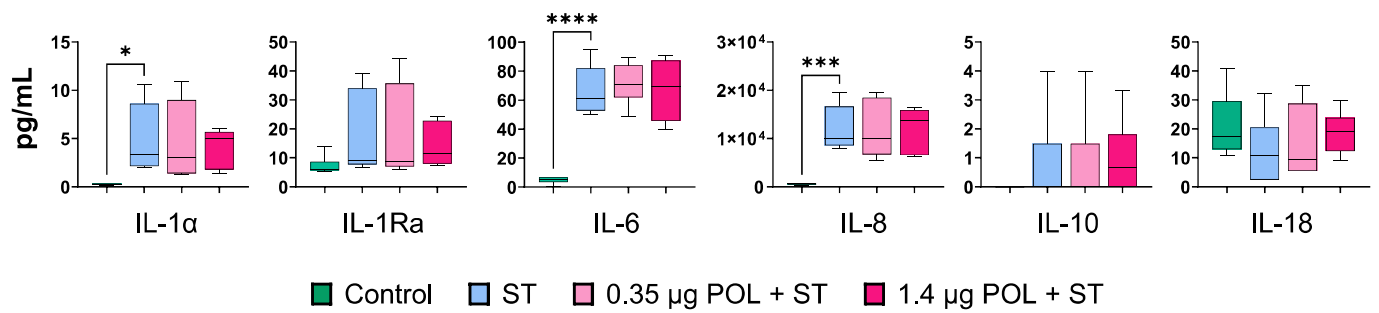


Figure 6. Cytokine release by IPEC-J2 cells in response to *S. typhimurium* infection after OMWW-extract polyphenol (OMWW-EP) pre-treatment. The tested conditions for IPEC-J2 cells were: uninfected and untreated cells (Control—dark green), infected with *S. typhimurium* (ST—blue), pre-treated with 0.35 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-κB pathway [45]. *CXCL8* is a pro-inflammatory chemokine (IL-8)-encoding gene whose expression could be regulated by the TLR4/MyD88/NF-κB pathway. In our study, OMWW-EP seemed to exert anti-inflammatory action by decreasing *MYD88* (a gene with a pivotal role in NF-κB 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- κ B activation, essential for the stimulation of pro-inflammatory gene expressions. Not surprisingly, we therefore observed the increased release of other pro-inflammatory cytokines (IL-1 α , 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- κ B [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- κ B translocation into the nucleus [49]. The activation of the nuclear factor NF- κ B 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 μ g 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 μ g 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 μ g OMWW-EP probably prevented the activation of NF- κ B 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-1 β and IL-18. During enteric *Salmonella* infection, the activation of caspase-1 and the production of IL-1 β 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 μ g), 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 IL-1 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-1 β [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-1 β , IL-12, TNF [89].

In our experiments, we observed that *S. typhimurium* infection determined a decrease in *TGFBI* 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 μ g) induced the up-regulation of *TGFBI* in infected cells compared to cells that were infected without OMWW-EP pre-treatment. The *TGFBI* 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 *TGFBI* increase. Moreover, in a study by Nallathambi et al. [93], a polyphenol-rich grape seed extract was able to enhance *TGFBI* 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 μ g 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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References

1. Capasso, R.; Cristinzio, G.; Evidente, A.; Scognamiglio, F. Isolation, Spectroscopy and Selective Phytotoxic Effects of Polyphenols from Vegetable Waste Waters. *Phytochemistry* **1992**, *31*, 4125–4128. [https://doi.org/10.1016/0031-9422\(92\)80426-F](https://doi.org/10.1016/0031-9422(92)80426-F).
2. Mekki, A.; Dhouib, A.; Sayadi, S. Changes in Microbial and Soil Properties Following Amendment with Treated and Untreated Olive Mill Wastewater. *Microbiol. Res.* **2006**, *161*, 93–101. <https://doi.org/10.1016/j.micres.2005.06.001>.
3. Ben Sassi, A.; Boularbah, A.; Jaouad, A.; Walker, G.; Boussaid, A. A Comparison of Olive Oil Mill Wastewaters (OMW) from Three Different Processes in Morocco. *Process Biochem.* **2006**, *41*, 74–78. <https://doi.org/10.1016/j.procbio.2005.03.074>.

4. Casa, R.; D'Annibale, A.; Pieruccetti, F.; Stazi, S.R.; Giovannozzi Sermanni, G.; Lo Cascio, B. Reduction of the Phenolic Components in Olive-Mill Wastewater by an Enzymatic Treatment and Its Impact on Durum Wheat (*Triticum durum* Desf.) Germinability. *Chemosphere* **2003**, *50*, 959–966. [https://doi.org/10.1016/S0045-6535\(02\)00707-5](https://doi.org/10.1016/S0045-6535(02)00707-5).
5. El Hadrami, A.; Belaqziz, M.; El Hassni, M.; Hanifi, S.; Abbad, A.; Capasso, R.; Gianfreda, L.; El Hadrami, I. Physico-chemical characterization and effects of olive oil mill wastewaters fertirrigation on the growth of some Mediterranean crops. *J. Agron.* **2004**, *3*, 247–254.
6. Quarantino, D.; D'Annibale, A.; Federici, F.; Cereti, C.F.; Rossini, F.; Fenice, M. Enzyme and Fungal Treatments and a Combination Thereof Reduce Olive Mill Wastewater Phytotoxicity on *Zea mays* L. Seeds. *Chemosphere* **2007**, *66*, 1627–1633. <https://doi.org/10.1016/j.chemosphere.2006.07.092>.
7. Rodis, P.S.; Karathanos, V.T.; Mantzavinou, A. Partitioning of Olive Oil Antioxidants between Oil and Water Phases. *J. Agric. Food Chem.* **2002**, *50*, 596–601. <https://doi.org/10.1021/jf010864j>.
8. Bertin, L.; Ferri, F.; Scoma, A.; Marchetti, L.; Fava, F. Recovery of High Added Value Natural Polyphenols from Actual Olive Mill Wastewater through Solid Phase Extraction. *Chem. Eng. J.* **2011**, *171*, 1287.
9. De Marco, E.; Savarese, M.; Paduano, A.; Sacchi, R. Characterization and Fractionation of Phenolic Compounds Extracted from Olive Oil Mill Wastewaters. *Food Chem.* **2007**, *104*, 858–867. <https://doi.org/10.1016/j.foodchem.2006.10.005>.
10. Varricchio, E.; Coccia, E.; Orso, G.; Lombardi, V.; Imperatore, R.; Vito, P.; Paolucci, M. Influence of Polyphenols from Olive Mill Wastewater on the Gastrointestinal Tract, Alveolar Macrophages and Blood Leukocytes of Pigs. *Ital. J. Anim. Sci.* **2019**, *18*, 574–586. <https://doi.org/10.1080/1828051X.2018.1548911>.
11. Branciarri, R.; Ranucci, D.; Ortenzi, R.; Roila, R.; Trabalza-Marinucci, M.; Servili, M.; Papa, P.; Galarini, R.; Valiani, A. Dietary Administration of Olive Mill Wastewater Extract Reduces *Campylobacter* Spp. Prevalence in Broiler Chickens. *Sustainability* **2016**, *8*, 837. <https://doi.org/10.3390/su8090837>.
12. Hume, D.A.; Whitelaw, C.B.A.; Archibald, A.L. The Future of Animal Production: Improving Productivity and Sustainability. *J. Agric. Sci.* **2011**, *149*, 9–16. <https://doi.org/10.1017/S0021859610001188>.
13. Obied, H.K.; Bedgood, D.R.; Prenzler, P.D.; Robards, K. Bioscreening of Australian Olive Mill Waste Extracts: Biophenol Content, Antioxidant, Antimicrobial and Molluscicidal Activities. *Food Chem. Toxicol.* **2007**, *45*, 1238–1248. <https://doi.org/10.1016/j.fct.2007.01.004>.
14. Servili, M.; Sordini, B.; Esposto, S.; Urbani, S.; Veneziani, G.; Di Maio, I.; Selvaggini, R.; Taticchi, A. Biological Activities of Phenolic Compounds of Extra Virgin Olive Oil. *Antioxidants* **2013**, *3*, 1–23. <https://doi.org/10.3390/antiox3010001>.
15. Veneziani, G.; Novelli, E.; Esposto, S.; Taticchi, A.; Servili, M. Applications of Recovered Bioactive Compounds in Food Products. In *Olive Mill Waste: Recent Advances for Sustainable Management*; Academic Press: Cambridge, MA, USA, 2017; pp. 231–253; ISBN 978-0-12-805314-0.
16. Salomone, R.; Ioppolo, G. Environmental Impacts of Olive Oil Production: A Life Cycle Assessment Case Study in the Province of Messina (Sicily). *J. Clean. Prod.* **2012**, *28*, 88–100. <https://doi.org/10.1016/j.jclepro.2011.10.004>.
17. Vastolo, A.; Calabrò, S.; Cutrignelli, M.I. A Review on the Use of Agro-Industrial CO-Products in Animals' Diets. *Ital. J. Anim. Sci.* **2022**, *21*, 577–594. <https://doi.org/10.1080/1828051X.2022.2039562>.
18. Branciarri, R.; Galarini, R.; Giusepponi, D.; Trabalza-Marinucci, M.; Forte, C.; Roila, R.; Miraglia, D.; Servili, M.; Acuti, G.; Valiani, A. Oxidative Status and Presence of Bioactive Compounds in Meat from Chickens Fed Polyphenols Extracted from Olive Oil Industry Waste. *Sustainability* **2017**, *9*, 1566. <https://doi.org/10.3390/su9091566>.
19. Sabino, M.; Cappelli, K.; Capomaccio, S.; Pascucci, L.; Biasato, I.; Verini-Supplizi, A.; Valiani, A.; Trabalza-Marinucci, M. Dietary Supplementation with Olive Mill Wastewaters

- Induces Modifications on Chicken Jejunum Epithelial Cell Transcriptome and Modulates Jejunum Morphology. *BMC Genom.* **2018**, *19*, 576. <https://doi.org/10.1186/s12864-018-4962-9>.
20. Ferlisi, F.; Tang, J.; Cappelli, K.; Trabalza-Marinucci, M. Dietary Supplementation with Olive Oil Co-Products Rich in Polyphenols: A Novel Nutraceutical Approach in Monogastric Animal Nutrition. *Front. Vet. Sci.* **2023**, *10*, 1272274. <https://doi.org/10.3389/fvets.2023.1272274>.
 21. Maranesi, M.; Dall'Aglio, C.; Acuti, G.; Cappelli, K.; Trabalza Marinucci, M.; Galarini, R.; Suvieri, C.; Zerani, M. Effects of Dietary Polyphenols from Olive Mill Waste Waters on Inflammatory and Apoptotic Effectors in Rabbit Ovary. *Animals* **2021**, *11*, 1727. <https://doi.org/10.3390/ani11061727>.
 22. Cappelli, K.; Ferlisi, F.; Mecocci, S.; Maranesi, M.; Trabalza-Marinucci, M.; Zerani, M.; Dal Bosco, A.; Acuti, G. Dietary Supplementation of Olive Mill Waste Water Polyphenols in Rabbits: Evaluation of the Potential Effects on Hepatic Apoptosis, Inflammation and Metabolism through RT-qPCR Approach. *Animals* **2021**, *11*, 2932. <https://doi.org/10.3390/ani11102932>.
 23. Lessard, M.; Talbot, G.; Bergeron, N.; Verso, L.L.; Morissette, B.; Yergeau, É.; Matte, J.J.; Bissonnette, N.; Blais, M.; Gong, J.; et al. Weaning Diet Supplemented with Health-Promoting Feed Additives Influences Microbiota and Immune Response in Piglets Challenged with *Salmonella*. *Vet. Immunol. Immunopathol.* **2023**, *255*, 110533. <https://doi.org/10.1016/j.vetimm.2022.110533>.
 24. Lian, S.; Lin, X.; Zhan, F.; Shen, X.; Liang, Y.; Li, C. Transcriptome Analysis Reveals the Multiple Functions of pBD2 in IPEC-J2 Cells against *E. coli*. *Int. J. Mol. Sci.* **2022**, *23*, 9754. <https://doi.org/10.3390/ijms23179754>.
 25. van den Bogaard, A.E.; Stobberingh, E.E. Antibiotic Usage in Animals. *Drugs* **1999**, *58*, 589–607. <https://doi.org/10.2165/00003495-199958040-00002>.
 26. Veldhuizen, E.J.A.; Koomen, I.; Ultee, T.; van Dijk, A.; Haagsman, H.P. *Salmonella* Serovar Specific Upregulation of Porcine Defensins 1 and 2 in a Jejunal Epithelial Cell Line. *Vet. Microbiol.* **2009**, *136*, 69–75. <https://doi.org/10.1016/j.vetmic.2008.09.072>.
 27. Farzan, A.; Friendship, R.M. A Clinical Field Trial to Evaluate the Efficacy of Vaccination in Controlling *Salmonella* Infection and the Association of *Salmonella*-Shedding and Weight Gain in Pigs. *Can. J. Vet. Res.* **2010**, *74*, 258–263.
 28. Naberhaus, S.A.; Krull, A.C.; Arruda, B.L.; Arruda, P.; Sahin, O.; Schwartz, K.J.; Burrough, E.R.; Magstadt, D.R.; Matias Ferreyra, F.; Gatto, I.R.H.; et al. Pathogenicity and Competitive Fitness of *Salmonella enterica* Serovar 4,[5],12:i:- Compared to *Salmonella* Typhimurium and *Salmonella* Derby in Swine. *Front. Vet. Sci.* **2019**, *6*, 502. <https://doi.org/10.3389/fvets.2019.00502>.
 29. Scott, M.B.; Styring, A.K.; McCullagh, J.S.O. Polyphenols: Bioavailability, Microbiome Interactions and Cellular Effects on Health in Humans and Animals. *Pathogens* **2022**, *11*, 770. <https://doi.org/10.3390/pathogens11070770>.
 30. Romier, B.; Schneider, Y.-J.; Larondelle, Y.; During, A. Dietary Polyphenols Can Modulate the Intestinal Inflammatory Response. *Nutr. Rev.* **2009**, *67*, 363–378. <https://doi.org/10.1111/j.1753-4887.2009.00210.x>.
 31. Zhang, Y.; Mu, T.; Deng, X.; Guo, R.; Xia, B.; Jiang, L.; Wu, Z.; Liu, M. New Insights of Biological Functions of Natural Polyphenols in Inflammatory Intestinal Diseases. *Int. J. Mol. Sci.* **2023**, *24*, 9581. <https://doi.org/10.3390/ijms24119581>.
 32. Axling, U.; Olsson, C.; Xu, J.; Fernandez, C.; Larsson, S.; Ström, K.; Ahrné, S.; Holm, C.; Molin, G.; Berger, K. Green Tea Powder and *Lactobacillus plantarum* Affect Gut Microbiota, Lipid Metabolism and Inflammation in High-Fat Fed C57BL/6J Mice. *Nutr. Metab.* **2012**, *9*, 105. <https://doi.org/10.1186/1743-7075-9-105>.
 33. Tassou, C.C.; Nychas, G.J.; Board, R.G. Effect of Phenolic Compounds and Oleuropein on the Germination of *Bacillus cereus* T Spores. *Biotechnol. Appl. Biochem.* **1991**, *13*, 231–237.

34. Anhê, F.F.; Pilon, G.; Roy, D.; Desjardins, Y.; Levy, E.; Marette, A. Triggering Akkermansia with Dietary Polyphenols: A New Weapon to Combat the Metabolic Syndrome? *Gut Microbes* **2016**, *7*, 146–153. <https://doi.org/10.1080/19490976.2016.1142036>.
35. Dueñas, M.; Muñoz-González, I.; Cueva, C.; Jiménez-Girón, A.; Sánchez-Patán, F.; Santos-Buelga, C.; Moreno-Arribas, M.V.; Bartolomé, B. A Survey of Modulation of Gut Microbiota by Dietary Polyphenols. *BioMed Res. Int.* **2015**, *2015*, e850902. <https://doi.org/10.1155/2015/850902>.
36. Hara, H.; Orita, N.; Hatano, S.; Ichikawa, H.; Hara, Y.; Matsumoto, N.; Kimura, Y.; Terada, A.; Mitsuoka, T. Effect of Tea Polyphenols on Fecal Flora and Fecal Metabolic Products of Pigs. *J. Vet. Med. Sci.* **1995**, *57*, 45–49. <https://doi.org/10.1292/jvms.57.45>.
37. Ishihara, N.; Chu, D.-C.; Akachi, S.; Juneja, L.R. Improvement of Intestinal Microflora Balance and Prevention of Digestive and Respiratory Organ Diseases in Calves by Green Tea Extracts. *Livest. Prod. Sci.* **2001**, *68*, 217–229. [https://doi.org/10.1016/S0301-6226\(00\)00233-5](https://doi.org/10.1016/S0301-6226(00)00233-5).
38. Liu, Y. Assessment of the Antimicrobial Activity of Olive Leaf Extract Against Foodborne Bacterial Pathogens. *Front. Microbiol.* **2017**, *8*, 113.
39. Sudjana, A.N.; D’Orazio, C.; Ryan, V.; Rasool, N.; Ng, J.; Islam, N.; Riley, T.V.; Hammer, K.A. Antimicrobial Activity of Commercial *Olea europaea* (Olive) Leaf Extract. *Int. J. Antimicrob. Agents* **2009**, *33*, 461–463. <https://doi.org/10.1016/j.ijantimicag.2008.10.026>.
40. Chen, J.; Yu, B.; Chen, D.; Huang, Z.; Mao, X.; Zheng, P.; Yu, J.; Luo, J.; He, J. Chlorogenic Acid Improves Intestinal Barrier Functions by Suppressing Mucosa Inflammation and Improving Antioxidant Capacity in Weaned Pigs. *J. Nutr. Biochem.* **2018**, *59*, 84–92. <https://doi.org/10.1016/j.jnutbio.2018.06.005>.
41. Chen, X.; Zeng, Z.; Huang, Z.; Chen, D.; He, J.; Chen, H.; Yu, B.; Yu, J.; Luo, J.; Luo, Y.; et al. Effects of Dietary Resveratrol Supplementation on Immunity, Antioxidative Capacity and Intestinal Barrier Function in Weaning Piglets. *Anim. Biotechnol.* **2021**, *32*, 240–245. <https://doi.org/10.1080/10495398.2019.1683022>.
42. Ahasan, A.S.M.L.; Invernizzi, G.; Farina, G.; Pilotto, A.; Barbé, F.; Bontempo, V.; Rossi, R.; Bellagamba, F.; Lecchi, C.; Savoini, G.; et al. The Effects of Superoxide Dismutase-Rich Melon Pulp Concentrate on Inflammation, Antioxidant Status and Growth Performance of Challenged Post-Weaning Piglets. *Animal* **2019**, *13*, 136–143. <https://doi.org/10.1017/S1751731118001234>.
43. Zhang, H.J.; Jiang, X.R.; Mantovani, G.; Lumbreras, A.E.V.; Comi, M.; Alborali, G.; Savoini, G.; Dell’Orto, V.; Bontempo, V. *Modulation of Plasma Antioxidant Activity in Weaned Piglets by Plant Polyphenols*; Taylor & Francis: Abingdon, UK, 2014.
44. Fiesel, A.; Gessner, D.K.; Most, E.; Eder, K. Effects of Dietary Polyphenol-Rich Plant Products from Grape or Hop on Pro-Inflammatory Gene Expression in the Intestine, Nutrient Digestibility and Faecal Microbiota of Weaned Pigs. *BMC Vet. Res.* **2014**, *10*, 196. <https://doi.org/10.1186/s12917-014-0196-5>.
45. Gessner, D.K.; Ringseis, R.; Eder, K. Potential of Plant Polyphenols to Combat Oxidative Stress and Inflammatory Processes in Farm Animals. *J. Anim. Physiol. Anim. Nutr.* **2017**, *101*, 605–628. <https://doi.org/10.1111/jpn.12579>.
46. Mahfuz, S.; Shang, Q.; Piao, X. Phenolic Compounds as Natural Feed Additives in Poultry and Swine Diets: A Review. *J. Anim. Sci. Biotechnol.* **2021**, *12*, 48. <https://doi.org/10.1186/s40104-021-00565-3>.
47. Mosele, J.I.; Macià, A.; Motilva, M.-J. Metabolic and Microbial Modulation of the Large Intestine Ecosystem by Non-Absorbed Diet Phenolic Compounds: A Review. *Molecules* **2015**, *20*, 17429–17468. <https://doi.org/10.3390/molecules200917429>.
48. Mecocci, S.; De Paolis, L.; Fruscione, F.; Pietrucci, D.; De Ciucis, C.G.; Giudici, S.D.; Franzoni, G.; Chillemi, G.; Cappelli, K.; Razzuoli, E. In Vitro Evaluation of Immunomodulatory Activities of Goat Milk Extracellular Vesicles (mEVs) in a Model of Gut Inflammation. *Res. Vet. Sci.* **2022**, *152*, 546–556. <https://doi.org/10.1016/j.rvsc.2022.09.021>.

49. Razzuoli, E.; Amadori, M.; Lazzara, F.; Bilato, D.; Ferraris, M.; Vito, G.; Ferrari, A. *Salmonella* Serovar-Specific Interaction with Jejunal Epithelial Cells. *Vet. Microbiol.* **2017**, *207*, 219–225. <https://doi.org/10.1016/j.vetmic.2017.07.002>.
50. Razzuoli, E.; Villa, R.; Amadori, M. IPEC-J2 Cells as Reporter System of the Anti-Inflammatory Control Actions of Interferon-Alpha. *J. Interferon Cytokine Res.* **2013**, *33*, 597–605. <https://doi.org/10.1089/jir.2012.0127>.
51. Sonntag, A.-K.; Bielaszewska, M.; Mellmann, A.; Dierksen, N.; Schierack, P.; Wieler, L.H.; Schmidt, M.A.; Karch, H. Shiga Toxin 2e-Producing *Escherichia coli* Isolates from Humans and Pigs Differ in Their Virulence Profiles and Interactions with Intestinal Epithelial Cells. *Appl. Environ. Microbiol.* **2005**, *71*, 8855–8863. <https://doi.org/10.1128/AEM.71.12.8855-8863.2005>.
52. De Ciucis, C.G.; Fruscione, F.; De Paolis, L.; Mecocci, S.; Zinellu, S.; Guardone, L.; Franzoni, G.; Cappelli, K.; Razzuoli, E. Toll-like Receptors and Cytokine Modulation by Goat Milk Extracellular Vesicles in a Model of Intestinal Inflammation. *Int. J. Mol. Sci.* **2023**, *24*, 11096. <https://doi.org/10.3390/ijms241311096>.
53. Mariani, V.; Palermo, S.; Fiorentini, S.; Lanubile, A.; Giuffra, E. Gene Expression Study of Two Widely Used Pig Intestinal Epithelial Cell Lines: IPEC-J2 and IPI-2I. *Vet. Immunol. Immunopathol.* **2009**, *131*, 278–284. <https://doi.org/10.1016/j.vetimm.2009.04.006>.
54. Schierack, P.; Nordhoff, M.; Pollmann, M.; Weyrauch, K.D.; Amasheh, S.; Lodemann, U.; Jores, J.; Tachu, B.; Kleta, S.; Blikslager, A.; et al. Characterization of a Porcine Intestinal Epithelial Cell Line for in Vitro Studies of Microbial Pathogenesis in Swine. *Histochem. Cell Biol.* **2006**, *125*, 293–305. <https://doi.org/10.1007/s00418-005-0067-z>.
55. Hill, D.A.; Artis, D. Intestinal Bacteria and the Regulation of Immune Cell Homeostasis. *Annu. Rev. Immunol.* **2010**, *28*, 623–667. <https://doi.org/10.1146/annurev-immunol-030409-101330>.
56. Dejban, P.; Nikravangolsefid, N.; Chamanara, M.; Dehpour, A.; Rashidian, A. The Role of Medicinal Products in the Treatment of Inflammatory Bowel Diseases (IBD) through Inhibition of TLR4/NF-kappaB Pathway. *Phytother. Res. PTR* **2021**, *35*, 835–845. <https://doi.org/10.1002/ptr.6866>.
57. Okumura, R.; Takeda, K. Roles of Intestinal Epithelial Cells in the Maintenance of Gut Homeostasis. *Exp. Mol. Med.* **2017**, *49*, e338. <https://doi.org/10.1038/emm.2017.20>.
58. Scharek, L.; Tedin, K. The Porcine Immune System—Differences Compared to Man and Mouse and Possible Consequences for Infections by *Salmonella* Serovars. *Berl. Munch. Tierarztl. Wochenschr.* **2007**, *120*, 347–354. <https://doi.org/10.2376/0005-9366-120-347>.
59. Skjolaas, K.A.; Burkey, T.E.; Dritz, S.S.; Minton, J.E. Effects of *Salmonella enterica* Serovars Typhimurium (ST) and Choleraesuis (SC) on Chemokine and Cytokine Expression in Swine Ileum and Jejunal Epithelial Cells. *Vet. Immunol. Immunopathol.* **2006**, *111*, 199–209. <https://doi.org/10.1016/j.vetimm.2006.01.002>.
60. Støy, A.C.F.; Heegaard, P.M.H.; Sangild, P.T.; Østergaard, M.V.; Skovgaard, K. Gene Expression Analysis of the IPEC-J2 Cell Line: A Simple Model for the Inflammation-Sensitive Preterm Intestine. *Int. Sch. Res. Not.* **2013**, *2013*, 980651. <https://doi.org/10.1155/2013/980651>.
61. Marks, H.; Grześkowiak, Ł.; Martinez-Vallespin, B.; Dietz, H.; Zentek, J. Porcine and Chicken Intestinal Epithelial Cell Models for Screening Phytogetic Feed Additives—Chances and Limitations in Use as Alternatives to Feeding Trials. *Microorganisms* **2022**, *10*, 629. <https://doi.org/10.3390/microorganisms10030629>.
62. Hejna, M.; Kovanda, L.; Rossi, L.; Liu, Y. Mint Oils: In Vitro Ability to Perform Anti-Inflammatory, Antioxidant, and Antimicrobial Activities and to Enhance Intestinal Barrier Integrity. *Antioxidants* **2021**, *10*, 1004. <https://doi.org/10.3390/antiox10071004>.
63. Li, E.; Horn, N.; Ajuwon, K.M. Mechanisms of Deoxynivalenol-Induced Endocytosis and Degradation of Tight Junction Proteins in Jejunal IPEC-J2 Cells Involve Selective Activation of the MAPK Pathways. *Arch. Toxicol.* **2021**, *95*, 2065–2079. <https://doi.org/10.1007/s00204-021-03044-w>.

64. Ling, K.-H.; Wan, M.L.Y.; El-Nezami, H.; Wang, M. Protective Capacity of Resveratrol, a Natural Polyphenolic Compound, against Deoxynivalenol-Induced Intestinal Barrier Dysfunction and Bacterial Translocation. *Chem. Res. Toxicol.* **2016**, *29*, 823–833. <https://doi.org/10.1021/acs.chemrestox.6b00001>.
65. Pomothly, J.M.; Barna, R.F.; Pászti, E.A.; Babiczky, Á.; Szóládi, Á.; Jerzsele, Á.; Gere, E.P. Beneficial Effects of Rosmarinic Acid on IPEC-J2 Cells Exposed to the Combination of Deoxynivalenol and T-2 Toxin. *Mediat. Inflamm.* **2020**, *2020*, 8880651. <https://doi.org/10.1155/2020/8880651>.
66. Singleton, V.L.; Rossi, J.A. Colorimetry of Total Phenolics with Phosphomolybdic-Phosphotungstic Acid Reagents. *Am. J. Enol. Vitic.* **1965**, *16*, 144–158. <https://doi.org/10.5344/ajev.1965.16.3.144>.
67. International Olive Council. Determination of Biophenols in Olive Oils by HPLC. Available online: <https://www.internationaloliveoil.org/wp-content/uploads/2022/10/COI-T20-Doc.-29-REV-1-2017-Eng.pdf> (accessed on 21 December 2023).
68. Livak, K.J.; Schmittgen, T.D. Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the $2^{-\Delta\Delta CT}$ Method. *Methods* **2001**, *25*, 402–408. <https://doi.org/10.1006/meth.2001.1262>.
69. Razzuoli, E.; Vencia, W.; Modesto, P.; Franzoni, G.; Giudici, S.D.; Parisi, E.; Ferrari, A.; Amadori, M. *Yersinia enterocolitica*-Specific Modulation of Innate Immune Responses in Jejunal Epithelial Cells. *Vet. Microbiol.* **2020**, *242*, 108596. <https://doi.org/10.1016/j.vetmic.2020.108596>.
70. Pistol, G.C.; Bulgaru, C.V.; Marin, D.E.; Oancea, A.G.; Taranu, I. Dietary Grape Seed Meal Bioactive Compounds Alleviate Epithelial Dysfunctions and Attenuates Inflammation in Colon of DSS-Treated Piglets. *Foods* **2021**, *10*, 530. <https://doi.org/10.3390/foods10030530>.
71. Li, T.; Li, F.; Liu, X.; Liu, J.; Li, D. Synergistic Anti-Inflammatory Effects of Quercetin and Catechin via Inhibiting Activation of TLR4–MyD88-Mediated NF- κ B and MAPK Signaling Pathways. *Phytother. Res.* **2019**, *33*, 756–767. <https://doi.org/10.1002/ptr.6268>.
72. Sahoo, M.; Ceballos-Olvera, I.; del Barrio, L.; Re, F. Role of the Inflammasome, IL-1 β , and IL-18 in Bacterial Infections. *Sci. World J.* **2011**, *11*, 2037–2050. <https://doi.org/10.1100/2011/212680>.
73. Dinarello, C.A. Overview of the IL-1 Family in Innate Inflammation and Acquired Immunity. *Immunol. Rev.* **2018**, *281*, 8–27. <https://doi.org/10.1111/imr.12621>.
74. Kaneko, N.; Kurata, M.; Yamamoto, T.; Morikawa, S.; Masumoto, J. The Role of Interleukin-1 in General Pathology. *Inflamm. Regen.* **2019**, *39*, 12. <https://doi.org/10.1186/s41232-019-0101-5>.
75. Olcum, M.; Tastan, B.; Ercan, I.; Eltutan, I.B.; Genc, S. Inhibitory Effects of Phytochemicals on NLRP3 Inflammasome Activation: A Review. *Phytomedicine* **2020**, *75*, 153238. <https://doi.org/10.1016/j.phymed.2020.153238>.
76. Serrano-López, J.; Martín-Antonio, B. Inflammaging, an Imbalanced Immune Response that Needs to Be Restored for Cancer Prevention and Treatment in the Elderly. *Cells* **2021**, *10*, 2562. <https://doi.org/10.3390/cells10102562>.
77. Wang, T.; Xu, H.; Dong, R.; Wu, S.; Guo, Y.; Wang, D. Effectiveness of Targeting the NLRP3 Inflammasome by Using Natural Polyphenols: A Systematic Review of Implications on Health Effects. *Food Res. Int.* **2023**, *165*, 112567. <https://doi.org/10.1016/j.foodres.2023.112567>.
78. Xiao, Y.-T.; Yan, W.-H.; Cao, Y.; Yan, J.-K.; Cai, W. Neutralization of IL-6 and TNF- α Ameliorates Intestinal Permeability in DSS-Induced Colitis. *Cytokine* **2016**, *83*, 189–192. <https://doi.org/10.1016/j.cyto.2016.04.012>.
79. Franchi, L. Role of Inflammasomes in *Salmonella* Infection. *Front. Microbiol.* **2011**, *2*, 8. <https://doi.org/10.3389/fmicb.2011.00008>.
80. Kelley, N.; Jeltema, D.; Duan, Y.; He, Y. The NLRP3 Inflammasome: An Overview of Mechanisms of Activation and Regulation. *Int. J. Mol. Sci.* **2019**, *20*, 3328. <https://doi.org/10.3390/ijms20133328>.

81. Gessner, D.K.; Ringseis, R.; Siebers, M.; Keller, J.; Kloster, J.; Wen, G.; Eder, K. Inhibition of the Pro-Inflammatory NF- κ B Pathway by a Grape Seed and Grape Marc Meal Extract in Intestinal Epithelial Cells. *J. Anim. Physiol. Anim. Nutr.* **2012**, *96*, 1074–1083. <https://doi.org/10.1111/j.1439-0396.2011.01222.x>.
82. González, R.; Ballester, I.; López-Posadas, R.; Suárez, M.D.; Zarzuelo, A.; Martínez-Augustin, O.; Medina, F.S.D. Effects of Flavonoids and Other Polyphenols on Inflammation. *Crit. Rev. Food Sci. Nutr.* **2011**, *51*, 331–362. <https://doi.org/10.1080/10408390903584094>.
83. Gupta, S.C.; Tyagi, A.K.; Deshmukh-Taskar, P.; Hinojosa, M.; Prasad, S.; Aggarwal, B.B. Downregulation of Tumor Necrosis Factor and Other Proinflammatory Biomarkers by Polyphenols. *Arch. Biochem. Biophys.* **2014**, *559*, 91–99. <https://doi.org/10.1016/j.abb.2014.06.006>.
84. Martín, A.R.; Villegas, I.; La Casa, C.; de la Lastra, C.A. Resveratrol, a Polyphenol Found in Grapes, Suppresses Oxidative Damage and Stimulates Apoptosis during Early Colonic Inflammation in Rats. *Biochem. Pharmacol.* **2004**, *67*, 1399–1410. <https://doi.org/10.1016/j.bcp.2003.12.024>.
85. Camuesco, D.; Comalada, M.; Concha, A.; Nieto, A.; Sierra, S.; Xaus, J.; Zarzuelo, A.; Gálvez, J. Intestinal Anti-Inflammatory Activity of Combined Quercitrin and Dietary Olive Oil Supplemented with Fish Oil, Rich in EPA and DHA (n-3) Polyunsaturated Fatty Acids, in Rats with DSS-Induced Colitis. *Clin. Nutr.* **2006**, *25*, 466–476. <https://doi.org/10.1016/j.clnu.2005.12.009>.
86. Salh, B.; Assi, K.; Templeman, V.; Parhar, K.; Owen, D.; Gómez-Muñoz, A.; Jacobson, K. Curcumin Attenuates DNB-Induced Murine Colitis. *Am. J. Physiol.-Gastrointest. Liver Physiol.* **2003**, *285*, G235–G243. <https://doi.org/10.1152/ajpgi.00449.2002>.
87. Taranu, I.; Habeanu, M.; Gras, M.A.; Pistol, G.C.; Lefter, N.; Palade, M.; Ropota, M.; Chedea, V.S.; Marin, D.E. Assessment of the Effect of Grape Seed Cake Inclusion in the Diet of Healthy Fattening-Finishing Pigs. *J. Anim. Physiol. Anim. Nutr.* **2018**, *102*, e30–e42. <https://doi.org/10.1111/jpn.12697>.
88. Britti, D.; Impellizzeri, D.; Procopio, A.; Cuzzocrea, S.; Britti, D.; Impellizzeri, D.; Procopio, A.; Cuzzocrea, S. Oleuropein an Olive Oil Compound in Acute and Chronic Inflammation Models: Facts and Perspectives. In *Olive Germplasm—The Olive Cultivation, Table Olive and Olive Oil Industry in Italy*; IntechOpen: London, UK, 2012; ISBN 978-953-51-0883-2.
89. Letterio, J.J.; Roberts, A.B. Regulation of Immune Responses by TGF-Beta. *Annu. Rev. Immunol.* **1998**, *16*, 137–161. <https://doi.org/10.1146/annurev.immunol.16.1.137>.
90. Qin, G.; Zhao, Y.; Gan, Y.; Yu, X.; Zhao, Y.; Peng, H.; Fang, S. Alterations in Gene Expressions of Caco-2 Cell Responses to LPS and Ploy(I:C) Stimulation. *PeerJ* **2023**, *11*, e15459. <https://doi.org/10.7717/peerj.15459>.
91. Huang, T.; Huang, X.; Shi, B.; Wang, F.; Feng, W.; Yao, M. Regulators of Salmonella-Host Interaction Identified by Peripheral Blood Transcriptome Profiling: Roles of TGFB1 and TRP53 in Intracellular *Salmonella* Replication in Pigs. *Vet. Res.* **2018**, *49*, 121. <https://doi.org/10.1186/s13567-018-0616-9>.
92. Howe, K.L.; Reardon, C.; Wang, A.; Nazli, A.; McKay, D.M. Transforming Growth Factor- β Regulation of Epithelial Tight Junction Proteins Enhances Barrier Function and Blocks Enterohemorrhagic *Escherichia coli* O157:H7-Induced Increased Permeability. *Am. J. Pathol.* **2005**, *167*, 1587–1597. [https://doi.org/10.1016/S0002-9440\(10\)61243-6](https://doi.org/10.1016/S0002-9440(10)61243-6).
93. Nallathambi, R.; Poulev, A.; Zuk, J.B.; Raskin, I. Proanthocyanidin-Rich Grape Seed Extract Reduces Inflammation and Oxidative Stress and Restores Tight Junction Barrier Function in Caco-2 Colon Cells. *Nutrients* **2020**, *12*, 1623. <https://doi.org/10.3390/nu12061623>.
94. Laurindo, L.F.; Santos, A.R. de O. dos; Carvalho, A.C.A. de; Bechara, M.D.; Guiguer, E.L.; Goulart, R. de A.; Vargas Sinatora, R.; Araújo, A.C.; Barbalho, S.M. Phytochemicals and Regulation of NF- κ B in Inflammatory Bowel Diseases: An Overview of In Vitro and In Vivo Effects. *Metabolites* **2023**, *13*, 96. <https://doi.org/10.3390/metabo13010096>.
95. Mecocci, S.; De Paolis, L.; Zoccola, R.; Fruscione, F.; De Ciucis, C.G.; Chiaradia, E.; Moccia, V.; Tognoloni, A.; Pascucci, L.; Zoppi, S.; et al. Antimicrobial and Immunomodulatory

Potential of Cow Colostrum Extracellular Vesicles (ColosEVs) in an Intestinal In Vitro Model.
Biomedicines **2022**, *10*, 3264. <https://doi.org/10.3390/biomedicines10123264>.

CHAPTER 3

Gastrointestinal health evaluation through amplicon-based metagenomics, histopathology and morphometry in finishing pigs fed an olive mill wastewater polyphenol extract

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Gastrointestinal health evaluation through amplicon-based metagenomics, histopathology and morphometry in finishing pigs fed an olive mill wastewater polyphenol extract

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Abstract

Pig microbiota influences overall animal health, and changes to the diet can modify gut microbiota composition and animal growth rate, bringing about environmental and economic advantages. Dietary polyphenols, due to their antimicrobial and antioxidant properties, may improve gut health. However, their use in monogastric diets is still emerging. The present study investigated the effects of polyphenols derived from an olive mill wastewater (OMWW) extract on gastrointestinal histopathology and morphometry and intestinal microbiota composition. One hundred thirty-five female Landrace × Duroc finishing pigs were randomly allocated to three experimental groups fed: a control (C) diet, or C diet supplemented with 74 ppm (P-LOW) or 225 ppm of OMWW polyphenols (P-HIGH). The experiment lasted 85 days, after which 15 animals per group were slaughtered. Final body weight was 148.1 ± 4.6 kg. The morphological analysis of the intestine demonstrated a significant increase in the villus height:crypt depth (VH/CD) ratio in the ileum of pigs from P-LOW and P-HIGH groups and in the jejunum of pigs from P-HIGH group vs C. 16S amplicon sequencing analysis showed an increase in two indexes of alpha diversity in the cecum of pigs from P-HIGH vs P-LOW. Differential abundance analysis showed that pigs from P-HIGH had a greater abundance of beneficial bacteria, especially *Eubacterium* and *Treponema*, and fewer harmful ones, including *Fusobacterium*, *Bacteroides helcogenes*, and *Corynebacterium urealiticum*. The results suggest that OMWW polyphenols may positively influence the pig gut microbiota and overall intestinal health. Further studies are needed to confirm these data.

Keywords: gut health, amplicon-based metagenomics, olive polyphenols, swine.

HIGHLIGHTS

- Olive mill wastewater (OMWW) extract contains a high concentration of 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) has led to several challenges for producers related to feed costs and the maintenance of growth performance and animal health status (Vasquez et al. 2022). Today's pig production sector demands the reduction of antibiotic use to avoid antimicrobial resistance and to promote animal welfare (Lessard et al. 2023; Papakonstantinou et al. 2023). In this context, phytogetic feed additives (PFAs) are considered potential alternatives to antibiotics for their antimicrobial actions (Papakonstantinou et al. 2023), and their antioxidant activities has been the subject of much research (Liu et al. 2021; Papatsiros et al. 2022; Papakonstantinou et al. 2023).

Diet, genetic factors, and intestinal microbial composition are all critical aspects affecting feed efficiency (FE) and utilization (Cheng et al. 2023). The pig intestinal microbiota exhibits high variability in bacterial species and plays 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, a novel high-throughput sequencing approach, can be used to investigate the relationship between intestinal bacteria and host characteristics (Mun et al. 2021). It has been employed to obtain distinct profiles 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 was recently studied using DNA sequencing techniques (Gardiner et al. 2020).

Dietary inclusion of bioactive compounds is currently considered one of the most efficient feeding interventions to targeting the gut microbiota, especially in growing pigs (Le Sciellour et al. 2018), due to their capacity to bring about beneficial changes to microbiota composition and, consequently, nutrient digestion, improving 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 use of agro-industrial by-products as supplements in animal diets is a novel strategy that contributes to lessening the impact of economic and environmental issues through the principles of circular economy (Altmann et al. 2018; Altmann et al. 2019). Of the natural feed additives recently subjected to investigation, olive oil by-products are of particular interest due to their being an efficient source of polyphenols. These by-products have higher concentrations of bioactive compounds compared with whole olive fruit (Silvan and Martinez-Rodriguez 2019; Altissimi et al. 2024). In particular, olive mill wastewater (OMWW)-a liquid waste produced by the olive milling industry- is particularly rich in phenolic compounds 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 livestock growth performances, gut microbiota, and overall animal health, potentially helping to decrease antibiotic use in several species (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 by stimulating the growth of beneficial intestinal bacteria and inhibiting that of pathogenic bacterial 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 been demonstrated to restore gut health through their anti-inflammatory action and modulation of the immune response (Selma et al. 2009; Zhang et al. 2020). Finally, it is known that microbiota interact with polyphenols, leading to the formation of beneficial bioactive compounds (metabolites such as phenolic acids and urolithins) (Luo et al. 2022). Indeed, previous studies have demonstrated 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 microbial 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 microbiota composition have never been analyzed to date. Therefore, this study assessed the effects of dietary supplementation with OMWW polyphenols in finishing pig diets on gut morphology, histopathological findings, and intestinal microbiota composition.

2. Materials and methods

2.1 Olive mill wastewater extract phenol content

OMWW extract was provided in powder form by Stymon Natural products, P.C., Patras, Greece (www.stymon.com). Total phenol content (TPC) was determined through 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 the results were expressed as gallic acid equivalents (mg GAE/kg). The total polyphenol content of OMWW extract was 36.99 ± 58.9 g/kg, whereas the content of hydroxytyrosol, tyrosol, and oleuropein were 9.39 ± 17.4 g/kg, 1.09 ± 3.2 g/kg, and 0.59 ± 0.4 g/kg, respectively.

2.2 RP-HPLC-DAD analysis of polyphenols

The hydroxytyrosol, tyrosol, and oleuropein content in OMWW extract were determined by reversed-phase high-performance liquid chromatography with diode array detector (RP-HPLC-DAD), according to Kyriakoudi et al. (Kyriakoudi et al. 2024). The RP-HPLC-DAD 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 conducted on a InfinityLab Poroshell 120 EC-C18 $4\mu\text{m}$ (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 follows: 0 min, 5% (B), 0-10 min, 20% (B); 10-15 min, 30% (B); 15-18 min, 30% (B); 18-20 min, 50% (B); 20-21 min, 100% (B); 21-25 min, 5% (B). The total run time was 25 min with a flow rate of 1 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 OpenLab CDS software, version 3.5 (2021, Agilent Technologies, Santa Clara, CA, USA). Peak identification was based on retention times and spectral characteristics (absorption maxima) with those of the available standards.

2.3 Animal welfare and ethic statement

The experiment was approved by the Bioethics Committee of the University of Perugia (protocol n. 399480) and performed in accordance with the ARRIVE guidelines. 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 conventional farm for pig production located in the region of Umbria, Italy. One hundred thirty-five female Landrace \times Duroc finishing pigs (body weight: 102.7 ± 6.8 kg), were randomly divided into three groups (three pens for each group, 15 pigs per pen) after 15 days of adaptation. The three groups were distinguished by the diet they received: a) control diet (C) currently used on the farm for the early finishing period (a commercial mash-form diet); b) C diet, supplemented with 74 ppm of polyphenols from OMWW (P-LOW diet); c) C diet, supplemented with 225 ppm of polyphenols from OMWW (P-HIGH diet). The phenolic dosages were according to previous studies (Rossi et al. 2009; Varricchio et al. 2019) in which the antioxidant activity of OMWW and other natural extracts containing polyphenols were evaluated. The powdered OMWW polyphenol extract was diluted in water (15 L of water per box) then mixed with the feed (2.8 kg/pig) such that the pigs in the P-LOW group received 210 mg polyphenols/day and those in the P-HIGH group received 630 mg polyphenols/day. The feed was supplied to pigs using an automated wet feeding system twice a day, and fresh water was provided *ad libitum*. The trial lasted 85 days. The composition and nutritional characteristics of the C diet are indicated in Table 1.

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

<i>Raw materials</i>	<i>% as fed basis</i>
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
<i>Analyzed nutrients</i>	<i>g/100 g</i>
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. ^a Mineral-vitamin supplement (mg or units/kg) : 216700 UI Vitamin A; 65.000 UI Vitamin D3; 4000 mg Vitamin K3; 86 mg Vitamin K3; 50 mg Vitamin B1; 160 mg Vitamin B2; 450 mg calcium D-pantothenate; 100 mg Vitamin B6; 1 mg Vitamin B12; 800 mg Vitamin B3; 5 mg Biotin; 40 mg Vitamin B9; 15000 mg Cholin chloride; 1.500 mg iron carbonate; 1500 mg iron(II) sulfate monohydrate; 50 mg calcium iodate; 1500 mg manganese oxide; 500 mg copper(II) sulfate pentahydrate; 8 mg sodium selenite; 3 mg selenomethionine hydroxylate; 1000 mg zinc oxide; 13000 methionin; 58000 mg L-lysine monohydrochloride; 16500 mg L-threonine; 18700 TXU endo-1,4-beta-xylanase; 16000 FTU endo-1,4-beta-glucanase; 16000 FTU 6-Phytase.

2.5 Feed analysis

Crude protein, crude fiber, ether extracts, ash, calcium, phosphorous concentrations, moisture, and starch were determined according to AOAC procedures (AOAC: Official Methods of Analysis (Volume 1). Analysis of the neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) was performed following the methods of Van Soest, Robertson, and Lewis (Van Soest et al. 1991).

2.6 Growth performance

Individual body weights (BW) were recorded at the beginning and the end of the trial. Average daily feed intake (ADFI) was calculated by measuring the total amount of feed consumed divided by the number of the days of the trial.

2.7 Sample collection

Feces were collected randomly from five pigs per pen at three sampling times: days (d) 0, 44,

and 85. Before testing the samples for the presence of intestinal parasites, fecal firmness and appearance were assessed and found to be similar across the experimental groups. Therefore, no scoring was assigned.

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 in accordance with European Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing. After slaughtering, the carcasses were weighed and stomach, jejunum, ileum, and colon were macroscopically inspected, sectioned, and fixed in formalin. For 16S amplicon sequencing, samples were collected by opening jejunum and ileum segments and taking 20–30 ml of well-homogenized intestinal contents. Jejunal and ileal contents were immediately frozen in dry ice and stored at -80°C until further processing.

2.8 Histopathological and morphometric findings

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), and the Kopinski and McKenzie scoring system (Kopinski and McKenzie 2007) was used to assess the grade of ulceration (from 0 to 3). Swabs from the pars oesophagea of the stomach were collected from all pigs to investigate the presence of *Helicobacter suis* and *H. pylori*. *H. suis* was examined due to its relevant etiological association with porcine gastric ulcers (De Witte et al. 2018), and due to its significance as a zoonotic agent (Haesebrouck et al. 2009), alongside *H. pylori*. DNA was extracted from the swabs using HiPurA® Multi-Sample DNA Purification (HiMedia, Germany) according to the manufacturer's protocol. PCR reactions for *H. suis* and *H. pylori* were performed in a final volume of 50 µl made up of: 25 µl of complete Taq 2x PCR Master Mix (ABM, Canada), 1 µl of forward and reverse primer (10 µM), and 200 ng of genomic DNA using a GeneAmp PCR System 9700 thermal cycler (Applied Biosystems, USA). The thermal cycling protocols and primers are reported in previous literature (Proietti et al. 2010). A 10 µl aliquot of PCR products was resolved in an ethidium bromide-stained 1.5% agarose gel and examined by transillumination.

Tissue samples from the stomach (transition between pars esophagea and cardiac gland region), jejunum (mid-jejunum), ileum (15 cm from the inlet in the colon) and colon (distal colon, 50 cm distal from anus) were collected and fixed in 10%-buffered formalin for 24 h. Samples were processed following standard protocols, and 3 µm tissue sections were obtained and stained with routine Hematoxylin and Eosin stain. Histological analysis on these sections was performed on an optic light microscope (Olympus BX50), and assessed for leukocyte infiltration, grading severity on a scale of 0 to 3 based on the percentage of affected tissue (where 0 = no tissue affected, 1 = less than 20%, 2 = 21–60%, and 3 = more than 61% affected). The same scale was also used to evaluate tissue epithelial degeneration, epithelial necrosis, fibrosis of the lamina propria and epithelial (for the stomach) and gastrointestinal-associated-lymphoid tissue (GALT) hyperplasia across all the examined segments of the GI tract (jejunum, ileum, colon). 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 analyzing 5 villi at 10× magnification (×100) and averaging the measurements. VH was measured from the apex to the base of the villus, and CD from the base of the villus to the base of the crypt (Supplementary Figure 1). The VH/CD ratio was calculated for both the jejunum and ileum.

2.9 Sequencing of 16S rRNA amplicons

2.9.1 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 analysis (final number of samples kept for analysis: 9 C, 11 P-LOW, and 9 P-HIGH). After thawing, total DNA was extracted from 350 mg of each sample of cecal and jejunal content using the DNeasy PowerSoil Pro Kit (Qiagen, Maryland, USA). After the sample was loaded into the PowerBead Pro tube, the CD1 solution was added. Then, samples were vortexed at maximum speed for 20 min using a Vortex Adapter for 24 tubes (1.5-2.0 ml), and DNA was isolated following the manufacturer's protocol. DNA was quantified using a NanoDrop 2000 spectrophotometer (Thermo Scientific, Waltham, USA). Amplicon sequencing of the 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 a NovaSeq6000 instrument (Illumina, San Diego, CA) using the 250-bp paired-end mode.

2.9.2 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 analysis was 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 the 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 using 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 using the ggplot2 package in R (Wickham 2016).

2.10 Statistical analysis

A two-way ANOVA model was applied, with the diet (C, P-LOW and P-HIGH) and pens as fixed variables, to analyze differences in pig body weights (BW).

Regarding the morphological and histopathological examinations, a Fisher's exact test was performed to evaluate the associations between binomial variables (yes/no) and the experimental groups. Differences in various histological parameters across groups were evaluated using the Kruskal-Wallis test. To test for differences between groups in the VH/CD ratio in the jejunum and ileum, a one-way ANOVA was performed. In case of significant differences between the groups, a Tukey's HSD test was applied for pairwise comparisons. All the statistical analyses were performed on Rstudio (2023.12.0+369 version). A chi-square test is used to compare the detection of *Helicobacter* between groups and associate the results to the macroscopic and histological findings in the pig stomach (macroscopic ulceration, leukocytes' infiltration, lamina propria fibrosis, and epithelial hyperplasia). Statistical significance was set at $p < 0.05$.

3. Results

3.1 Growth performance and animal health

Body weights were in accordance with the standard weights for the pigs and there were no statistically significant differences between the experimental groups. The average initial and final body weights were 102.7 ± 6.8 kg and 148.1 ± 4.6 kg, respectively. Feed intake was the same for all the experimental groups, as the pigs always ingested the entire amount of feed provided throughout the trial. All fecal samples taken during the experiment were negative for intestinal parasites and the consistency of the feces was uniform across the experimental groups. Furthermore, with the exception of a single pig, no episodes of diarrhea are recorded during the whole experimental period.

3.2 Histopathological and morphometric findings

Gastric ulceration was recorded macroscopically for all experimental groups. A lower frequency of gastric ulcerations was observed in both the P-LOW ($n = 7/14$) and P-HIGH ($n = 5/11$) groups compared with C ($n = 11/14$). However, the association between gastric ulceration and experimental group was not significant ($p > 0.05$). All samples were negative for *H. pylori*, whereas *H. suis* was identified in 53.3%, 46.7%, and 63.6% of the animals in the C, P-LOW, and P-HIGH group, respectively. There was no statistically significant difference in *H. suis* detection between groups ($p > 0.05$). Moreover, there was no statistically significant association between the presence of *H. suis* with any of the pathological parameters evaluated for the stomach (ulceration, leukocyte infiltration, fibrosis of lamina propria, and epithelial hyperplasia). No significant differences were found between experimental groups in the histological parameters assessed in the stomach (grade of gastric ulceration), jejunum, ileum, and colon (tissue epithelial degeneration, epithelial necrosis, GALT hyperplasia, fibrosis of the lamina propria, and leukocyte infiltration) ($p > 0.05$). All data regarding the histopathological assessments are reported in Supplementary Table 1.

Regarding the morphometric analysis, mean values of VH, CD, and VH/CD ratio for the three groups are reported in Table 2. The VH/CD ratio for the jejunal was found to be significant different between groups: $F(2,36) = 3.487$, $p < 0.05$ (Figure 1A). The post-hoc comparisons (Tukey HSD test) indicated that the mean differences between P-LOW vs C and between P-HIGH vs P-LOW were not significant ($p > 0.05$), whereas the mean difference between P-HIGH vs Group C (0.671) was significant (95% CI, 0.014–1.328; $p < 0.05$). The VH/CD ratio was also found to be significantly different between groups for the ileum: $F(2,36) = 9.323$, $p < 0.001$ (Figure 1B). The post-hoc comparisons (Tukey HSD test) indicated the following group differences: a significant ($p < 0.01$) mean difference (0.949) between P-LOW and C (95% CI, 0.221 to 1.676), a significant ($p < 0.001$) mean difference (1.291) between P-HIGH and C (95% CI, 0.515 to 2.066). The mean difference between P-HIGH vs 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. Villous height (VH), crypt dept (CD), and VH/CD ratio in the jejunum and ileum of pigs receiving the C (control), P-LOW and P-HIGH OMWW phenolic diets.

	C	P-LOW	P-HIGH	p-value
Jejunum				
VH	$376.30 \pm 74.80 \mu\text{m}$	$417.05 \pm 52.80 \mu\text{m}$	$424.45 \pm 54.79 \mu\text{m}$	
CD	$152.96 \pm 38.88 \mu\text{m}$	$145.06 \pm 39.22 \mu\text{m}$	$135.73 \pm 29.17 \mu\text{m}$	
VH/CD	2.60	3.25	3.40	$p < 0.05$
Ileum				
VH	$264.73 \pm 47.19 \mu\text{m}$	$330.82 \pm 69.39 \mu\text{m}$	$310.48 \pm 41.99 \mu\text{m}$	
CD	$146.53 \pm 25.22 \mu\text{m}$	$123.33 \pm 26.46 \mu\text{m}$	$109.55 \pm 35.79 \mu\text{m}$	
VH/CD	1.70	2.67	3.26	$p < 0.001$

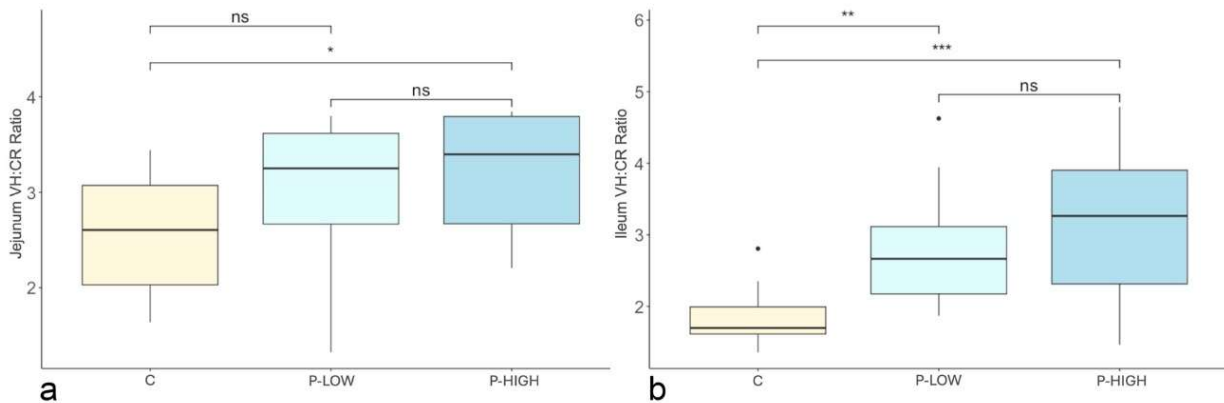


Figure 1. Boxplot summarizing jejunum (a) and ileum (b) villous height to crypt dept (VH/CD) ratio according to dietary treatment. *** $p < 0.001$, ** $p < 0.01$, * $p < 0.05$. Note: ns = not significant; VH= villous height; CD= crypt depth.

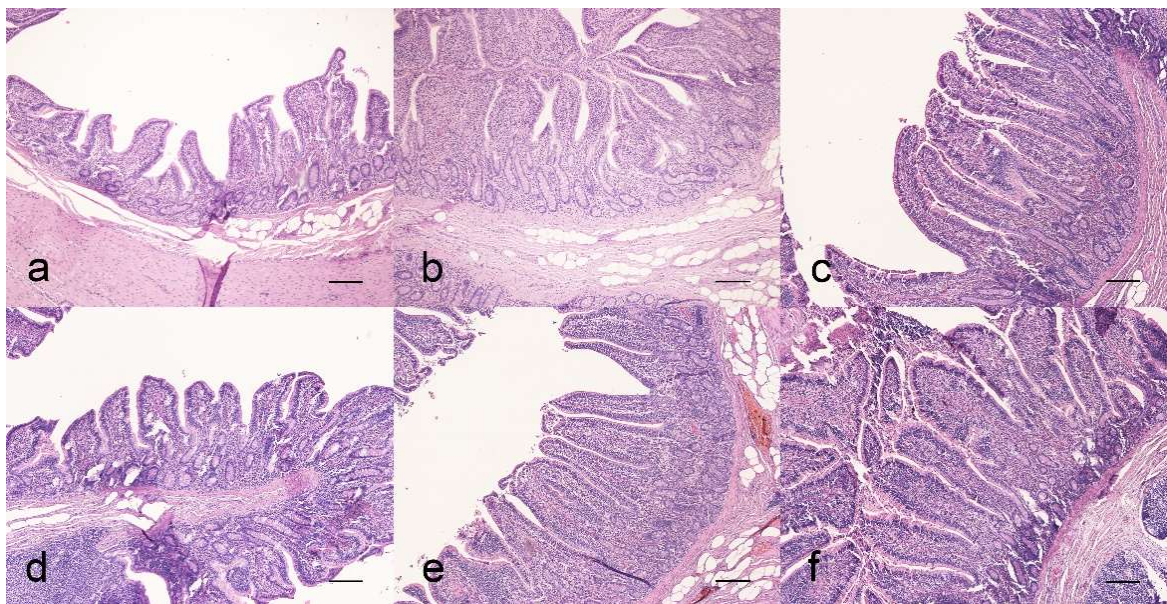


Figure 2. The panel presents histological sections (Hematoxylin and Eosin) of the jejunum (a-c) and ileum (d-f) (x100); scale bar = 100 μm . The jejunal mucosal architecture of sections from group C (a) shows short, intact villi and crypts. In Group P-LOW (b) and P-HIGH (c), the villi and crypts are notably longer, indicating visible changes. The villi in the ileum from group C animals (d) are shorter with deeper crypts compared with those from P-LOW (e) and P-HIGH (f), where the villi are longer and the crypts are shallower, reflecting differences in mucosal architecture across groups

3.3 Sequencing 16S rRNA amplicons

Regarding the microbial content of the cecum, metabarcoding library preparation for sequencing was carried out for all 45 subjects (15 C, 15 P-LOW, and 15 P-HIGH); however, unfortunately, the sequencing of 4 samples from the P-HIGH group failed. After each bioinformatic analysis step, over

141 K, on average, high-quality reads per sample (about 75% of raw sequences) (Supplementary Table 2). The histogram used for downstream analysis depicting the read depth of cleaned reads for each sample is reported in Supplementary Figure 2A. After filtering, 879 amplicon sequencing variants (ASV) were retrieved from the 6767 sequences. Rarefaction curves are reported in Supplementary Figure 2B, which allowed the best sample size to be chosen for rarefaction. The agglomeration step performed on rarefied data grouped these ASVs into 145 species, 112 genera, and 44 families. Figure 3A shows the top 15 most abundant genera and the relative families for each sample. The calculated relative abundances did not reveal any significant differences between the experimental groups at the genus and family level (Figure 3A).

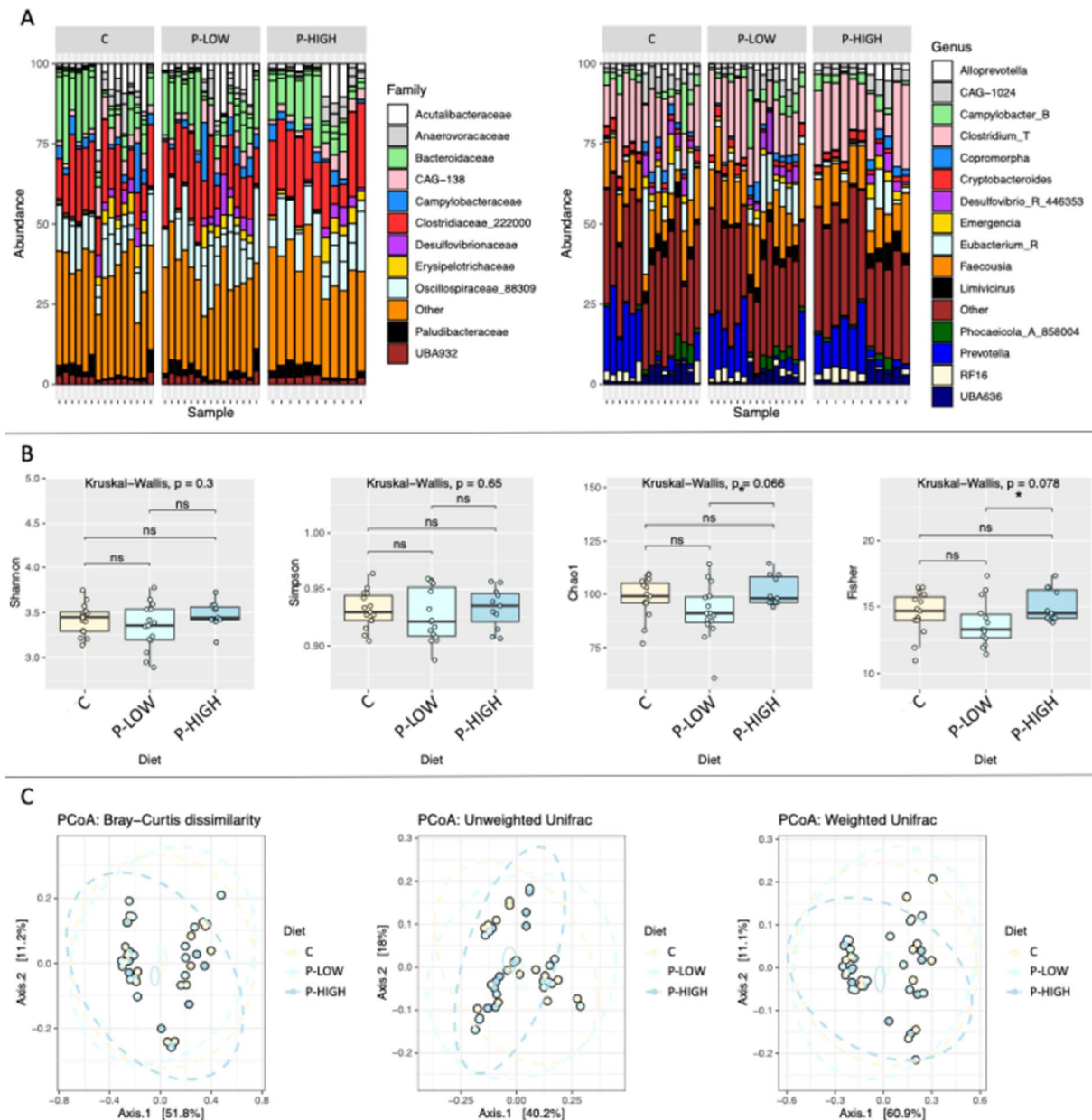


Figure 3. Cecal content characterization and differences between experimental groups (Control C, olive mill waste water phenolic diets P-LOW and P-HIGH). A) Relative abundances of the 15 families and genera. B) Alpha diversity indices (Shannon, Simpson, Chao1, and Fisher), testing relative statistical significance through Kruskal-Wallis test and Wilcox test for pairwise comparison;

(C) principal coordinate analysis graphs for Bray-Curtis dissimilarity index, unweighted UniFrac distance, and weighted UniFrac distance. *Note*: ns= not significant; * $p < 0.05$.

Regarding the 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 with the P-LOW group (Figure 3B). Concerning beta diversity, the phylogenetic tree used to calculate UniFrac distances is shown in Supplementary Figure 2C. Figure 3C showed that the samples from the three experimental groups did not form separate groups in relation to any of the indexes, indicating that the general microbial content of the cecum was similar across the three experimental groups. The results of PERMANOVA test confirmed the lack of any 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 and P-HIGH 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

Concerning the microbial content of the jejunum, metabarcoding sequencing was carried out for the 27 available samples (9 controls, 11 P-LOW, and 9 P-HIGH). On average, a total of 116 K high-quality reads were obtained per sample (71% of raw sequences), and the distribution was reported in Supplementary Figure 3A. Sequencing statistics are reported in Supplementary Table 3.

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 3B. Three samples from the P-LOW group (2T, 4T and 9T; Supplementary Table 2) 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 the relative families for each sample. No differences were found in all the alpha diversity indexes between the three experimental groups (Figure 4B) and in beta diversity (Figure 4C). Indeed, the plots indicate that samples from the three groups were quite homogeneous, and no statistically significant differences were found (Table 4). The phylogenetic tree used to calculate UniFrac distances is shown in Supplementary Figure 3C.

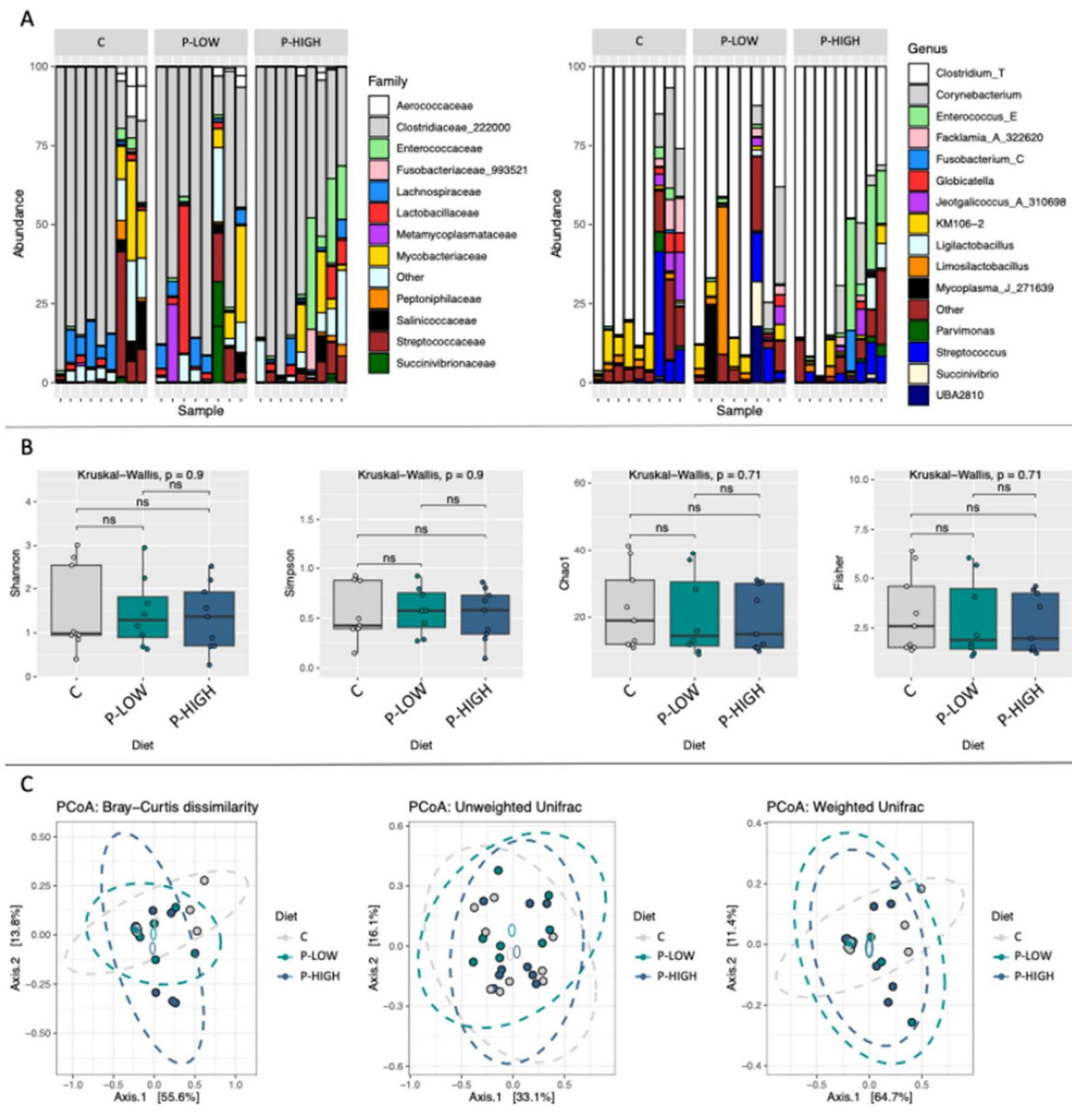


Figure 4. Jejunum content characterization and differences between experimental groups (Control C, olive mill waste water phenolic diets P-LOW and P-HIGH). A) Relative abundances of the 15 most common families and genera. B) Alpha diversity indices (Shannon, Simpson, Chao1 and Fisher), testing relative statistical significance through Kruskal Wallis test and Wilcox test for pairwise comparison; (C) Principal coordinate analysis graphs for Bray-Curtis dissimilarity index, unweighted UniFrac distance, and weighted UniFrac distance. *Note*: ns= not significant.

Table 4. Statical significance by PERMANOVA test for the beta diversity calculation among control (C) and treated groups P-LOW and P-HIGH 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

Finally, the differentially abundant (DA) genera and species in the cecal and jejunal contents are reported in Figures 5 and 6, respectively. Details for the cecum are reported in Supplementary Tables 4 and 5, and for the jejunum in Supplementary Tables 6 and 7, while relative DA families are reported in Supplementary Figures 4A and 4B for the cecal and jejunal substrates, respectively.

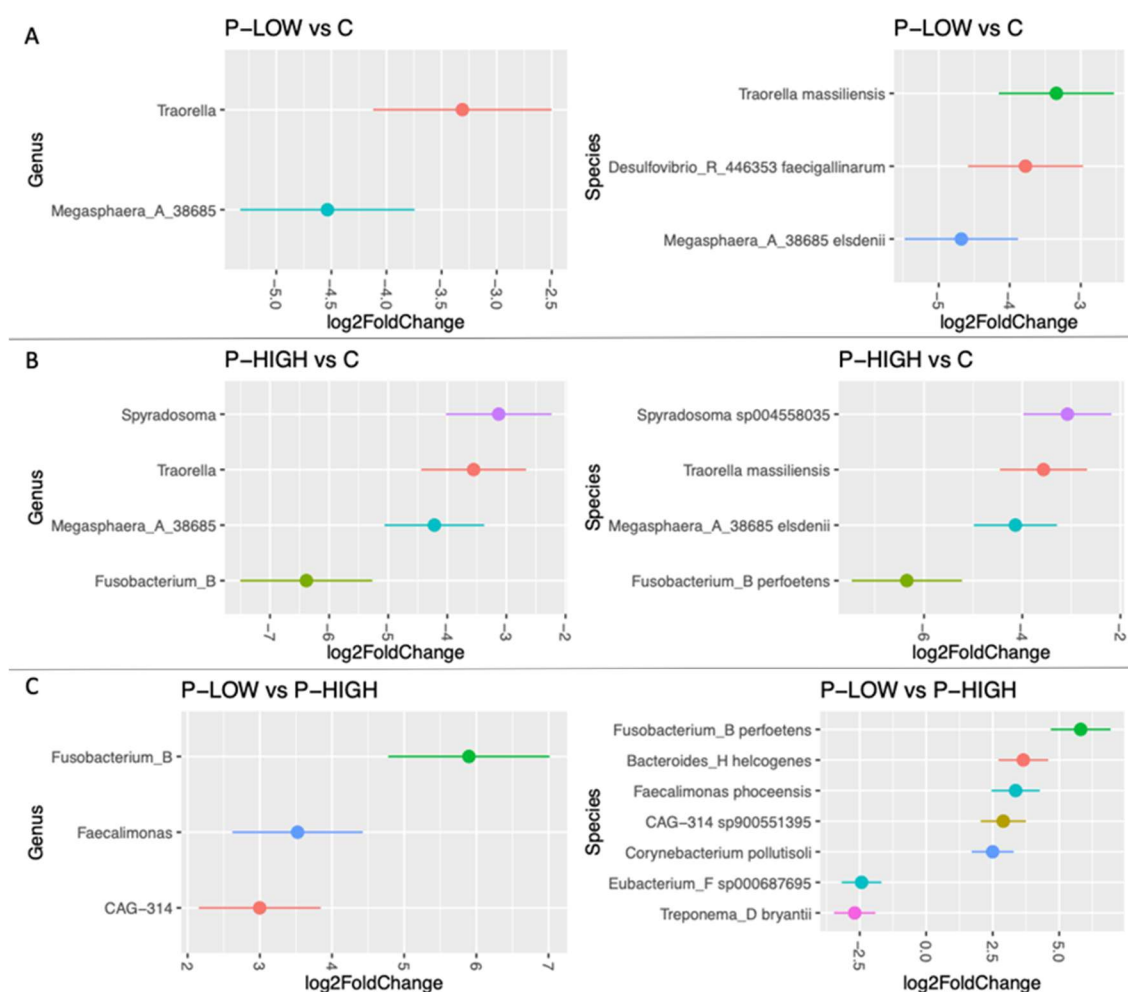


Figure 5. Differentially abundant (DA) genus and species identified in cecum contents from the three experimental groups (Control C, olive mill waste water phenolic diets P-LOW and P-HIGH). For each comparison, genera (left panel) and species (right panel), are shown with a multiple testing corrected p-value ≤ 0.05 . Statistically significant features (false discovery rate – FDR < 0.05; and log₂ 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 P-LOW. B) P-HIGH vs Control, taxa with a fold change greater than 0 are more abundant in P-HIGH. C) P-LOW vs P-HIGH, taxa with a fold change greater than 0 are more abundant in P-LOW. The colors of the dots indicate the same family, and bars show the standard error of the log fold change.

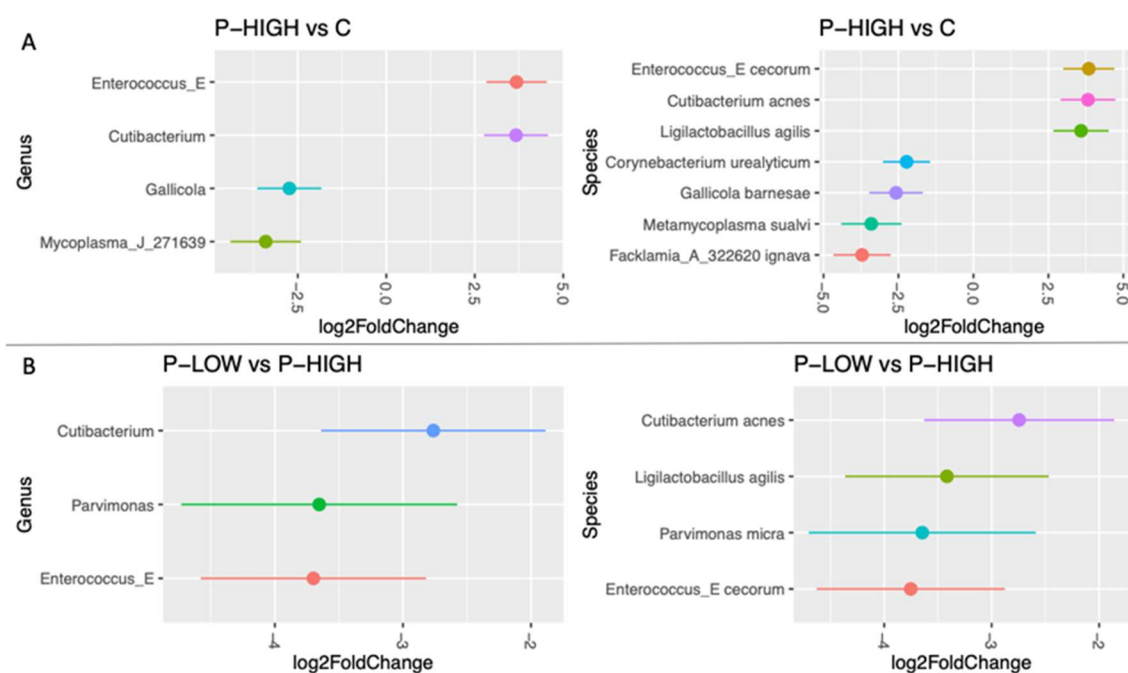


Figure 6. Differentially abundant (DA) genus and species identified in jejunum contents from the three experimental groups (Control C, olive mill waste water phenolic diets P-LOW and P-HIGH). Statistically significant features (false discovery rate – FDR < 0.05; and log₂ fold change - |log₂ FC| > 1.5) are reported for A) P-LOW vs C, taxa with a fold change greater than 0 are more abundant in P-LOW ; B) P-HIGH vs C, taxa with a fold change greater than 0 are more abundant in P-HIGH ; and C) P-LOW vs P-HIGH, taxa with a fold change greater than 0 are more abundant in P-LOW ; in P-LOW vs C comparison no statistical significance resulted. The colors of the dots indicate the same family, and bars show the standard error of the log fold change.

4. Discussion

The interest in exploring the use of agro-industrial by-products, such as those obtained from the olive oil industry, in monogastric diets is increasing, necessitating research into the potential impact of such products on animal growth, production efficiency, 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 obtained from OMWW on the morphology and histopathological features of the gastrointestinal tract and intestinal microbial composition in finishing pigs.

4.1 Growth performances and animal health

Phenolic dietary treatments (74 and 225 ppm) did not influence pig body weight and feed intake parameters, similar to other studies reporting no changes in swine growth performances after supplementation with olive by-products (Ferrer et al. 2020; Sánchez et al. 2022). This was not surprising as *in vivo* studies in other species have generally shown phenolic compounds to have limited positive effects on product quality (Branciari et al. 2017; Branciari et al. 2020; Branciari et al. 2021). Moreover, throughout the trial, all the pigs maintained good health status, and did not present any signs of illness (presence of intestinal parasites or diarrhea).

4.2 Histopathological and morphometric findings

The macroscopic evaluation for the presence of gastric ulceration revealed a lower frequency of ulcers in the P-LOW and P-HIGH groups compared with the control group. The association between experimental group and presence of gastric ulcer was non-significant. None of the examined histopathological parameters revealed a significant association and/or a difference in the grade of distribution with the three experimental groups. Interestingly, the finding of no significant differences in the semiquantitative scoring of leukocyte infiltration in the intestinal lamina propria differs from that of a previous study (Varricchio et al. 2019); specifically, although the authors reported varying degrees of leukocyte infiltration, as similarly observed in our study, they also found a significantly higher number of infiltrating leukocytes in the group receiving a dietary supplement of polyphenol extracted from OMWW.

The impact of the OMWW phenolic extract on mucosa health was additionally assessed by calculating the VH/CD ratio, which revealed noteworthy differences across the different groups in both the jejunum and ileum. In the jejunum, the P-HIGH group demonstrated a higher VH/CD ratio compared with the C group; the same effect was noted in the ileum in both the P-LOW and P-HIGH groups. The higher VH/CD ratio in the treated groups could imply an enhanced absorptive capacity or a more favorable mucosal structure, which might be beneficial for nutrient absorption or overall gut health. The effects of polyphenol supplementation were particularly marked in the ileal tract, where the differences with the C group were greater compared with those in the jejunum. Consistent with our results, two previous studies reported that dietary supplementation with the polyphenol resveratrol increased the VH/CD ratio in the small intestines of piglets (Chen et al. 2021; Xun et al. 2021). Additionally, feeding piglets with olive cake extract appeared to restore the VH/CD ratio in the intestines following a challenge with lipopolysaccharides (Zhang et al. 2021). The results of the current study sustain the lack of any major side effects or lesions to the gastrointestinal system of pigs at the examined dosages of OMWW extract supplementation. These results agree with the current literature, suggesting potential beneficial effects of olive oil-processing products on intestinal health (Varricchio et al. 2019; Zhang et al. 2021; Sánchez et al. 2022; Belloumi et al. 2024).

4.3 Relative abundances and microbial diversity

Concerning the cecal and jejunal microbial compositions, the present study did not detect any major differences between the three experimental groups in the relative abundances of genera and families (Figures 3A and 4A). The alpha diversity indexes were also generally comparable between groups, indicating similar levels of biodiversity and richness; the only exceptions being the Chao1 and Fisher indexes in the cecum of the P-HIGH group, which were significantly higher with respect to those of the P-LOW group (Figure 3B). No significant differences were found in beta diversity between the groups in either the cecum or jejunum, indicating that supplementation with OMWW extract had no effect on the overall microbial composition of the pig gut. Similarly, Belloumi et al. (2024) found no significant differences in the alpha diversity indexes for the fecal microbiota of pigs fed an olive cake by-product dietary supplement (Belloumi et al. 2024).

It is known that maturation of the intestinal communities correlates with increases in microbial diversity, and that the latter also contributes towards a more stable bacterial ecosystem, preventing stress conditions that could lead to dysbiosis (Luise et al. 2024). Although alpha and beta indexes of gut microbiota diversity were similar across experimental groups, significant variations in the abundances of many bacteria at the genera and species level were found between the three experimental groups in both the cecum and jejunum.

4.4 Differential abundances in the cecum

Analysis of cecum content reported higher levels of two species belonging to the *Eubacterium* and *Treponema* genera in the P-HIGH group compared with P-LOW (Figure 5, $\log_2FC < 0$ in P-LOW vs P-HIGH). *Eubacterium* belongs to the phylum Firmicutes and is one of the most common butyrate-producing bacteria in the pig gut (Xu et al. 2021). A study by Bergamaschi et al. (2020) found a positive correlation between the genus *Eubacterium* and feed efficiency (Bergamaschi et al. 2020). Furthermore, hydroxytyrosol, the most represented polyphenol in OMWW extract, is known to increase the abundance of several beneficial bacteria, including *Eubacterium* (Han et al. 2022). Thus, the greater abundance of *Eubacterium* F sp000687695 in the P-HIGH group suggests that the beneficial effects of polyphenols on the pig cecum may be dose-dependent. *Treponema* is a fiber-degrading bacterium playing a significant role in the digestion of complex carbohydrates in pig cecum (Cwyk and Canale-Parola 1979; Stanton and Canale-Parola 1980; Nordhoff et al. 2005). In particular, the *Treponema* genus in both the small and large intestine is associated with the production of short-chain fatty acids, including acetic acid (Belloumi et al. 2024), and with growth and fatness parameters (Gardiner et al., 2020). Quan et al. (2020) demonstrated that the *Treponema* genus, specifically *T. bryantii*, was more abundant in the cecum of pigs demonstrating higher feed efficiencies (Quan et al. 2020). Our results are coherent with those reported by Zhang and colleagues, which showed the abundance of the *Treponema* genus to be greater in piglets supplemented with an olive cake extract (Zhang et al. 2021), suggesting a positive impact of this by-product supplementation on pig production efficiency.

Additionally, the present analysis found that *Fusobacterium* was less abundant in terms of genus and species (e.g. *Fusobacterium perfoetens*) in P-HIGH compared with P-LOW and C, suggesting that the highest phenolic dosage could decrease the risk of intestinal disorders. Indeed, an increase in this bacterium has been associated with the etiology of diarrhea in piglets (Yang et al. 2017). Interestingly, P-HIGH pigs also showed lower levels of *Bacteroides helcogenes* compared with the P-LOW. Although *B. helcogenes* has rarely been identified as the causative agent of intestinal damage in animals raised in intensive systems, this pathogenic bacterium has been isolated from pig feces and from abscesses (Benno and Mitsuoka 1984; Pati et al. 2011); thus its reduction following the administration of a P-HIGH diet has the potential to have a positive impact on pig gut health. Compared with P-LOW, the P-HIGH group also showed a lower abundance of *Faecalimonas*. 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). Supplementation with magnolol, a plant polyphenol with health-promoting functions in the pig intestines, has been shown to reduce *Faecalimonas* concentration, confirming our findings (Li et al. 2024).

A further result was the unexpected lower abundances of two ‘controversial’ bacteria, namely *Desulfovibrio* and *Megasphaera*, in the treated groups compared with controls. The genus *Desulfovibrio* belongs to the group of sulfate-reducing bacteria, the action of which is fundamental for removing H_2 and maintaining a healthy pig gut (Ran et al. 2019). However, their action can lead to an excess of hydrogen sulfide (the product of sulfate reduction), which is associated with intestinal damage (Deplancke et al. 2000). Overall, the diversity of sulfate-reducing bacteria in the pig gut microbiota remains unclear and more data are needed to understand the role of this microbial group (Ran et al. 2019). Concerning *Megasphaera* and *Megasphaera*_A_38685_elsdenii, their abundances were lower in the two treatment groups compared with controls. *Megasphaera* belongs to the phylum Firmicutes and is one of the anaerobic bacteria dominating the pig large intestine (Grosu et al. 2019) and which plays a central role in fermentation, helping to maintain the intestinal pH and preventing 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 mucosal atrophy and increase protection in small and large intestine in weaned piglets (Yoshida et al. 2009). Two studies in the literature sustain that the abundance of *Megasphaera* in pig microbiota can be increased through dietary supplementation with grape seed meal, due to its high level of polyphenol flavonoids (Grosu et al.

2019; Grosu et al. 2020). These findings contrast with our results, potentially due to the different chemical nature of olive polyphenols.

4.5 Differential abundances in the jejunum

Analysis of jejunum contents revealed higher levels of *Enterococcus* at the genus and species level (*Enterococcus cecorum*) in P-HIGH compared with both C and P-LOW. The genus *Enterococcus* comprises harmful bacteria as well as 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 the chicken gut microbiota, and has been identified as an emerging avian pathogen (EFSA Panel on Animal Health and Welfare (AHAW) et al. 2022); however, it has also been isolated from the healthy intestines of livestock animals, including pigs (Jung et al. 2017). Increased levels of this species were also found in the feces of diarrheic piglets (Sun et al. 2019). However, the pigs in the present trial did not present any episodes of diarrhea (with the exception of a single animal), and according to the literature *E. cecorum* has not been associated with any specific swine diseases (Jung et al. 2017). In addition, a grape pomace extract rich in polyphenols (and apigenin in particular) stimulated an increase in *Enterococcus* in an *in vitro* model of human gut microbiota (Sánchez et al. 2016; Wang et al. 2017), and the same source of phenolic compounds was found to increase this bacterium in the intestinal content of broilers (Viveros et al. 2011).

The abundance of *Ligilactobacillus agilis* increased in the jejunum of the pigs receiving the P-HIGH diet. This microbial species is an important member of the Firmicutes phylum (Wiese et al. 2021). Interestingly, an increase of *L. agilis* was observed after dietary supplementation with a fermented herbal feed additive in piglets and with tea polyphenols in laying hens (Wang et al. 2024; Xiao et al. 2024). Considering the higher abundance of this helpful microorganism in the P-HIGH group compared with P-LOW and control animals, it is possible to assume that the 225 ppm dosage of polyphenols may have had a positive impact on gut health in these pigs. The *Metamycoplasma* genus includes pathogenic microorganisms associated with severe chronic infections and negative impacts on animal health (Chae et al., 2020). In the present study, the abundance of *Mycoplasma suis* was significantly lower in the P-HIGH group compared with C, however, *M. suis* is generally considered less pathogenic compared with others. Members of the *Mycoplasma* genus can cause an increase in oxidative stress in pigs (Helm et al. 2018), thus, the lower abundance of *Mycoplasma* in the P-HIGH group may suggest a protective effect on the swine gut compared with controls. Finally, lower levels of *Corynebacterium urealiticum* were detected in the jejunum of pigs receiving the P-HIGH diet. *C. urealiticum* has previously been identified in pigs (Vela 2003; Eiamsam-ang et al. 2023) and may be associated with the urinary tract infections via the ascending route (Carlton et al. 2010).

5. Conclusion

The role of dietary polyphenols in maintaining intestinal health in monogastric livestock species has yet to be explored in depth, especially in relation to the use of olive by-products as a natural source of polyphenols. Integrating these industrial by-products into livestock diets may help limit the use of antimicrobials and their associated high costs for producers, as well as reduce the environmental impact of the olive oil industry. In our study, supplementation of the finishing pig diet with a polyphenol extract from OMWW seemed to stimulate a number of beneficial changes in the intestinal environment, with an improvement in the VH/CD ratio and in the microbiota (namely, as increase in beneficial intestinal bacteria and a reduction in harmful ones). These two outcomes may be linked to beneficial effects of polyphenols on intestinal morphology and microbiota composition. 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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7. 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 Mourtzinis, Anastasia Kyriakoudi and Luca Mechelli reviewed and edited the last version. Funding acquisition: Massimo Trabalza-Marinucci, Ioannis Mourtzinis, Luca Mechelli. All authors read and approved the final manuscript.

8. Disclosure statement

No potential conflict of interest was reported by the authors.

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10. 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).

References

- Almuhayawi MS, Alruhaili MH, Gattan HS, Alharbi MT, Nagshabandi MK, Almehayawi MS, Jaouni SKA, Selim S, Alqahtani FS, El-Saadony MT, Alagawany M. 2023. Evaluation of antimicrobial effect of olive leaves powder and its role in improving the broiler productivity, carcass traits, blood metabolites, and caecal microbiota. *Poult Sci.* 102(11):103054. <https://doi.org/10.1016/j.psj.2023.103054>
- Altissimi C, Roila R, Ranucci D, Branciarri R, Cai D, Paulsen P. 2024. Preventing Microbial Growth in Game Meat by Applying Polyphenolic Extracts from Olive Mill Vegetation Water. *Foods.* 13(5):658. <https://doi.org/10.3390/foods13050658>

- Altmann BA, Neumann C, Rothstein S, Liebert F, Mörlein D. 2019. Do dietary soy alternatives lead to pork quality improvements or drawbacks? A look into micro-alga and insect protein in swine diets. *Meat Science*. 153:26–34. <https://doi.org/10.1016/j.meatsci.2019.03.001>
- Altmann BA, Neumann C, Velten S, Liebert F, Mörlein D. 2018. Meat Quality Derived from High Inclusion of a Micro-Alga or Insect Meal as an Alternative Protein Source in Poultry Diets: A Pilot Study. *Foods*. 7(3):34. <https://doi.org/10.3390/foods7030034>
- AOAC: Official Methods of Analysis (Volume 1).
- Belloumi D, García-Rebollar P, Calvet S, Francino MP, Reyes-Prieto M, González-Garrido J, Piquer L, Jiménez-Belenguer AI, Bermejo A, Cano C, Cerisuelo A. 2024. Impact of including two types of destoned olive cakes in pigs' diets on fecal bacterial composition and study of the relationship between fecal microbiota, feed efficiency, gut fermentation, and gaseous emissions. *Front Microbiol*. 15:1359670. <https://doi.org/10.3389/fmicb.2024.1359670>
- Benno Y, Mitsuoka T. 1984. Incidence and Isolation of Three New *Bacteroides* spp. from Abscesses in pigs. *The Japanese Journal of Veterinary Science*. 46(5):749–752. <https://doi.org/10.1292/jvms1939.46.749>
- Bergamaschi M, Tiezzi F, Howard J, Huang YJ, Gray KA, Schillebeeckx C, McNulty NP, Maltecca C. 2020. Gut microbiome composition differences among breeds impact feed efficiency in swine. *Microbiome*. 8(1):110. <https://doi.org/10.1186/s40168-020-00888-9>
- Biagi E, Mengucci C, Barone M, Picone G, Lucchi A, Celi P, Litta G, Candela M, Manfreda G, Brigidi P, et al. 2020. Effects of Vitamin B2 Supplementation in Broilers Microbiota and Metabolome. *Microorganisms*. 8(8):1134. <https://doi.org/10.3390/microorganisms8081134>
- Bokulich NA, Subramanian S, Faith JJ, Gevers D, Gordon JI, Knight R, Mills DA, Caporaso JG. 2013. Quality-filtering vastly improves diversity estimates from Illumina amplicon sequencing. *Nat Methods*. 10(1):57–59. <https://doi.org/10.1038/nmeth.2276>
- Bolyen E, Rideout JR, Dillon MR, Bokulich NA, Abnet CC, Al-Ghalith GA, Alexander H, Alm EJ, Arumugam M, Asnicar F, et al. 2019. Reproducible, interactive, scalable and extensible microbiome data science using QIIME 2. *Nat Biotechnol*. 37(8):852–857. <https://doi.org/10.1038/s41587-019-0209-9>
- Bonos E, Skoufos I, Petrotos K, Giavasis I, Mitsagga C, Fotou K, Vasilopoulou K, Giannenas I, Gouva E, Tsinas A, et al. 2022. Innovative Use of Olive, Winery and Cheese Waste By-Products as Functional Ingredients in Broiler Nutrition. *Vet Sci*. 9(6):290. <https://doi.org/10.3390/vetsci9060290>
- Branciarri R, Galarini R, Giusepponi D, Trabalza-Marinucci M, Forte C, Roila R, Miraglia D, Servili M, Acuti G, Valiani A. 2017. Oxidative Status and Presence of Bioactive Compounds in Meat from Chickens Fed Polyphenols Extracted from Olive Oil Industry Waste. *Sustainability*. 9(9):1566. <https://doi.org/10.3390/su9091566>
- Branciarri R, Galarini R, Miraglia D, Ranucci D, Valiani A, Giusepponi D, Servili M, Acuti G, Pauselli M, Trabalza-Marinucci M. 2020. Dietary Supplementation with Olive Mill Wastewater in Dairy Sheep: Evaluation of Cheese Characteristics and Presence of Bioactive Molecules. *Animals*. 10(11):1941. <https://doi.org/10.3390/ani10111941>

- Branciarri R, Galarini R, Trabalza-Marinucci M, Miraglia D, Roila R, Acuti G, Giusepponi D, Dal Bosco A, Ranucci D. 2021. Effects of Olive Mill Vegetation Water Phenol Metabolites Transferred to Muscle through Animal Diet on Rabbit Meat Microbial Quality. *Sustainability*. 13(8):4522. <https://doi.org/10.3390/su13084522>
- Buzoianu SG, Walsh MC, Rea MC, O’Sullivan O, Crispie F, Cotter PD, Ross RP, Gardiner GE, Lawlor PG. 2012. The Effect of Feeding Bt MON810 Maize to Pigs for 110 Days on Intestinal Microbiota. *PLOS ONE*. 7(5):e33668. <https://doi.org/10.1371/journal.pone.0033668>
- Buzoianu SG, Walsh MC, Rea MC, Quigley L, O’Sullivan O, Cotter PD, Ross RP, Gardiner GE, Lawlor PG. 2013. Sequence-Based Analysis of the Intestinal Microbiota of Sows and Their Offspring Fed Genetically Modified Maize Expressing a Truncated Form of *Bacillus thuringiensis* Cry1Ab Protein (Bt Maize). *Applied and Environmental Microbiology*. 79(24):7735–7744. <https://doi.org/10.1128/AEM.02937-13>
- Callahan BJ, McMurdie PJ, Rosen MJ, Han AW, Johnson AJA, Holmes SP. 2016. DADA2: High-resolution sample inference from Illumina amplicon data. *Nat Methods*. 13(7):581–583. <https://doi.org/10.1038/nmeth.3869>
- Carlton L. Gyles, John F. Prescott, J. Glenn Songer, Charles O. Thoen. 2010. Pathogenesis of bacterial infections in animals. 4th ed. Ames (Iowa): Wiley-Blackwell.
- Chen X, Zeng Z, Huang Z, Chen D, He J, Chen H, Yu B, Yu J, Luo J, Luo Y, Zheng P. 2021. Effects of dietary resveratrol supplementation on immunity, antioxidative capacity and intestinal barrier function in weaning piglets. *Animal Biotechnology*. 32(2):240–245. <https://doi.org/10.1080/10495398.2019.1683022>
- Cheng Y, Ding S, Azad MdAK, Song B, Kong X. 2023. Small Intestinal Digestive Functions and Feed Efficiency Differ in Different Pig Breeds. *Animals*. 13(7):1172. <https://doi.org/10.3390/ani13071172>
- Cwyk WM, Canale-Parola E. 1979. *Treponema succinifaciens* sp. nov., an anaerobic spirochete from the swine intestine. *Arch Microbiol*. 122(3):231–239. <https://doi.org/10.1007/BF00411285>
- Davies LC, Vilhena AM, Novais JM, Martins-Dias S. 2004. Olive mill wastewater characteristics: modelling and statistical analysis. *Grasas y Aceites*. 55(3):233–241. <https://doi.org/10.3989/gya.2004.v55.i3.171>
- De Witte C, Ducatelle R, Haesebrouck F. 2018. The role of infectious agents in the development of porcine gastric ulceration. *Vet J*. 236:56–61. <https://doi.org/10.1016/j.tvjl.2018.04.015>
- Deplancke B, Hristova KR, Oakley HA, McCracken VJ, Aminov R, Mackie RI, Gaskins HR. 2000. Molecular ecological analysis of the succession and diversity of sulfate-reducing bacteria in the mouse gastrointestinal tract. *Appl Environ Microbiol*. 66(5):2166–2174. <https://doi.org/10.1128/AEM.66.5.2166-2174.2000>
- EFSA Panel on Animal Health and Welfare (AHAW), Nielsen SS, Bicout DJ, Calistri P, Canali E, Drewe JA, Garin-Bastuji B, Gonzales Rojas JL, Gortázar C, Herskin M, et al. 2022. Assessment of listing and categorisation of animal diseases within the framework of the Animal Health Law (Regulation (EU) No 2016/429): antimicrobial-resistant *Enterococcus cecorum* in poultry. *EFSA Journal*. 20(2):e07126. <https://doi.org/10.2903/j.efsa.2022.7126>

- Eiamsam-ang T, Tadee P, Buddhasiri S, Chuammitri P, Kittiwon N, Pascoe B, Patchanee P. 2023. Investigation of swine caecal microbiomes in the northern region of Thailand [Internet]. [accessed 2024 Sep 14]. <https://doi.org/10.1101/2023.07.03.547425>
- FAOSTAT. [accessed 2024 Oct 24]. <https://www.fao.org/faostat/en/#data/QCL>
- Ferlisi F, Tang J, Cappelli K, Trabalza-Marinucci M. 2023. Dietary supplementation with olive oil co-products rich in polyphenols: a novel nutraceutical approach in monogastric animal nutrition. *Front Vet Sci.* 10:1272274. <https://doi.org/10.3389/fvets.2023.1272274>
- Ferrer P, Calvet S, García-Rebollar P, De Blas C, Jiménez-Belenguer AI, Hernández P, Piquer O, Cerisuelo A. 2020. Partially defatted olive cake in finishing pig diets: implications on performance, faecal microbiota, carcass quality, slurry composition and gas emission. *Animal.* 14(2):426–434. <https://doi.org/10.1017/S1751731119002040>
- Fiesel A, Gessner DK, Most E, Eder K. 2014. Effects of dietary polyphenol-rich plant products from grape or hop on pro-inflammatory gene expression in the intestine, nutrient digestibility and faecal microbiota of weaned pigs. *BMC Veterinary Research.* 10(1):196. <https://doi.org/10.1186/s12917-014-0196-5>
- García Casco JM, Muñoz M, Martínez Torres JM, García López A, González Barroso MÁ, González-Sánchez E. 2017. Alternative Feeding in Iberian Pigs during Growth Period: Incorporation of Olive Cake in a Dry or Wet (silage) Form. *Agriculturae Conspectus Scientificus.* 82(2):147–150.
- Gardiner GE, Metzler-Zebeli BU, Lawlor PG. 2020. Impact of Intestinal Microbiota on Growth and Feed Efficiency in Pigs: A Review. *Microorganisms.* 8(12):1886. <https://doi.org/10.3390/microorganisms8121886>
- Goel A, Ncho C-M, Jeong C-M, Gupta V, Jung J-Y, Ha S-Y, Yang J-K, Choi Y-H. 2022. Effects of Dietary Supplementation of Solubles from Shredded, Steam-Exploded Pine Particles on the Performance and Cecum Microbiota of Acute Heat-Stressed Broilers. *Microorganisms.* 10(9):1795. <https://doi.org/10.3390/microorganisms10091795>
- Grosu IA, Pistol GC, Marin DE, Cișmileanu A, Palade LM, Țăranu I. 2020. Effects of Dietary Grape Seed Meal Bioactive Compounds on the Colonic Microbiota of Weaned Piglets With Dextran Sodium Sulfate-Induced Colitis Used as an Inflammatory Model. *Front Vet Sci.* 7:31. <https://doi.org/10.3389/fvets.2020.00031>
- Grosu IA, Pistol GC, Taranu I, Marin DE. 2019. The Impact of Dietary Grape Seed Meal on Healthy and Aflatoxin B1 Afflicted Microbiota of Pigs after Weaning. *Toxins.* 11(1):25. <https://doi.org/10.3390/toxins11010025>
- Haesebrouck F, Pasmans F, Flahou B, Chiers K, Baele M, Meys T, Decostere A, Ducatelle R. 2009. Gastric helicobacters in domestic animals and nonhuman primates and their significance for human health. *Clin Microbiol Rev.* 22(2):202–223, Table of Contents. <https://doi.org/10.1128/CMR.00041-08>
- Han H, Zhong R, Zhou Y, Xiong B, Chen L, Jiang Y, Liu L, Sun H, Tan J, Tao F, et al. 2022. Hydroxytyrosol Benefits Boar Semen Quality via Improving Gut Microbiota and Blood Metabolome. *Front Nutr.* 8:815922. <https://doi.org/10.3389/fnut.2021.815922>

- Helm ET, Outhouse AC, Schwartz KJ, Lonergan SM, Curry SM, Dekkers JCM, Gabler NK. 2018. Metabolic adaptation of pigs to a *Mycoplasma hyopneumoniae* and *Lawsonia intracellularis* dual challenge¹. *Journal of Animal Science* [Internet]. [accessed 2024 Sep 14]. <https://doi.org/10.1093/jas/sky220>
- Joven M, Pintos E, Latorre MA, Suárez-Belloch J, Guada JA, Fondevila M. 2014. Effect of replacing barley by increasing levels of olive cake in the diet of finishing pigs: Growth performances, digestibility, carcass, meat and fat quality. *Animal Feed Science and Technology*. 197:185–193. <https://doi.org/10.1016/j.anifeedsci.2014.08.007>
- Jung A, Metzner M, Ryll M. 2017. Comparison of pathogenic and non-pathogenic *Enterococcus cecorum* strains from different animal species. *BMC Microbiol*. 17(1):33. <https://doi.org/10.1186/s12866-017-0949-y>
- Kim J, Nguyen SG, Guevarra RB, Lee I, Unno T. 2015. Analysis of swine fecal microbiota at various growth stages. *Arch Microbiol*. 197(6):753–759. <https://doi.org/10.1007/s00203-015-1108-1>
- Kopinski JS, McKenzie RA. 2007. Oesophagogastric ulceration in pigs: a visual morphological scoring guide. *Aust Vet J*. 85(9):356–361. https://doi.org/10.1111/j.1751-0813.2007.196_1.x
- Kyriakoudi A, Mourtzinou I, Tyśkiewicz K, Milovanovic S. 2024. An Eco-Friendly Supercritical CO₂ Recovery of Value-Added Extracts from *Olea europaea* Leaves. *Foods*. 13(12):1836. <https://doi.org/10.3390/foods13121836>
- Le Sciellour M, Labussière E, Zemb O, Renaudeau D. 2018. Effect of dietary fiber content on nutrient digestibility and fecal microbiota composition in growing-finishing pigs. Looor JJ, editor. *PLoS ONE*. 13(10):e0206159. <https://doi.org/10.1371/journal.pone.0206159>
- Lessard M, Talbot G, Bergeron N, Lo Verso L, Morissette B, Yergeau É, Matte JJ, Bissonnette N, Blais M, Gong J, et al. 2023. Weaning diet supplemented with health-promoting feed additives influences microbiota and immune response in piglets challenged with *Salmonella*. *Veterinary Immunology and Immunopathology*. 255:110533. <https://doi.org/10.1016/j.vetimm.2022.110533>
- Li Y, Liu Y, Mu C, Zhang C, Yu M, Tian Z, Deng D, Ma X. 2024. Magnolol-driven microbiota modulation elicits changes in tryptophan metabolism resulting in reduced skatole formation in pigs. *Journal of Hazardous Materials*. 467:133423. <https://doi.org/10.1016/j.jhazmat.2024.133423>
- Liehr M, Mereu A, Pastor JJ, Quintela JC, Staats S, Rimbach G, Ipharraguerre IR. 2017. Olive oil bioactives protect pigs against experimentally-induced chronic inflammation independently of alterations in gut microbiota. Smidt H, editor. *PLoS ONE*. 12(3):e0174239. <https://doi.org/10.1371/journal.pone.0174239>
- Liotta L, Chiofalo V, Lo Presti V, Chiofalo B. 2019. In Vivo Performances, Carcass Traits, and Meat Quality of Pigs Fed Olive Cake Processing Waste. *Animals*. 9(12):1155. <https://doi.org/10.3390/ani9121155>
- Liu G, Li P, Hou L, Niu Q, Pu G, Wang B, Du T, Kim SW, Niu P, Li Q, Huang R. 2021. Metagenomic Analysis Reveals New Microbiota Related to Fiber Digestion in Pigs. *Front Microbiol*. 12:746717. <https://doi.org/10.3389/fmicb.2021.746717>

- Love MI, Huber W, Anders S. 2014. Moderated estimation of fold change and dispersion for RNA-seq data with DESeq2. *Genome Biol.* 15(12):550. <https://doi.org/10.1186/s13059-014-0550-8>
- Luise D, Correa F, Cestonaro G, Sattin E, Conte G, Mele M, Archetti I, Viridis S, Negrini C, Galasso I, et al. 2024. Effect of different doses of camelina cake inclusion as a substitute of dietary soyabean meal on growth performance and gut health of weaned pigs. *Br J Nutr.* 131(12):1962–1974. <https://doi.org/10.1017/S0007114524000722>
- Luo Y, Ren W, Smidt H, Wright A-DG, Yu B, Schyns G, McCormack UM, Cowieson AJ, Yu J, He J, et al. 2022. Dynamic Distribution of Gut Microbiota in Pigs at Different Growth Stages: Composition and Contribution. Kao C-Y, editor. *Microbiol Spectr.* 10(3):e00688-21. <https://doi.org/10.1128/spectrum.00688-21>
- McDonald D, Jiang Y, Balaban M, Cantrell K, Zhu Q, Gonzalez A, Morton JT, Nicolaou G, Parks DH, Karst SM, et al. 2024. Greengenes2 unifies microbial data in a single reference tree. *Nature Biotechnology.* 42(5):715–718. <https://doi.org/10.1038/s41587-023-01845-1>
- McMurdie PJ, Holmes S. 2013. phyloseq: an R package for reproducible interactive analysis and graphics of microbiome census data. *PLoS One.* 8(4):e61217. <https://doi.org/10.1371/journal.pone.0061217>
- Mun D, Kim H, Shin M, Ryu S, Song M, Oh S, Kim Y. 2021. Decoding the intestinal microbiota repertoire of sow and weaned pigs using culturomic and metagenomic approaches. *J Anim Sci Technol.* 63(6):1423–1432. <https://doi.org/10.5187/jast.2021.e124>
- Chae, C., Gomes-Neto, J.C., Segalés, J., Sibila, M., 2020. Diversity of *Mycoplasma hyopneumoniae* strains. In: *Mycoplasmas in Swine.*, [ed. by Maes, D., Sibila, M., Pieters, M.]. Leuven, Belgium: Acco Publishers. 147-161
- Nenadis N, Kyriakoudi A, Tsimidou MZ. 2013. Impact of alkaline or acid digestion to antioxidant activity, phenolic content and composition of rice hull extracts. *LWT - Food Science and Technology.* 54(1):207–215. <https://doi.org/10.1016/j.lwt.2013.05.005>
- Nordhoff M, Taras D, Macha M, Tedin K, Busse H-J, Wieler LH. 2005. *Treponema berlinense* sp. nov. and *Treponema porcinum* sp. nov., novel spirochaetes isolated from porcine faeces. *Int J Syst Evol Microbiol.* 55(Pt 4):1675–1680. <https://doi.org/10.1099/ijs.0.63388-0>
- Oksanen J, Simpson GL, Blanchet FG, Kindt R, Legendre P, Minchin PR, O'Hara RB, Solymos P, Stevens MHH, Szoecs E, et al. 2024. vegan: Community Ecology Package [Internet]. [accessed 2024 Sep 18]. <https://cran.r-project.org/web/packages/vegan/index.html>
- Paiva-Martins F, Barbosa S, Pinheiro V, Mourão JL, Outor-Monteiro D. 2009. The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. *Meat Sci.* 82(4):438–443. <https://doi.org/10.1016/j.meatsci.2009.02.014>
- Papakonstantinou GI, Meletis E, Petrotos K, Kostoulas P, Tsekouras N, Kantere MC, Voulgarakis N, Gougoulis D, Filippopoulos L, Christodoulopoulos G, et al. 2023. Effects of a Natural Polyphenolic Product from Olive Mill Wastewater on Oxidative Stress and Post-Weaning Diarrhea in Piglets. *Agriculture.* 13(7):1356. <https://doi.org/10.3390/agriculture13071356>
- Papatsiros VG, Katsogiannou EG, Papakonstantinou GI, Michel A, Petrotos K, Athanasiou LV. 2022. Effects of Phenolic Phytogetic Feed Additives on Certain Oxidative Damage

Biomarkers and the Performance of Primiparous Sows Exposed to Heat Stress under Field Conditions. *Antioxidants* (Basel). 11(3):593. <https://doi.org/10.3390/antiox11030593>

- Pati A, Gronow S, Zeytun A, Lapidus A, Nolan M, Hammon N, Deshpande S, Cheng J-F, Tapia R, Han C, et al. 2011. Complete genome sequence of *Bacteroides helcogenes* type strain (P 36-108T). *Stand Genomic Sci.* 4(1):45–53. <https://doi.org/10.4056/sigs.1513795>
- Pereira AC, Bandeira V, Fonseca C, Cunha MV. 2020. Egyptian Mongoose (*Herpestes ichneumon*) Gut Microbiota: Taxonomical and Functional Differences across Sex and Age Classes. *Microorganisms.* 8(3):392. <https://doi.org/10.3390/microorganisms8030392>
- Proietti PC, Bietta A, Brachelente C, Lepri E, Davidson I, Franciosini MP. 2010. Detection of *Helicobacter* spp. in gastric, fecal and saliva samples from swine affected by gastric ulceration. *J Vet Sci.* 11(3):221–225. <https://doi.org/10.4142/jvs.2010.11.3.221>
- Quan J, Wu Z, Ye Y, Peng L, Wu J, Ruan D, Qiu Y, Ding R, Wang X, Zheng E, et al. 2020. Metagenomic Characterization of Intestinal Regions in Pigs With Contrasting Feed Efficiency. *Front Microbiol.* 11:32. <https://doi.org/10.3389/fmicb.2020.00032>
- Ran S, Mu C, Zhu W. 2019. Diversity and community pattern of sulfate-reducing bacteria in piglet gut. *J Animal Sci Biotechnol.* 10(1):40. <https://doi.org/10.1186/s40104-019-0346-5>
- Rebollada-Merino A, Bárcena C, Ugarte-Ruiz M, Porrás N, Mayoral-Alegre FJ, Tomé-Sánchez I, Domínguez L, Rodríguez-Bertos A. 2019. Effects on Intestinal Mucosal Morphology, Productive Parameters and Microbiota Composition after Supplementation with Fermented Defatted Alperujo (FDA) in Laying Hens. *Antibiotics.* 8(4):215. <https://doi.org/10.3390/antibiotics8040215>
- Rossi R, Corino C, Pastorelli G, Durand P, Prost M. 2009. Assessment of antioxidant activity of natural extracts. *Italian Journal of Animal Science.* 8(sup2):655–657. <https://doi.org/10.4081/ijas.2009.s2.655>
- Sakamoto M, Ikeyama N, Yuki M, Ohkuma M. 2018. Draft Genome Sequence of *Faecalimonas umbilicata* JCM 30896^T, an Acetate-Producing Bacterium Isolated from Human Feces. Denney JJ, editor. *Microbiol Resour Announc.* 7(9):10.1128/mra.01091-18. <https://doi.org/10.1128/mra.01091-18>
- Sánchez CJ, Barrero-Domínguez B, Martínez-Miró S, Madrid J, Baños A, Aguinaga MA, López S, Hernández F. 2022. Use of Olive Pulp for Gestating Iberian Sow Feeding: Influence on Performance, Health Status Indicators, and Fecal Microbiota. *Animals.* 12(22):3178. <https://doi.org/10.3390/ani12223178>
- Sánchez I, Duran B, gonzalez manzano S, Santos Buelga C, Cueva C, Martín-Cabrejas M, Sanz-Buenhombre M, Guadarrama A, Moreno-Arribas MV, Bartolomé B. 2016. Chemical characterization and in vitro colonic fermentation of grape pomace extracts. *Journal of the Science of Food and Agriculture.* 97. <https://doi.org/10.1002/jsfa.8197>
- Selma MV, Espín JC, Tomás-Barberán FA. 2009. Interaction between phenolics and gut microbiota: role in human health. *J Agric Food Chem.* 57(15):6485–6501. <https://doi.org/10.1021/jf902107d>
- Silva-Guillen YV, Arellano C, Boyd RD, Martinez G, van Heugten E. 2020. Growth performance, oxidative stress and immune status of newly weaned pigs fed peroxidized lipids with or

without supplemental vitamin E or polyphenols. *J Anim Sci Biotechnol.* 11:22.
<https://doi.org/10.1186/s40104-020-0431-9>

Silvan JM, Martinez-Rodriguez AJ. 2019. Food By-products as Natural Source of Bioactive Compounds Against *Campylobacter*. In: *Encyclopedia of Food Security and Sustainability* [Internet]. [place unknown]: Elsevier; [accessed 2024 Jun 10]; p. 336–350.
<https://doi.org/10.1016/B978-0-08-100596-5.22139-2>

Stanton TB, Canale-Parola E. 1980. *Treponema bryantii* sp. nov., a rumen spirochete that interacts with cellulolytic bacteria. *Arch Microbiol.* 127(2):145–156.
<https://doi.org/10.1007/BF00428018>

Sun J, Du L, Li X, Zhong H, Ding Y, Liu Z, Ge L. 2019. Identification of the core bacteria in rectums of diarrheic and non-diarrheic piglets. *Sci Rep.* 9(1):18675.
<https://doi.org/10.1038/s41598-019-55328-y>

Tian X, Li D, Zhao X, Xiao Z, Sun J, Yuan T, Wang Y, Zuo X, Yang G, Yu T. 2023. Dietary grape pomace extract supplementation improved meat quality, antioxidant capacity, and immune performance in finishing pigs. *Front Microbiol.* 14:1116022.
<https://doi.org/10.3389/fmicb.2023.1116022>

Torres C, Alonso CA, Ruiz-Ripa L, León-Sampedro R, Del Campo R, Coque TM. 2018. Antimicrobial Resistance in *Enterococcus* spp. of animal origin. Aarestrup FM, Schwarz S, Shen J, Cavaco L, editors. *Microbiol Spectr.* 6(4):6.4.24.
<https://doi.org/10.1128/microbiolspec.ARBA-0032-2018>

Van Boeckel TP, Pires J, Silvester R, Zhao C, Song J, Criscuolo NG, Gilbert M, Bonhoeffer S, Laxminarayan R. 2019. Global trends in antimicrobial resistance in animals in low- and middle-income countries. *Science.* 365(6459):eaaw1944.
<https://doi.org/10.1126/science.aaw1944>

Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science.* 74(10):3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)

Varricchio E, Coccia E, Orso G, Lombardi V, Imperatore R, Vito P, Paolucci M. 2019. Influence of polyphenols from olive mill wastewater on the gastrointestinal tract, alveolar macrophages and blood leukocytes of pigs. *Italian Journal of Animal Science.* 18(1):574–586.
<https://doi.org/10.1080/1828051X.2018.1548911>

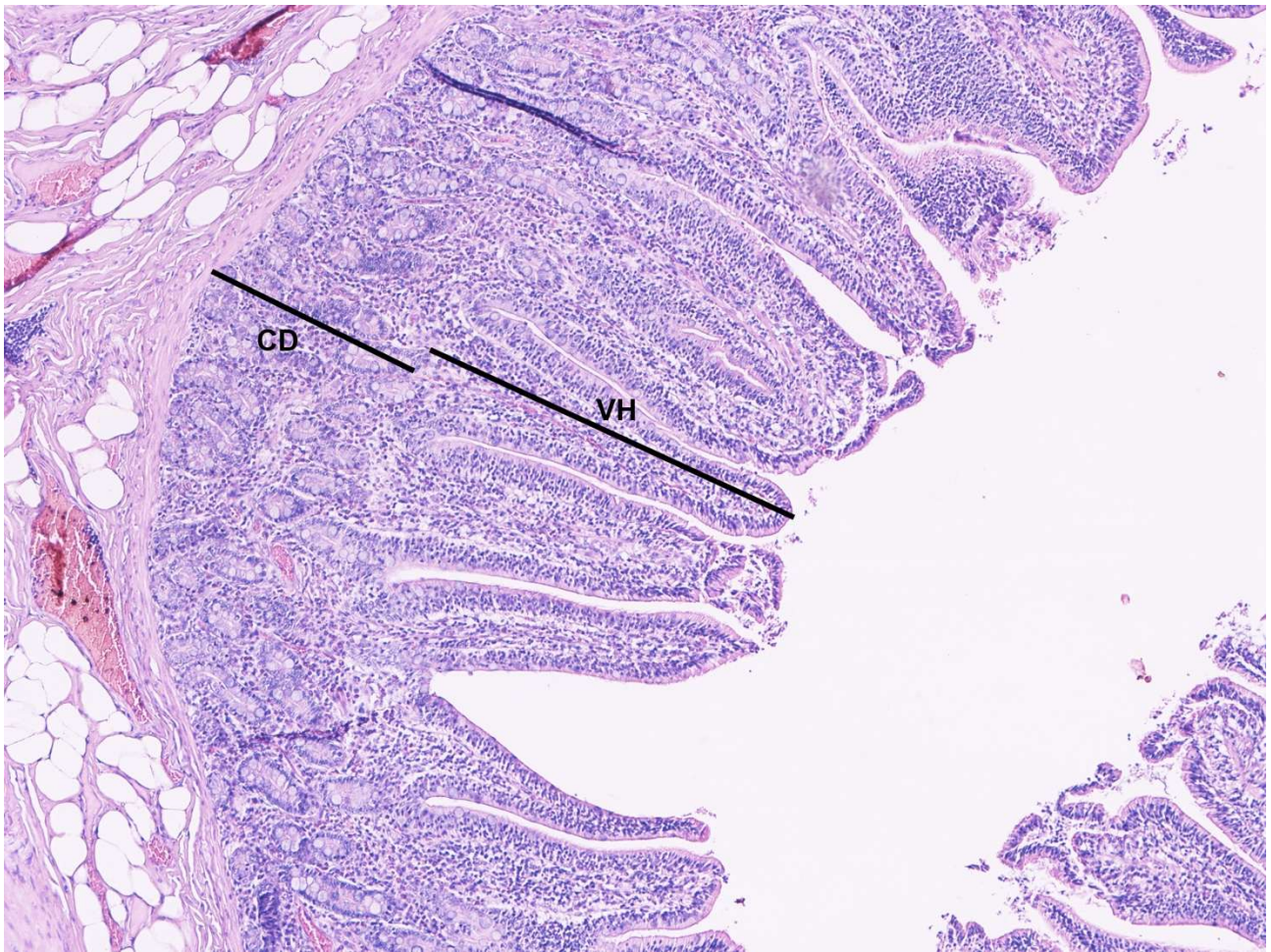
Vasquez R, Oh JK, Song JH, Kang D-K. 2022. Gut microbiome-produced metabolites in pigs: a review on their biological functions and the influence of probiotics. *J Anim Sci Technol.* 64(4):671–695. <https://doi.org/10.5187/jast.2022.e58>

Vela AI. 2003. *Corynebacterium suicordis* sp. nov., from pigs. *INTERNATIONAL JOURNAL OF SYSTEMATIC AND EVOLUTIONARY MICROBIOLOGY.* 53(6):2027–2031.
<https://doi.org/10.1099/ijs.0.02645-0>

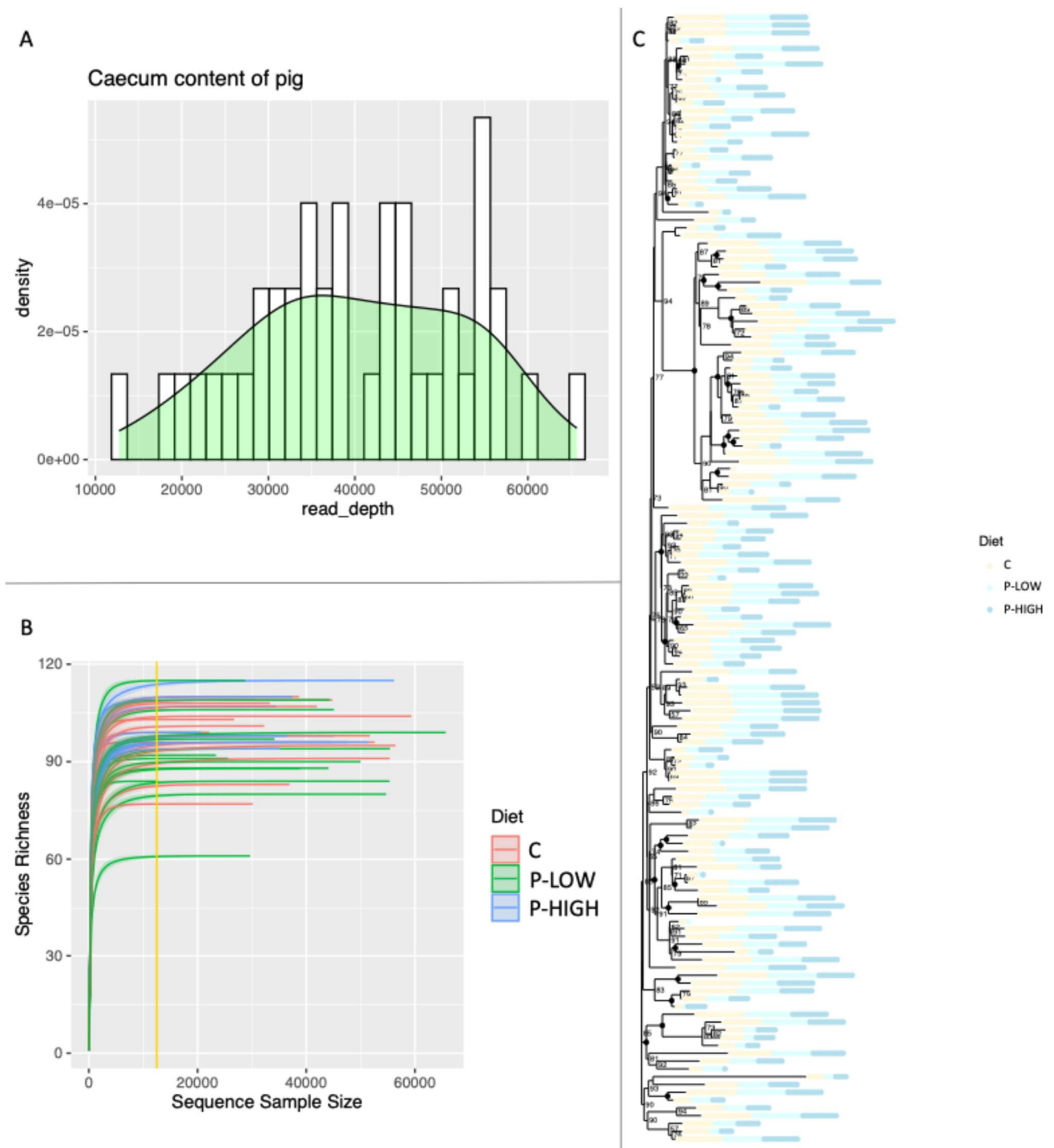
Verhelst R, Schroyen M, Buys N, Niewold T. 2014. Dietary polyphenols reduce diarrhea in enterotoxigenic *Escherichia coli* (ETEC) infected post-weaning piglets. *Livestock Science.* 160:138–140. <https://doi.org/10.1016/j.livsci.2013.11.026>

- Viveros A, Chamorro S, Pizarro M, Arija I, Centeno C, Brenes A. 2011. Effects of dietary polyphenol-rich grape products on intestinal microflora and gut morphology in broiler chicks. *Poultry Science*. 90(3):566–578. <https://doi.org/10.3382/ps.2010-00889>
- Wang M, Firrman J, Zhang L, Arango-Argoty G, Tomasula P, Liu L, Xiao W, Yam K. 2017. Apigenin Impacts the Growth of the Gut Microbiota and Alters the Gene Expression of *Enterococcus*. *Molecules*. 22(8):1292. <https://doi.org/10.3390/molecules22081292>
- Wang Q, Wang Z, Shang B, Li Y, Zhou F, Zeng X, Liu Z, Yang H, Zhu M. 2024. Tea polyphenols improve lipid deposition via modulation of gut microbiota in rats and Ningxiang pigs. *Journal of Functional Foods*. 113:106049. <https://doi.org/10.1016/j.jff.2024.106049>
- Wickham H. 2016. *ggplot2* [Internet]. Cham: Springer International Publishing; [accessed 2024 Sep 27]. <https://doi.org/10.1007/978-3-319-24277-4>
- Wiese M, Hui Y, Holck J, Sejberg JJP, Daures C, Maas E, Kot W, Borné JM, Khakimov B, Thymann T, Nielsen DS. 2021. High throughput in vitro characterization of pectins for pig(let) nutrition. *anim microbiome*. 3(1):69. <https://doi.org/10.1186/s42523-021-00129-w>
- Xiao R, Wang L, Tang Z, Qian X, Wang J, Lian Y, Tang J, Xu J, Lin Y, Shi B, et al. 2024. Metabolomics profiling reveals association of fermented herbal feed additive, growth performance and gut microbiota in piglets [Internet]. [accessed 2024 Sep 14]. <https://doi.org/10.1101/2024.07.21.604508>
- Xu E, Yang H, Ren M, Wang Y, Xiao M, Tang Q, Zhu M, Xiao Y. 2021. Identification of Enterotype and Its Effects on Intestinal Butyrate Production in Pigs. *Animals*. 11(3):730. <https://doi.org/10.3390/ani11030730>
- Xu X, Hua H, Wang L, He P, Zhang L, Qin Q, Yu C, Wang X, Zhang G, Liu Y. 2020. Holly polyphenols alleviate intestinal inflammation and alter microbiota composition in lipopolysaccharide-challenged pigs. *Br J Nutr*. 123(8):881–891. <https://doi.org/10.1017/S0007114520000082>
- Xun W, Fu Q, Shi L, Cao T, Jiang H, Ma Z. 2021. Resveratrol protects intestinal integrity, alleviates intestinal inflammation and oxidative stress by modulating AhR/Nrf2 pathways in weaned piglets challenged with diquat. *International Immunopharmacology*. 99:107989. <https://doi.org/10.1016/j.intimp.2021.107989>
- Yang Q, Huang X, Zhao S, Sun W, Yan Z, Wang P, Li S, Huang W, Zhang S, Liu L, Gun S. 2017. Structure and Function of the Fecal Microbiota in Diarrheic Neonatal Piglets. *Front Microbiol* [Internet]. [accessed 2024 Sep 14] 8. <https://doi.org/10.3389/fmicb.2017.00502>
- Yoshida Y, Tsukahara T, Ushida K. 2009. Oral administration of *Lactobacillus plantarum* Lq80 and *Megasphaera elsdenii* iNP-001 induces efficient recovery from mucosal atrophy in the small and the large intestines of weaning piglets. *Animal Science Journal*. 80(6):709–715. <https://doi.org/10.1111/j.1740-0929.2009.00692.x>
- Zhang Y, Deng Z-X, He M-L, Luo XM, Liu J-X, Wang H. 2020. Olive Extract Ameliorates Oxidative Stress and Inflammation, and Protects Intestinal Villus and Microbiota in Piglets Induced by Lipopolysaccharides [Internet]. [accessed 2024 Jul 24]. <https://doi.org/10.21203/rs.3.rs-101171/v1>

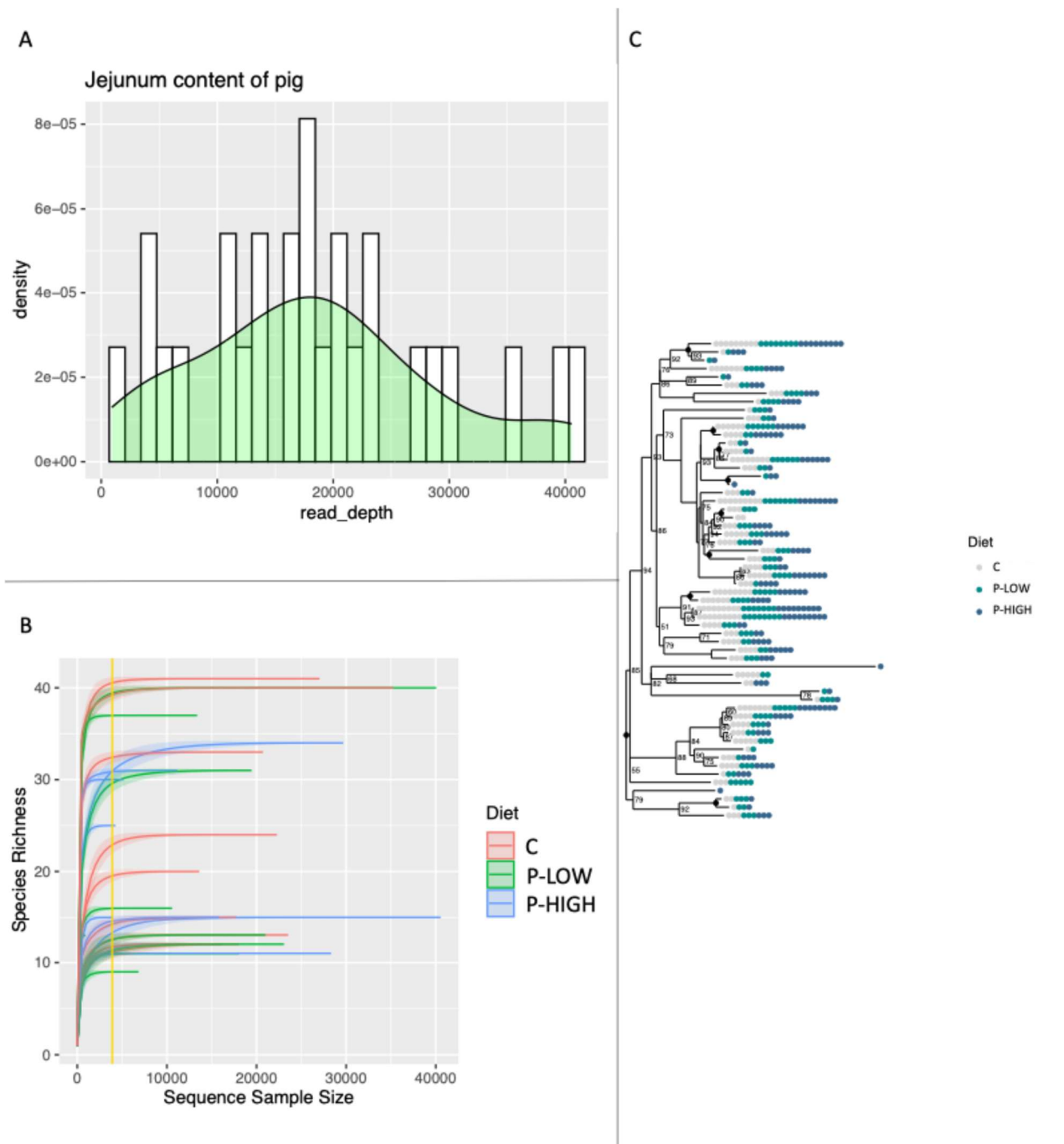
Zhang Y, Deng Z-X, He M-L, Pastor JJ, Tedo G, Liu J-X, Wang H-F. 2021. Olive oil cake extract stabilizes the physiological condition of lipopolysaccharide-challenged piglets by reducing oxidative stress and inflammatory responses and modulating the ileal microbiome. *Food Funct.* 12(20):10171–10183. <https://doi.org/10.1039/D0FO03012K>



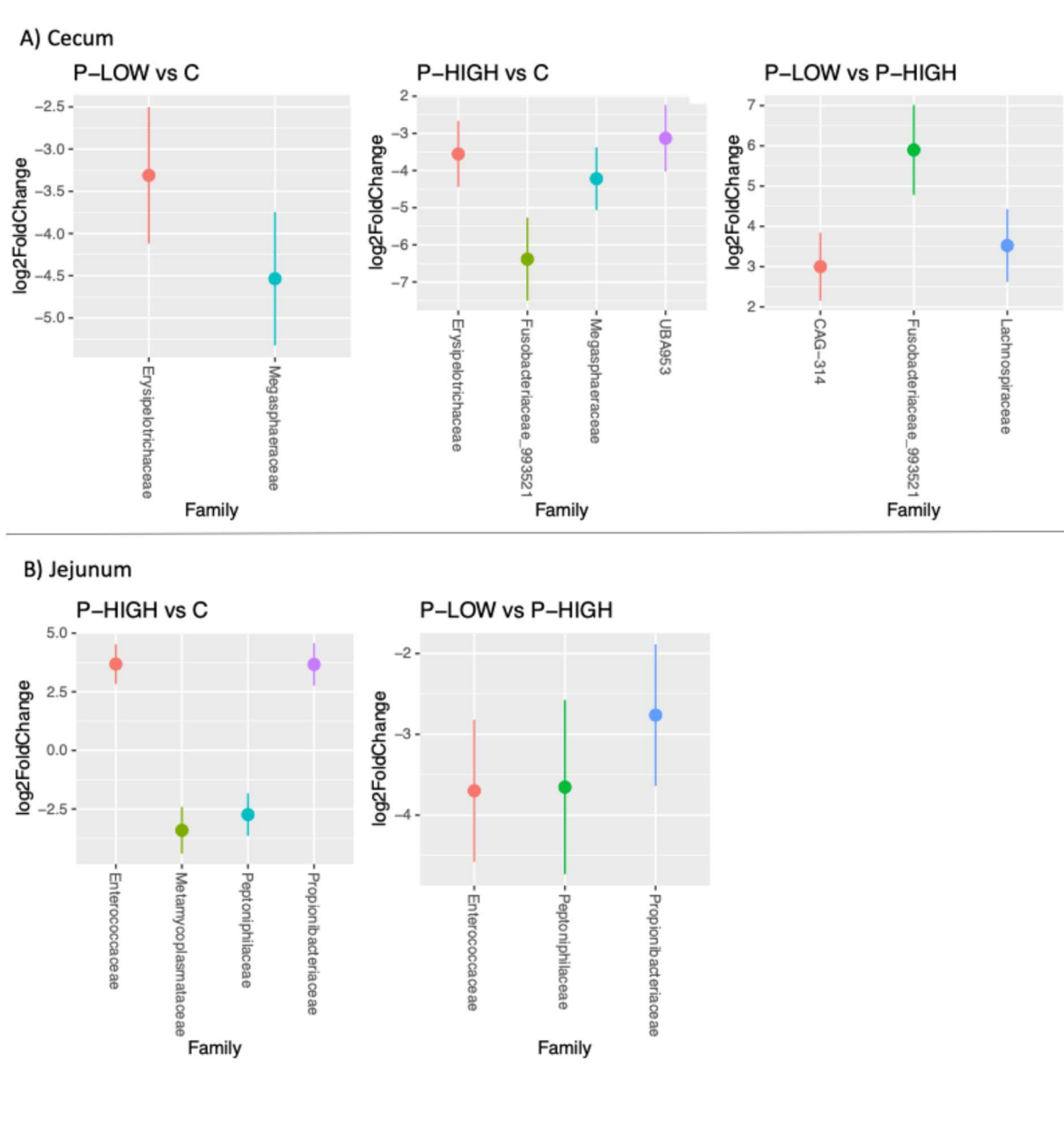
Supplementary Figure 1. Histology of the jejunum showing the method used for the morphometric analysis on villous height (VH) and crypt dept (CD) (H&E; x100). Measurements were obtained from the apex to the base of the villus (VH) and from the base of the villus to the base of the crypt (CD).



Supplementary Figure 2. 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 phylogenetic tree used to calculate unfrac distances (C); C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).



Supplementary Figure 3. 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 phylogenetic tree used to calculate unfrac distances (C); C (control), P-LOW (74 ppm), and P-HIGH (225 ppm).



Supplementary Figure 4. Differentially abundant families (False Discovery rate – FDR < 0.05; and log₂ Fold Change - |log₂ 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. Macroscopic assessment of gastric ulcer and histopathologic assessment of all intestinal segments.

Case	Liver					Stomach			Jejunum				Ileum				Colon			
	Gross		Histology			Gross		Histology			Gross		Histology			Gross		Histology		
	Deg.	Necr.	Leuk.	Ulcer	Ulcer grade	Leuk.	Fibrosis	Epithelial. Hyperpl.	Leuk.	Epith. Deg.	Epith. Necr.	GALT. Hyperpl.	Leuk.	Epith. Deg.	Epith. Necr.	GALT. Hyperpl.	Leuk.	Epith. Deg.	Epith. Necr.	GALT. Hyperpl.
Control	0	0	0	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	1
	0	0	1	yes	1	1	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	yes	1	0	0	1	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	yes	2	1	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	1	1	0	2	2	0	0	0	2	0	0	0	1	0	0	1
	0	0	0	yes	1	1	1	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	1
	0	0	2	yes	2	2	0	0	2	0	0	0	3	0	0	0	2	0	0	0
	1	0	0	no	0	0	0	1	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	yes	3	2	1	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	no	0	1	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	1
	1	0	0	no	0	0	0	2	2	0	0	0	2	0	0	0	2	0	0	0
0	0	0	yes	3	2	1	2	2	0	0	0	2	0	0	0	1	0	0	0	
P-LOW	0	0	1	yes	2	2	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	1	1	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	1	1	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	no	0	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	yes	1	1	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	1	0	0	yes	2	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	no	0	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	1	no	0	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0

	0	0	0	yes	3	2	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	no	0	1	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	1	0	0	no	0	0	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	1	0	0	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	1	0	1	no	0	0	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	1	0	1	no	0	0	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	yes	1	1	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	no	0	2	0	1	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	1	0	0	yes	1	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	1	no	0	1	0	2	2	0	0	0	2	0	0	0	1	0	0	0
P- HIGH	1	0	1	no	0	1	0	2	0	0	0	0	2	0	0	0	1	0	0	0
	0	0	1	yes	3	2	1	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	no	0	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	no	0	1	0	2	2	0	0	0	2	0	0	0	1	0	0	0
	0	0	0	yes	3	2	1	2	2	0	0	0	2	0	0	0	2	0	0	0
	0	0	0	no	0	1	0	2	2	0	0	0	2	0	0	0	2	0	0	0

Supplementary Table 2. 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- chime ric
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	49,660	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 3. 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- chime ric
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 4. Differentially abundant genus (False Discovery rate – FDR < 0.05; and log₂ Fold Change - |log₂ 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 5. Differentially abundant species (False Discovery rate – FDR < 0.05; and log₂ Fold Change - |log₂ 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 6. Differentially abundant genus (False Discovery rate – FDR < 0.05; and log₂ Fold Change - |log₂ 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 7. Differentially abundant species (False Discovery rate – FDR < 0.05; and log₂ Fold Change - |log₂ 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

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

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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, 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 ($n = 15 \times 3$ 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, histopathology of liver, ovaries, uterus, fat, and muscle and morphometry of liver, ovaries and uterus). Furthermore, the antioxidant power of these molecules was evaluated in blood, meat, liver, and dry-cured ham, as well as their protective role in the quality traits and physico-chemical characteristics of the fresh and cured product. Texture properties, fatty acid composition and sensory analysis were also evaluated in the dry-cured ham. The present study demonstrated no significant differences among treatments in growth performance traits, histopathological and morphometric findings and backfat thickness. An increase in 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 decreased, while yellowness (b^*) increased in the polyphenol-supplemented animals. Dry-cured hams of the P-HIGH group showed a decrease in yellowness in all the analyzed tissues (*semimembranosus* and *biceps femoris* muscles and subcutaneous fat). Furthermore, protein carbonylation and lipid oxidation decreased in the dry-cured hams of the two polyphenols-supplemented groups, even after *in vitro* gastric digestion (P-HIGH group). The development of several volatile compounds, whose excess may be related to sensory defects in ham, was decreased by the dietary treatment. The observed effects on fresh meat cooking loss and *biceps femoris* moisture content of dry-cured ham showed a potential influence of the polyphenols on meat structure, water retention capacity, and salt concentration and distribution. These outcomes, together with the decrease in secondary products of lipid oxidation, were associated to differences in the sensory characteristics of the final products. No differences were reported in the fatty acid composition of the two dry-cured ham muscles. Further research is required to better understand the role of dietary OMWW polyphenols in *in vivo* and post-slaughtering characteristics of heavy pigs, especially in relation to the technological quality and antioxidant state of meat and dry-cured ham.

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, 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 molecules 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, and potentially reducing bacterial infections thanks to their 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). However, research on OMWW extracts is scarce. 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 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.

2.1.1 Reagents and solvents

Trolox (6-hydroxy-2,5,7,8-tetramethyl-chroman-2-carboxylic acid) (97%) was from Aldrich Chemical Co. (Steinheim, Germany). DPPH[•] (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).

2.1.2 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-C184 μ m (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 μ L. Extracts were analyzed after filtration through 0.45 μ m 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.

2.2 Animal welfare and ethic statement

The experiment was approved by the Bioethics Committee of the University of Perugia (protocol n. 399480) and performed under the ARRIVE guidelines. 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.

2.3 Animals and diet

The study was carried out on a conventional farm in a hilly area of Umbria, Italy. One hundred thirty-five female Landrace \times 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). 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. Water was given *ad libitum*. All pigs underwent a period of adaptation of 15 days prior the experiment. The trial lasted 85 days. Table 1 shows the composition of the control diet.

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

<i>Raw materials</i>	<i>% as fed basis</i>
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
<i>Analyzed nutrients</i>	<i>g/100 g</i>
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. ^a Mineral-vitamin supplement (mg or units/kg): 216700 UI Vitamin A; 65.000 UI Vitamin D3; 4000 mg Vitamin K3; 86 mg Vitamin K3; 50 mg Vitamin B1; 160 mg Vitamin B2; 450 mg calcium D-pantothenate; 100 mg Vitamin B6; 1 mg Vitamin B12; 800 mg Vitamin B3; 5 mg Biotin; 40 mg Vitamin B9; 15000 mg Cholin chloride; 1.500 mg iron carbonate; 1500 mg iron(II) sulfate monohydrate; 50 mg calcium iodate; 1500 mg manganese oxide; 500 mg copper(II) sulfate pentahydrate; 8 mg sodium selenite; 3 mg selenomethionine hydroxylate; 1000 mg zinc oxide; 13000 methionin; 58000 mg L-lysine monohydrochloride; 16500 mg L-threonine; 18700 TXU endo-1,4-beta-xylanase; 16000 FTU endo-1,4-beta-glucanase; 16000 FTU 6-Phytase.

2.4 Feed analysis

Crude protein, crude fiber, ether extracts, ash, calcium, phosphorous concentrations, moisture, and starch were determined according to AOAC procedures (AOAC: Official Methods of Analysis (Volume 1)). Analysis of the neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) was performed following the methods of Van Soest, Robertson, and Lewis (Van Soest et al. 1991).

2.5 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 ($CW/BW \times 100$).

2.6 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. (Cisneros et al. 1996), the transducer was set at 4.0 MHz and 10 cm-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), one-side assessment with three fat measurements on the single-scan image was chosen as representative of the backfat deposition.

2.7 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.

2.7.1 Histopathological and morphometric findings

Macroscopic analysis was performed on the liver, ovaries and uterus for the reproductive tract. Tissue samples from the liver, ovaries, uterus, fat, and skeletal muscle were collected and fixed in 10%-buffered formalin for 24 h for subsequent histological analysis. Formalin-fixed and paraffin embedded (FFPE) samples were processed according to standard protocols, and 3- μ m tissue sections were obtained and stained with Hematoxylin and Eosin. A scoring system was developed to assess the severity of tissue changes. The scoring system was established following recommendations reported by Gibson-Corley et al. (Gibson-Corley et al. 2013). Specifically, leukocyte infiltration was assessed in all the examined tissues (liver, ovary, uterus, fat, skeletal muscle) and its severity was established on a scale of 0 to 3, based on the percentage of affected tissue: 0 = no tissue affected, 1 = less than 20%, 2 = 21-60%, and 3 = more than 61%. The same grading scale (0 to 3 based on the percentage of affected tissue) was applied to assess hepatocellular degeneration and necrosis and hepatic fibrosis, as well as fat atrophy and necrosis. Additionally, the same scoring system was applied to investigate muscle atrophy, hypertrophy and regeneration, as well as epithelial degeneration, epithelial necrosis, mucosal glandular hyperplasia and lamina propria fibrosis of the uterus. Moreover, for the reproductive tract, the presence of luteal bodies in the ovary was recorded as a

binomial factor (either yes or no), similarly to the presence of adenomyosis in the uterus. 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.

2.7.2 Oxidative status in blood and tissues

Liver and *sternocleidomastoid* muscle tissues were collected for the evaluation of the antioxidant status and 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 $\mu\text{mol Trolox/g dry sample} \pm \text{SD}$ 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 \times 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).

2.8 Curing process and sampling of dry-cured ham

Six animals from each group (the same animals selected for meat analyses) were chosen for ham production. The right hind legs underwent 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. 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 72-78%), hams were subjected to two larding processes on the upper part of the leg, closer to the surface. The entire curing process lasted 12 months. Details about ham characteristics are explained in Figure 4 (Section 3.7). Then, the hams were weighed, deboned and divided in three pieces (front, upper back, lower part) (Figure 1). A 3-cm thick slice was taken from the lower part, including at least 3 muscle portions (*semimembranosus* - SM, *biceps femoris* - BF and *semitendinosus* - ST) and subcutaneous covering fat (Figure 2). SM, BF and the subcutaneous covering fat were used for the quality, proximate and texture analyses. The lower portion of the dry-cured ham was also used for the sensory analysis. The front part (including the *quadriceps femoris* muscle) was used for the analyses of the oxidation products (i.e. protein carbonylation, lipid oxidation, *in vitro* digestion and volatile compounds).



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

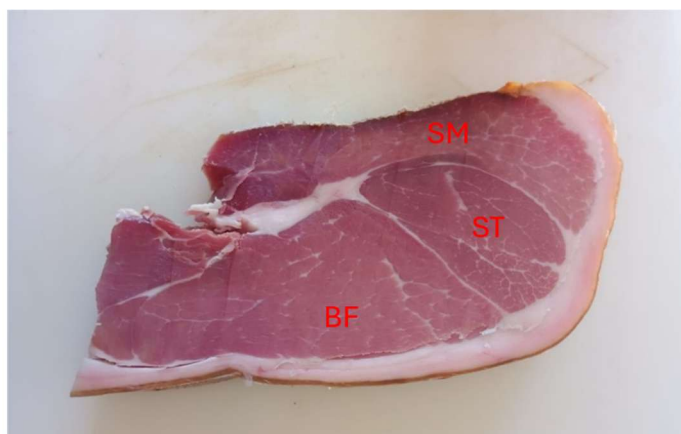


Figure 2. Muscles of the lower part of the dry-cured ham: *semimembranosus* (SM), *biceps femoris* (BF) and *semitendinosus* (ST).

2.9 Proximate analysis in meat and dry-cured ham

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 Fatty acid composition in dry-cured ham

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 addition 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 Branciarri 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 gas chromatograph (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 program 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).

2.11 Meat quality measurements

2.11.1 Raw meat analysis

Six animals (2 from each replicate) of each group (C, P-LOW, and P-HIGH) were chosen for the analysis of meat. All the measurements were performed on *gluteus medius*, due to its anatomical comparability to the dry-cured ham. 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 color measurements were conducted 24 h after slaughtering on raw meat samples following a 1 hour bloom period at the refrigeration temperature ($4 \pm 1^\circ\text{C}$) (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^*).

2.11.2 Cooked meat analysis

Cooking loss was performed on the same muscle type as reported by Honikel (Honikel 1998). For cooking loss determination, meat samples ($6.0 \times 6.0 \times 2.5\text{cm}$; average weight: $83.32 \pm 1.67\text{ g}$, $84.46 \pm 4.15\text{ g}$ and $84.05 \pm 2.92\text{ g}$ 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 $100 \times (\text{initial weight} - \text{final weight}) / \text{initial weight}$ (Honikel 1998). The Warner–Bratzler shear force measurement was also performed according to the Honikel (Honikel 1998) method. Three cylindrical cores ($\varnothing 1.25\text{ cm}$), 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/ $\frac{1}{2}$ inches was converted in kg/cm^2 .

2.11.3 Subcutaneous fat

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 24 h) and colour measurements (L^* , a^* , b^*) were carried out following the same protocols for meat analysis but without the bloom period.

2.12 Dry-cured ham quality and texture analysis

At sampling, pH and color (CIE L^* , a^* , b^* ,) were measured in the SM, BF and subcutaneous fat of dry-cured ham (lower part), with the same protocol used for raw meat and subcutaneous fat. Three cubes of $1 \times 1 \times 1\text{ cm}$ 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).

2.13 Detection of carbonylated proteins in dry-cured ham

Sarcoplasmic proteins were extracted from the dry-cured ham (front part) according to Gallego et al. (2016) with some modifications. Briefly, 1 mg of each sample, taken from the same location, were minced in 1 mL of 50 mM Tris-HCl pH 8.0 and mixed with a vortex for 2 minutes. The samples were incubated on ice for 15 minutes, followed by three cycles of sonication, each lasting 30 seconds. The samples were then incubated on ice for an additional 15 minutes. The homogenates were centrifuged at $13,000 \times g$ for 10 minutes at 4°C, and the supernatant, containing the proteins was collected. The protein concentration was determined using the Bradford protein assay (Biorad, Hercules, USA), with bovine serum albumin (BSA) as the protein standard.

The levels of carbonylated proteins were evaluated by indirect labeling on PVDF membranes with 2,4-dinitrophenylhydrazine (DNPH) (Chiaradia et al. 2023). Briefly, 25 µg of total proteins were electrophoresed on 12% T SDS-page and electrotransferred onto PVDF membranes. The membrane was washed for 15 minutes in PBS-T and incubated in 2 N HCl for 5 minutes and then incubated for 5 minutes in a DNPH solution (0.1 mg/mL in 2 N HCl) to derivatize the protein carbonyl groups. After washing and blocking steps, the membrane was incubated with an anti-DNP antibody (Molecular Probes, Eugene, OR, USA) O.N. at 4 °C and then with HRP-conjugated secondary antibodies (Cell Signaling, Beverly, USA). The immunoreactive proteins were finally visualized by chemiluminescence using the ECL system (Biorad, Hercules, USA). Images were acquired using ChemiDoc Imaging System (Biorad, Hercules, USA).

2.14 Lipid oxidation of dry-cured ham

Lipid peroxidation was measured in the cured product by thiobarbituric reactive substances (TBARS) determination, according to Tarladgis et al. (1960). TBARS concentrations were expressed as mg MDA/kg meat.

2.15 *In vitro* digestion of dry-cured ham and lipid oxidation

Digestive enzymes (α -amylase from porcine pancreas, pepsin from porcine gastric mucosa and pancreatin from porcine pancreas) and reagents for digestion and analytical determinations were purchased from Sigma (Milan, Italy). Six dry-cured ham samples for experimental group (C, P-LOW, P-HIGH) were chosen for the analysis. The *in vitro* digestion was performed following the protocol within the COST Action INFOGEST and Minekus, Alvinger, Alvito, Balance, and Bohn (Minekus et al. 2014). The advanced lipoxidation end-products were measured as TBARS as reported by Buege and Aust (Buege and Aust 1978) with minor modifications and adapted to a microplate reader (Martini et al. 2018). The results were expressed as µmol malondialdehyde (MDA) equivalent per 100 g of dry-cured ham.

2.16 Volatile compounds in dry-cured ham

The evaluation of volatile compounds in ham samples was performed by headspace solid-phase microextraction, followed by gas chromatography-mass spectrometry (HS-SPME/GC-MS), according to the method of Taticchi et al. (2021), with some modifications. 1 g of sample was added with 2 mL of saturated NaCl solution and 50 μ L of 4-methyl-2-pentanol as internal standard (4-methyl-2-pentanol at a concentration of 625 μ g/L). The SPME sampling of volatile compounds was performed by exposing the fiber consisting of Carboxen/divinylbenzene/polydimethylsiloxane 50/30 μ m, 2 cm long (Supelco Inc., Bellefonte, PA, USA). Before adsorption, the sample was stirred (750 rpm) for 30 min at 40 °C. The adsorbed compounds were then thermally desorbed for 5 min by inserting the fiber into the GC injector maintained at 250 °C in splitless mode. The analyses were performed with an Agilent Technologies 7890B GC, equipped with a 7693A Multimode Injector (MMI) (Agilent Technologies, Santa Clara, CA, USA) and a thermostated PAL3 RSI 120 autosampler with fiber conditioning module and stirrer (CTC Analytics AG, Zwingen, Switzerland), coupled to a single MS 5977B quadrupole (MSD) with an XTR (Extractor Ion Source) electron impact source (Agilent Technologies, Santa Clara, CA, USA). A DB-WAXetr fused silica capillary column with a length of 50 m, an i.d. of 1.5 mm, was used for the separation of volatile compounds. of 0.32 mm and a film thickness of 1 μ m (Agilent Technologies, Santa Clara, CA, USA). Helium was used as carrier gas at a flow rate of 1.7 mL/min, which was kept constant throughout the analysis time by an electronic flow control (EFC). The GC column oven temperature was set according to the following program: initial temperature 35 °C held for 4 min, then increased up to 45 °C with a rate of 5 °C/min, further increased up to 150 °C with a rate of 4 °C/min, increased again up to 180 °C with a rate of 8 °C/min and held for 2 min, finally brought to 210 °C with a rate of 11 °C/min and held for 13.77 min; under these conditions the total analysis time was 55 min. The injector was set to a temperature of 250 °C. The transfer line temperature was set to 215 °C; for the experimental conditions of the mass spectrometer, the source temperature was 190 °C and that of the quadrupole 150 °C. The electron impact (EI) mass spectrum was recorded with an ionization energy of 70 eV in the mass range 25-350 a.m.u., 4.3 scan/s, the MS spectra were acquired in scan mode. The processing of the collected data was carried out using the Agilent MassHunter B.08.00 software with the Unknown Analysis module. The identification of volatile compounds was carried out by comparing the mass spectra and retention times thus obtained with those of pure analytical standards and with the spectra of the NIST-2014 library. Volatile compounds were quantified, and expressed in μ g/kg, by comparing the area of the extracted ion of each peak to the area of the peak of the internal standard ion (4-methyl-2-pentanol), as reported by Xiao et al. (2014).

2.17 Sensory analysis of dry-cured ham

A consumer test was performed at the Department of Veterinary Medicine, University of Perugia. Participants were mainly represented by students and staff members and were asked to complete a questionnaire that included information regarding their age, sex and country of origin. The consumer test was performed under two different conditions (blind and expected). In the two sessions, three hams for dietary group (C, P-LOW and P-HIGH) were used and each consumer tasted 1 sample (1 mm slice) deriving from 1 ham of the three groups. Consumers have been educated before the test to become familiar with the use of a nine-point hedonic scale (from 1, “dislike extremely” to 9, “like extremely”) and binomial scale (yes/no). Participants also had the opportunity to provide personal comments about the products. The samples (1 mm slices) were served on white plastic plates identified by three random shape-based codes (for C, P-LOW and P-HIGH). In the first session, participants did not receive information regarding the product (blind experimental condition) and were asked to assess sensory attributes using a nine-point hedonic scale for appearance, taste, texture

and overall liking and a yes/no binomial scale for the consumer and purchase intention and acceptable price. In the second session, consumers evaluated the same sensory attributes similarly to the blind test, but receiving information about the pigs' diet (standard diet; P-LOW diet supplemented with a low dosage of polyphenols; P-HIGH diet supplemented with a high dosage of polyphenols).

2.18 Statistical analysis

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. In particular, data of growth performances, were analyzed by the following linear model:

$$y_{ik} = \mu + D_i + I_k(D) + e_{ikj}$$

where y_{ik} is the observation, μ is the overall mean, D_i is the fixed effect of the i^{th} diet ($i = 1$ to 3), I_k is the random effect of the replicate nested within the treatment ($k = 1$ to 3) and e_{ikj} is the residual error (Rstudio).

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), fatty acid composition, protein carbonylation, lipid oxidation, lipid oxidation during *in vitro* digestion, volatile compounds and sensory traits (appearance, taste, texture and overall liking) for dry-cured ham were analysed through a one-way ANOVA, followed by a Tukey's test. The other sensory traits (consumer's willingness to consume, purchase and pay) were evaluated through a chi-square 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 ($p > 0.05$) differences were observed for BW, ADG, FCR, hot carcass weight (HCW) and dressing out percentage values between the experimental groups (Table 2). Feed intake was the same for all the experimental groups, as the pigs always ingested the entire amount of feed provided throughout the trial.

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 ± 3.5	103.5 ± 6.3	109.09 ± 4.3	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 ± 0.1	80.79 ± 0.3	83.79 ± 0.2	0.09

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

3.2 Fat deposition

Regarding backfat thickness, no differences ($p = 0.35$) 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 treated (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

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

3.3 Histopathology and morphometry

For the reproductive tract, the association between all binomial factors (presence of luteal bodies, presence of uterine adenomyosis) assessed and the experimental groups was not significant ($p > 0.05$).

Similarly, no differences across groups ($p > 0.05$) were detected for the tissue changes graded in liver, muscle, fat and reproductive tract.

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 of pubertal and prepubertal gilts at the final stage 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 = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C 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 tocopherols were evaluated in pig serum, together with a number of markers of liver function (total proteins, albumins, globulins, cholesterol, ceruloplasmin, GOT, GGT, bilirubin, retinol). There was a tendency ($p = 0.06$) for group P-HIGH, when compared to group P-LOW, to have higher values of PON and GOT (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. More in detail, high values of bilirubin and albumins allow to discriminate the C group, while high values of 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 = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

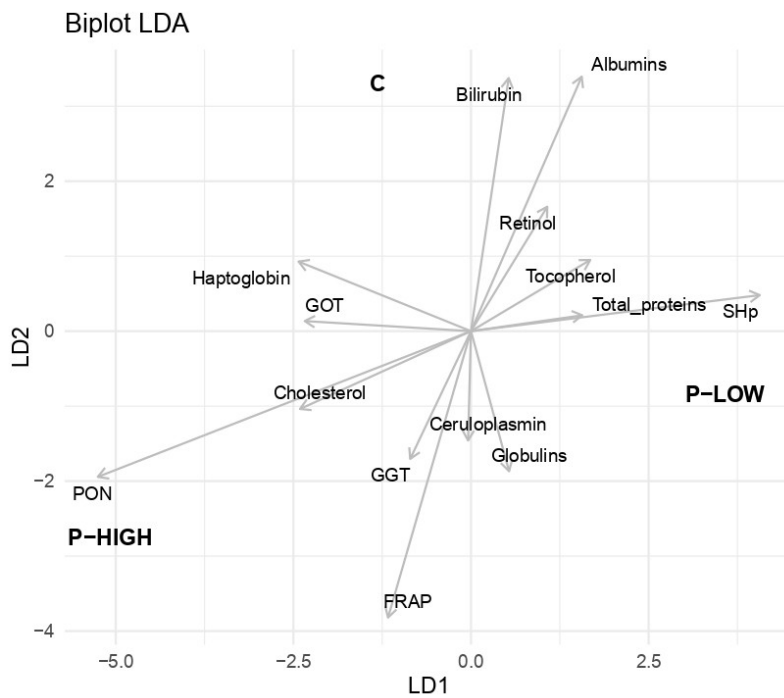


Figure 3. Linear Discriminant Analysis (LDA) diagram representing differentiations of blood serum parameters by the three experimental groups: C (control), P-LOW (C diet supplemented with 74 ppm OMWW polyphenols), and P-HIGH (C diet supplemented with 225 ppm OMWW polyphenols).

3.6 Antioxidant activity in muscle and liver

3.6.1 DPPH assay

In the liver, the DPPH assay showed significant ($p = 0.019$) differences in radical scavenging activity (% RSA) among the three experimental groups, with an increase of RSA value in pigs of the 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			p-value
	C	P-LOW	P-HIGH	
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 = C diet with 74 ppm OMWW polyphenols; P-HIGH = C diet with 225 ppm OMWW polyphenols.

3.6.2 CUPRAC assay

As for 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			p-value
	C	P-LOW	P-HIGH	
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 = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C 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 for the *sternocleidomastoid* muscle. (Table 8).

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

	Treatments			p-value
	C	P-LOW	P-HIGH	
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 = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols. ^{a-c} within a row, means without a common superscript differ ($p \leq 0.05$).

3.7 Dry-cured ham parameters

Figure 4 shows the progression of the ham yield during three stages of processing (final salting phase, 8-month curing phase, final curing phase), observed in the three experimental groups (C group, P-LOW and P-HIGH). No differences among groups were detected.

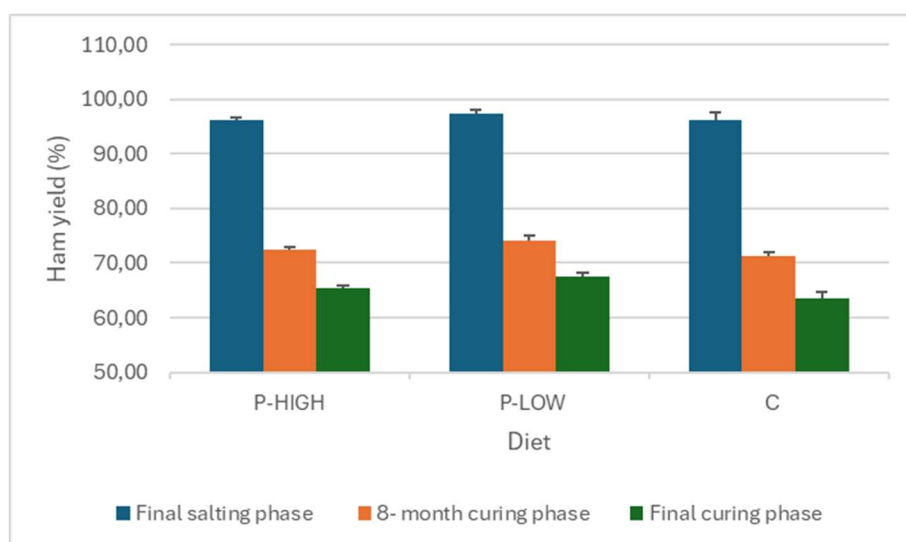


Figure 4. Ham yield parameters evaluated in the three experimental groups (C, P-LOW and P-HIGH) during three different phases of dry-cured ham processing. C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

3.8 Proximate composition

Proximate composition (protein, lipid, moisture and ashes content) of meat was not affected by dietary treatment. In dry-cured ham, the BF muscle showed higher ($p = 0.005$) moisture and lower ($p = 0.01$) ash contents for the P-HIGH group when compared to C.

Table 9. Proximate composition analyses of meat (*sternocleidomastoid*) and dry-cured ham (*semimembranosus* SM and *biceps femoris* BF) from pigs fed control or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
Meat					
<i>Sternocleidomastoid</i>					
Protein (%)	21.10	21.27	20.94	0.163	0.392
Lipid (%)	1.14	1.13	1.16	0.019	0.648
Moisture (%)	70.52	70.26	70.37	0.202	0.687
Ashes (%)	7.23	7.32	7.52	0.094	0.121
Dry-cured ham					
<i>Semimembranosus</i>					
Protein (%)	33.79	34.55	34.89	0.400	0.181
Lipid (%)	4.17	4.00	3.67	0.144	0.083
Moisture (%)	54.30	54.18	54.36	0.378	0.947
Ashes (%)	7.76	7.26	7.08	0.189	0.065
<i>Biceps femoris</i>					
Protein (%)	31.18	30.55	30.61	0.366	0.434
Lipid (%)	3.21	3.24	3.01	0.117	0.355
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	0.012

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

3.9 Fatty acid profile in dry-cured ham

Results of fatty acid composition in BF and SM are reported in Tables 10 and 11. 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 individual 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 ($p = 0.053$) to a decrease with the increase of polyphenol supplementation.

Table 10. Fatty acid profile of intramuscular fat of *biceps femoris* muscle in dry-cured ham from pigs fed control or polyphenol-supplemented diets.

Fatty acids	C	P-LOW	P-HIGH	SEM	p-value
<i>Biceps femoris</i>					
C14:0	1.45	1.56	1.43	0.090	0.579
C14:1	0.00	0.02	0.00	0.008	0.136
C15:1	0.01	0.00	0.00	0.004	0.141
C16:0	22.36	22.95	21.55	0.501	0.194
C16:1	3.08	3.41	3.25	0.111	0.180
C17:0	0.18	0.19	0.24	0.023	0.242
C18:0	10.54	10.52	10.33	0.556	0.955
C18:1n-9c	44.72	44.58	0.00	0.303	0.684
C18:1n-7c	4.55	4.40	4.75	0.113	0.153
C18:2n-6c	10.33	9.88	10.75	0.43	0.394
C18:3n-6	0.02	0.00	0.00	0.006	0.141
C18:3n-3	0.70	0.66	0.75	0.041	0.362
C20:0	0.14	0.12	0.13	0.010	0.525
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	0.502
C20:4 n-6	0.51	0.48	0.55	0.05	0.680
ΣSFA	34.76	35.34	33.67	0.55	0.152
ΣMUFA	53.07	53.00	53.57	0.230	0.216
ΣPUFA	12.26	11.66	12.76	0.473	0.306
PUFA/SFA	0.35	0.33	0.38	0.018	0.234

SEM: Standard Error of the Mean. SFA, Saturated fatty acid; MUFA, Monounsaturated fatty acid; PUFA, Polyunsaturated fatty acid. C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

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

Fatty acids	C	P-LOW	P-HIGH	SEM	p-value
<i>Semimembranosus</i>					
C12:0	0.11	0.13	0.11	0.008	0.130
C14:0	1.60	1.64	1.65	0.050	0.779
C16:0	22.69	22.85	22.50	0.139	0.253
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	0.396
C18:1n-9t	0.17	0.17	0.17	0.003	0.206
C18:1n-9c	43.88	44.06	44.24	0.215	0.525
C18:1n-7c	4.27	4.23	4.32	0.182	0.945
C18:2n-6c	10.07	10.02	9.99	0.228	0.968
C18:3n-6	0.04	0.05	0.05	0.008	0.546
C18:3n-3	0.63	0.64	0.64	0.016	0.873
C20:0	0.10	0.09	0.09	0.016	0.837
C20:1n-9	0.66	0.67	0.67	0.007	0.534
C20:2	0.40	0.38	0.39	0.038	0.960
C20:3 n-6	0.12	0.12	0.12	0.009	0.989
C20:4 n-6	0.41	0.40	0.49	0.038	0.279
ΣSFA	36.06	35.74	35.34	0.339	0.365
ΣMUFA	52.27	52.65	52.98	0.37	0.436
ΣPUFA	11.67	11.61	11.68	0.27	0.982
PUFA/SFA	0.32	0.32	0.33	0.009	0.837

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

3.10 Meat and subcutaneous fat quality

The pH and color parameters of meat evaluated on *gluteus medius* are reported in Table 12. No differences ($p > 0.05$) between the experimental groups were found for pH and lightness. Regarding the redness, a decrease in the polyphenol-supplemented groups was observed (with a significant difference between P-LOW and C), while the yellowness followed an opposite trend. Cooking loss decreased with the polyphenol supplementation. No differences between groups ($p > 0.05$) were reported for WB shear force. As for the subcutaneous fat (Table 12), pH values did not exhibit significant changes, while L^* increased ($p = 0.015$) and a^* values decreased in P-LOW compared to C ($p = 0.011$). No differences were reported for yellowness (Table 12).

Table 12. Quality traits of meat and subcutaneous fat of pigs fed control or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
<i>Gluteus medius</i>					
pH (24 h)	5.47	5.47	5.45	0.031	0.919
L^*	50.76	50.96	52.36	0.765	0.282
a^*	9.24 ^a	7.59 ^b	8.99 ^{ab}	0.426	0.018
b^*	4.59 ^a	5.36 ^{ab}	5.77 ^b	0.269	0.011
Cooking loss (%)	34.50 ^a	32.20 ^b	33.72 ^{ab}	0.535	0.025
WB shear force (kg/cm ²)	62.90	69.11	60.97	3.956	0.323
<i>Subcutaneous fat</i>					
pH (24 h)	5.83	5.80	5.90	0.030	0.067
L^*	71.37 ^{ab}	73.86 ^a	70.67 ^b	0.756	0.015
a^*	6.07 ^a	4.37 ^b	5.19 ^{ab}	0.370	0.011
b^*	6.56	5.74	6.03	0.285	0.137

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

3.11 Dry-cured ham and subcutaneous fat quality

The pH and color parameters of dry-cured ham evaluated in SM and BF are reported in Table 13. In SM, no differences ($p > 0.05$) between the experimental groups were found for pH, L^* and a^* values. Decreased values ($p = 0.01$) of yellowness, for both SM and BF, were found in the P-HIGH group. In the BF muscle, decreased ($p = 0.02$) values of L^* were also found in the same experimental group and no differences were found for the pH and a^* values. In the subcutaneous fat, b^* and L^* values decreased ($p = 0.02$) in the P-HIGH group (Table 13), while the pH increased ($p = 0.005$). Fat redness decreased in the P-LOW group ($p = 0.02$), when compared to C.

Table 13. Quality traits of dry-cured ham measured on *semimembranosus* and *biceps femoris* muscles and subcutaneous fat from pigs fed control or polyphenol-supplemented diets.

Item	C	P-LOW	P-HIGH	SEM	p-value
<i>Semimembranosus</i>					
pH	6.06	6.02	6.04	0.017	0.207
L*	38.8	36.7	36.2	0.812	0.081
a*	12.3	12.6	11.3	0.397	0.107
b*	8.02 ^{ab}	8.59 ^a	6.81 ^b	0.368	0.012
<i>Biceps femoris</i>					
pH	6.10	6.07	6.09	0.015	0.410
L*	40.8 ^{ab}	41.5 ^a	39.3 ^b	0.517	0.023
a*	13.1	13.01	13.3	0.227	0.650
b*	5.47 ^a	5.52 ^a	4.74 ^b	0.138	0.002
<i>Subcutaneous fat</i>					
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	0.029
a*	5.85 ^a	4.37 ^b	5.19 ^{ab}	0.317	0.016
b*	6.87 ^a	6.02 ^{ab}	5.74 ^b	0.256	0.018

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

3.12 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, except for SM which showed a lower value of springiness after supplementation with the highest dosage of polyphenols ($p = 0.007$).

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

Item	C	P-LOW	P-HIGH	SEM	p-value
<i>Semimembranosus</i>					
Peak force A (g)	8957.08	10004.90	7185.20	1188.457	0.268
Springiness	0.66 ^a	0.63 ^a	0.52 ^b	0.029	0.007
Chewiness	3305.08	4006.10	2306.30	505.219	0.089
Gumminess	5088.42	6257.40	4199.60	772.025	0.201
Cohesiveness	0.560	0.62	0.56	0.025	0.159
<i>Biceps femoris</i>					
Peak force A (g)	2150.08	2250.30	2496.80	343.194	0.767
Springiness	0.55	0.53	0.51	0.024	0.588
Chewiness	726.50	656.90	708.50	144.647	0.940
Gumminess	1256.17	1189.50	1333.20	216.25	0.896
Cohesiveness	0.57	0.52	0.52	0.016	0.067
<i>Subcutaneous fat</i>					
Peak force A (g)	2851.56	2492.10	2634.50	508.561	0.881
Springiness	0.53	0.59	0.53	0.025	0.274
Chewiness	669.33	716.10	718.00	151.103	0.967
Gumminess	1366.25	1251.00	1355.40	266.085	0.945
Cohesiveness	0.47	0.51	0.51	0.015	0.207

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

3.13 Carbonylated proteins levels

As shown in Figure 4A and 4B, hams from P-LOW and P-HIGH groups presented a lower content of carbonylated proteins, when compared to the C group ($p < 0.0001$).

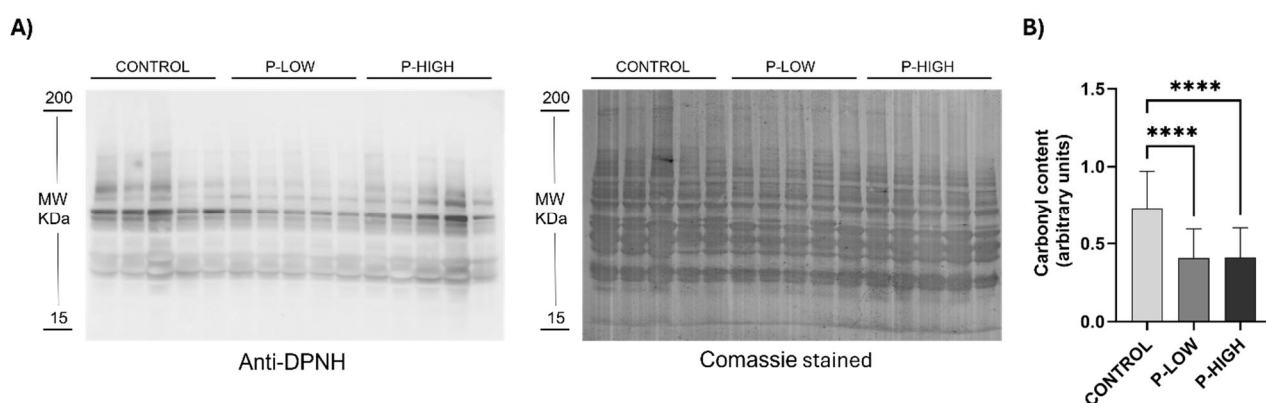


Figure 5. Protein carbonylation in dry-cured ham. A) Immunoblot of 2,4-dinitrophenylhydrazine (DNPH)-derivatized protein carbonyls and Coomassie-stained PVDF membrane (WB); B) Bar graph showing carbonylated protein levels expressed as the ratio of optical density (OD) of the immunoreactive lane to the OD of the corresponding lane in the Coomassie-stained PVDF membrane. **** $p < 0.0001$. C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

3.14 Lipid peroxidation in dry-cured ham

The levels of thiobarbituric acid reactive substances were lower in the P-LOW and P-HIGH groups compared to the C group (Table 17).

Table 15. Thiobarbituric acid reactive substances (TBARS) in dry-cured ham.

Item	C	P-LOW	P-HIGH	p-value
TBARS (mg MDA/kg)	2.51 ± 1.4^a	1.84 ± 1.4^b	1.37 ± 1.4^b	0.05

Data are expressed as means \pm standard deviation. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

3.15 Lipid peroxidation in dry-cured ham after *in vitro* gastro-intestinal digestion

Results of *in vitro* digestion of dry-cured ham reported a gradual increase in TBARS levels throughout the different phases of digestion (pre-digestive, gastric and intestinal phase). Moreover, during gastric digestion, lower TBARS levels ($p = 0.034$) were detected in the P-HIGH group (Figure 6).

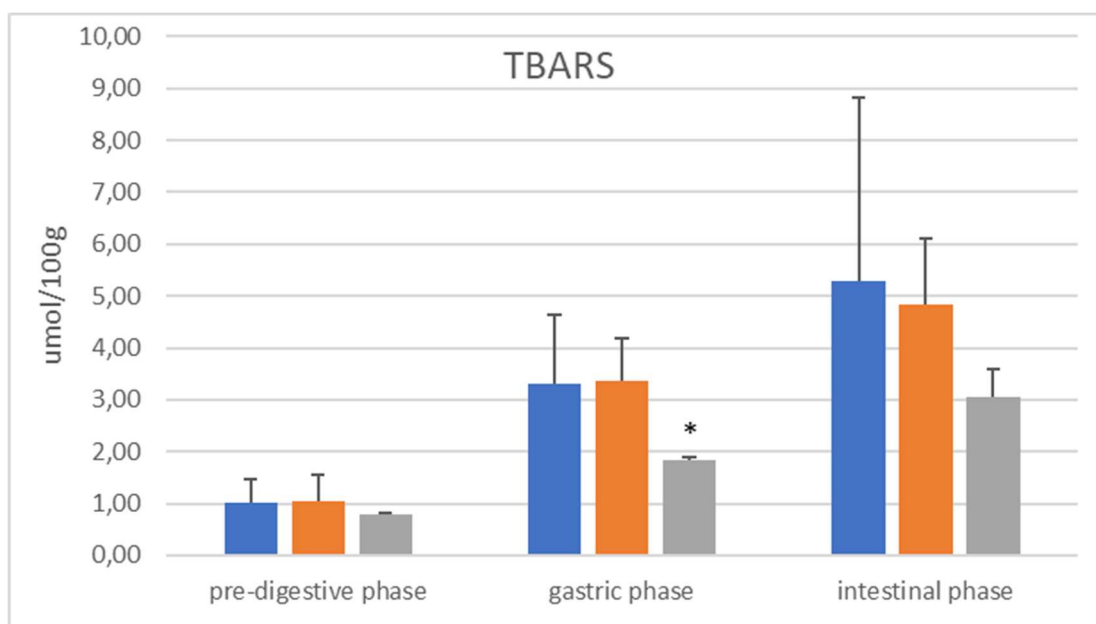


Figure 6. Effect of the different pig diets on the amount of lipid oxidation end-products (quantified as thiobarbituric acid reactive substances - TBARS) during *in vitro* digestion of pork dry-cured ham. TBARS were measured in the pre-digestive phase and at the end of the gastric and intestinal phase. Blue bars = Control; orange bars = control diet supplemented with 225 ppm OMWW polyphenols; gray bars = control diet supplemented with 74 ppm OMWW polyphenols. * $p < 0.05$.

3.16 Volatile compounds in dry-cured ham

Five aldehydes (hexanal, nonanal, octanal, 2-methylbutanal, 3-methyl-butanal) and one alcohol (3-methyl-1-butanol) were detected in dry-cured hams. Results showed a significant decrease in hexanal, nonanal, octanal and 3-methyl-butanal in the P-LOW and P-HIGH groups compared to C (Table 16). Concerning 2-methylbutanal and 3-methyl-1-butanol levels, differences between the groups were not significant ($p > 0.05$).

Table 16. Volatile compounds (μg of 4-methyl-2-pentanol equivalent/1000 g) in dry-cured ham. Data are expressed as means \pm standard deviation. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

Volatile compounds	C	P-LOW	P-HIGH	p-value
<i>Aldehydes</i>				
Hexanal	4.06 \pm 0.5 ^a	0.86 \pm 0.6 ^b	0.33 \pm 0.6 ^b	< 0.0001
Nonanal	7.44 \pm 0.5 ^a	1.81 \pm 0.5 ^b	1.44 \pm 0.5 ^b	< 0.0001
Octanal	4.06 \pm 0.5 ^a	0.86 \pm 0.6 ^b	0.33 \pm 0.6 ^b	< 0.0001
2-methyl-butanal	14.47 \pm 0.8	12.27 \pm 0.8	13.37 \pm 0.8	> 0.05
3-methyl-butanal	105.97 \pm 12.2 ^a	48.3 \pm 13.3 ^b	29.28 \pm 13.3 ^b	< 0.001
<i>Alcohols</i>				
3-methyl-1-butanol	46.79 \pm 5.9	62.19 \pm 6.4	49.2 \pm 6.4	> 0.05

3.17 Sensory evaluation

The P-LOW and P-HIGH dry-cured hams showed higher scores regarding appearance, taste, texture and overall liking compared to controls in both blind (Table 15) and informed (Table 16) tests. Furthermore, the blind test revealed a higher consumption ($p < 0.0001$) and purchase intention ($p < 0.0001$) for both P-LOW and P-HIGH hams compared to C (Figures 7A and 7B). The same trend was observed in the informed test (Figures 8A and 8B). In the blind test, consumers selected the intermediate price for hams of the P-HIGH group, while the highest price was chosen by a limited number of participants, regardless of the dietary treatment (Figure 9A). After receiving information regarding the dietary treatments, a higher number of consumers selected the lowest price for the C hams (Figure 9B). Finally, many consumers rated the control hams as saltier than the OMMW polyphenols products.

Table 15. Mean of the consumers' scores based on a 9- points hedonic scale (1= dislike extremely, 9 = like extremely) in the blind test. SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

Item	C	P-LOW	P-HIGH	SEM	p-value
<i>Blind</i>					
Appearance	6.92 ^b	7.38 ^a	7.38 ^a	0.157	< 0.05
Taste	5.89 ^b	6.75 ^a	7.20 ^a	0.168	< 0.0001
Texture	6.46 ^b	7.01 ^a	7.26 ^a	0.144	< 0.01
Overall liking	6.24 ^b	6.93 ^a	7.29 ^a	0.157	< 0.0001

Table 16. Mean of the consumers' scores based on a 9- points hedonic scale (1= dislike extremely, 9 = like extremely) in the informed test. SEM: Standard Error of the Mean. Values in the same row with different superscripts differ significantly ($p \leq 0.05$). C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

Item	C	P-LOW	P-HIGH	SEM	p-value
Informed					
Appearance	6.50 ^b	7.34 ^a	7.69 ^a	0.136	< 0.0001
Taste	5.96 ^b	7.09 ^a	7.56 ^a	0.145	< 0.0001
Texture	6.26 ^b	7.19 ^a	7.58 ^a	0.144	< 0.0001
Overall liking	6.06 ^b	7.27 ^a	7.70 ^a	0.133	< 0.0001

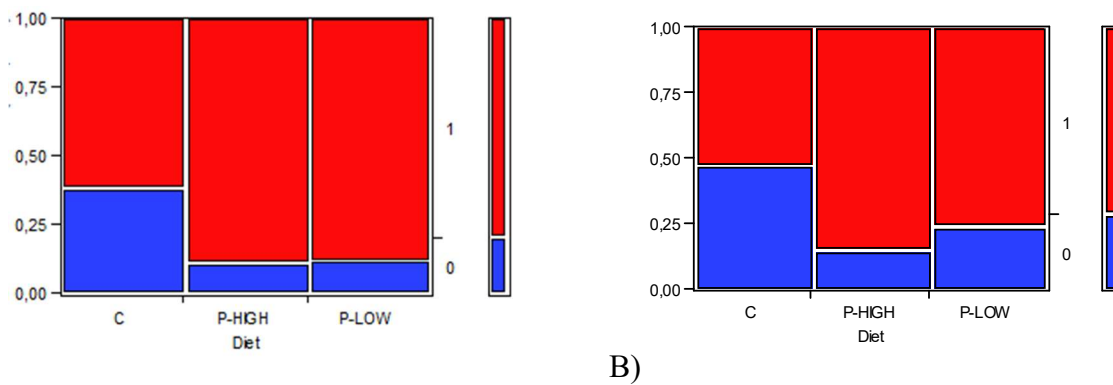


Figure 7. Blind consumer test on consumption (A) and purchase intention (B) as affected by dietary treatment (1 = “yes”; 0 = “no”). C= Control; P-LOW = C diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = C diet supplemented with 225 ppm OMWW polyphenols.

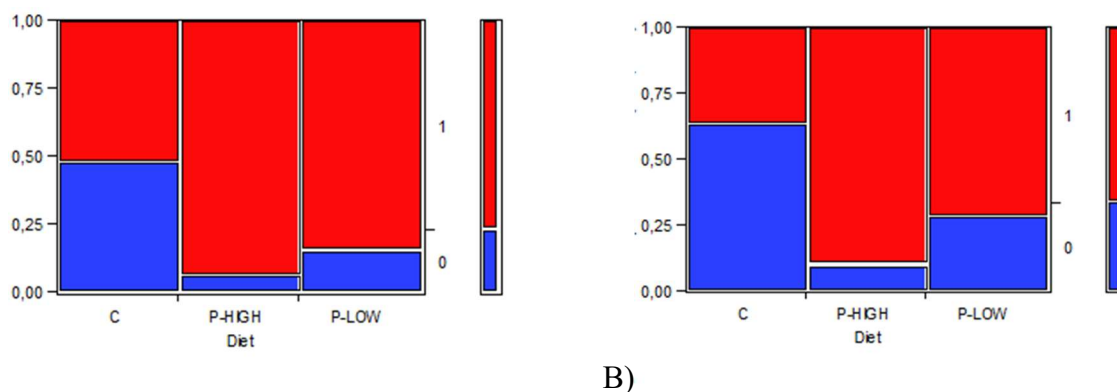


Figure 8. Informed consumer test on consumption (A) and purchase intention (B) as affected by dietary treatment (1 = “yes”; 0 = “no”). C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

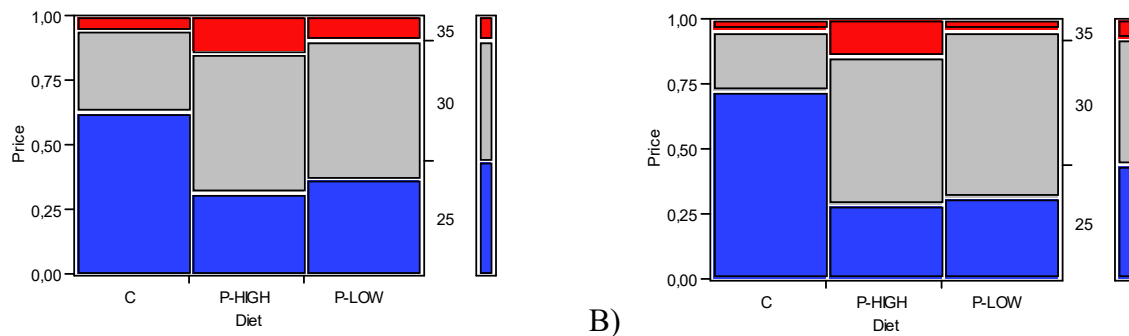


Figure 9. Acceptable price range determined in blind (A) and informed (B) consumer tests as affected by dietary treatment. C= Control; P-LOW = diet supplemented with 74 ppm OMWW polyphenols; P-HIGH = diet supplemented with 225 ppm OMWW polyphenols.

4. Discussion

Olive mill waste water and phenolic extracts deriving from this matrix have been recently 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 as food supplements in the diet of livestock animals, including pigs and poultry. The importance of phenolic compounds is mainly related to their antioxidant role (Branciari et al. 2017). Furthermore, olive polyphenols are highly water soluble and, because of that, mainly dispersed in the wastewater (Bertin et al. 2011). 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, despite a tendency ($p = 0.07$) toward a statistical difference in final BW, with pigs of the P-HIGH group showing the highest value (+5.3 % compared to C). Several studies reported that olive cake, one of the most used olive by-product in animal nutrition, failed to influence growth performance 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). It is also relevant to consider that the effects of natural antioxidants in animals are neither consistent nor easily predictable, as they are related to several factors, including molecule type, dosage, diet, animal health status and environmental conditions. 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

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).

4.3 Histopathological and morphometric findings

The absence of macroscopic and microscopic tissue changes in all examined tissues (liver, ovaries, uterus, adipose tissue, and skeletal muscle) in treated and untreated animals suggests a potentially limited effect of polyphenol supplementation on tissue morphology. Concerning the macroscopical assessment of pubertal status, there were no differences in the onset of puberty between pigs from the three experimental groups (Table 4). However, 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).

4.4 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 the oxidative environment (Aviram et al., 1998). 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 was associated to the P-HIGH group is a potential indication of the increased antioxidant activity in blood (Figure 3).

4.5 Antioxidant activity in liver and muscle

In the present study, the antioxidant activity of *sternocleidomastoid* muscle and liver was evaluated through a series of assays (DPPH, CUPRAC, and ABTS). The 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, in the same tissue, a difference in the RSA values between the P-HIGH and the P-LOW groups was shown, suggesting that the increase of the dosage can improve the effect of the OMWW phenolic extract. No significant differences among 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 was 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 significant changes in the antioxidant activity of poultry meat after dietary supplementation with an olive freeze-dried powder containing 2.5% of hydroxytyrosol (King et al. 2014). Other studies such as the one from Branciaro 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*) (Branciaro 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 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 any increase of the antioxidant activity in the liver of pigs following dietary supplementation with a plant mixture rich in 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 values was relatively small (Table 8). One possible reason could be related to the muscular matrix itself. 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 the literature findings, the variations in the outcomes of these three assays could be related to the different antioxidant capacity of each compound (including polyphenols) that interacts with the radicals used in the analyses (Echegaray et al. 2020). Moreover, very few antioxidant assays have been performed so far on pig tissues, especially the liver, suggesting the need for further detailed studies to expand the application of these analyses in monogastric animals.

4.6 Proximate composition

In dry-cured ham, particularly in the BF muscle, an increase in moisture and a decrease in ash content was reported for the P-HIGH group (Table 9). 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 these factors 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 the BF muscle, which is more protected by skin and subcutaneous fat, compared to SM (Marušić Radovčić et al. 2021). The increase in water retention capacity by polyphenols is supported by the outcomes of the sensorial analysis, which showed that hams from pigs of the polyphenol-supplemented groups were more appreciated and considered less salty than the controls (Section 4.15).

4.7 Fatty acid profile in dry-cured ham

The dietary OMWW polyphenols did not seem to influence the acidic composition of the SM and BF muscles, in terms of total SFA, MUFA, PUFA and PUFA/SFA ratio and the single fatty acids investigated, despite the decrease in yellowness observed in these muscles following dietary supplementation with the highest polyphenol dosage.

4.8 Meat and subcutaneous 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 no differences between experimental groups were detected (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 become more prominent during preservation rather than in raw meat (Garrido et al. 2011; Branciarri et al. 2015). Dietary antioxidants are usually known for their protective role on myoglobin, whose oxidation and reduction are related to meat discoloration (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 and thus redness color. In addition, our analysis showed higher b^* values with the increasing dosage of OMWW polyphenols. This change in the b^* value could also be related to the natural pigmentation of the phenolic OMWW extract, that gives a brownish color 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, cooking loss decreased with polyphenols' supplementation (although the statistical significance was reached only for the P-LOW group). Xu et al. (2021) exhibited that certain water-soluble polyphenols (gallic acid, epigallocatechin gallate and tannic acid) enhanced the stability of myofibrillar proteins in gel and confirmed these outcomes on pork meatballs (Xu et al. 2021). Similarly, hydroxytyrosol and tyrosol, the main polyphenols contained in OMWW extract, can interact with water and other polar compounds through hydrogen bonds and can potentially reduce the hydrophobic surface of myofibrils, maintaining their strength and finally reducing meat cooking loss. Despite the observed effect of the phenolic dietary treatments on cooking loss, meat lightness values were not affected, 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). Finally, the lightness and redness parameters have shown variations in subcutaneous fat that need further investigation (Table 12).

4.9 Dry-cured ham and subcutaneous fat quality

In the dry-cured ham, the pH of the two analyzed muscles (SM and BF) did not change among experimental groups. Although the dietary addition of polyphenols stimulated an increase in moisture in the P-HIGH BF muscle, this group showed lower L^* values (Table 13). This suggests that lightness can vary in meat products independently of water content, as observed in several meat products (Gimeno et al. 2001). In both SM and BF muscles, redness values were consistent among experimental groups, and this is relevant for the consumers' acceptability (Morales et al. 2013).

Parolari et al. (2016) reported, in both SM and BF muscles of 12 months-seasoning hams, a^* values that are in line with our findings (Parolari et al. 2016). The stability of the a^* value indicates a protection of the myoglobin redox state and red color in the ham, which may be correlated to the observed lower yellowness in the P-HIGH group (Serra et al. 2018; Iftikhar et al. 2024). During ham seasoning, color changes are related to the increased concentration of the pigments that is induced by salt addition (Møller et al. 2003; Giovanelli et al. 2016). The concentration of pigments stimulates myoglobin transformation and thus the typical red color of dry-cured ham, which is darker compared to the fresh meat (Chizzolini et al. 1996) as confirmed in our analyses (Tables 12 and 13). Moreover, results corroborated that a^* values were higher in BF compared to SM, as demonstrated by other authors (Pérez-Alvarez et al. 1999; García-Esteban et al. 2003; Marušić Radovčić et al. 2021), and that BF had a greater lightness than SM, due to the higher moisture and protection of the former muscle (Tables 12 and 13) (Giovanelli et al. 2016). The yellowness of the dry-cured ham depends on the development of rancidity caused by lipid oxidation (Ruiz et al. 2002). Polyphenols, thanks to their antioxidant properties, may protect from lipid oxidation and its negative consequences in meat and derived products (Vasilopoulos et al. 2022; Bolletta et al. 2024; Jovanovichs et al. 2025). In the present study, the decrease in b^* value in SM and BF hams of the P-HIGH group could be related to the protection of oxidation of intra-muscular lipids induced by the highest phenolic dosage. Similarly, Serra et al. (2018) reported a decrease of the b^* value in Cinta Senese sausages after a dietary supplementation with olive pomace (Serra et al. 2018).

Interestingly, the same effect is reported on subcutaneous fat, where b^* values decreased in the group supplemented with the highest dosage of polyphenols. The P-HIGH group also presented an increase in pH which can be correlated to the lower fat lightness (Lawrie's Meat Science 2022) and higher a^* values (Table 13).

To conclude, the results of our study are not easily comparable with the literature, because quality parameters, including color of dry-cured ham, depend on several internal (animal genotype and age, pigment concentrations in the muscle, fatty acid content and composition) and external factors (processing of the cured products) (Gilles 2009; Parolari et al. 2016).

4.10 Texture properties

The two dietary dosages did not seem to affect the texture properties of dry-cured ham. However, springiness (i.e. elasticity) in the SM muscle decreased with the 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 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 treatments and processing (Zhou et al. 2020).

4.11 Carbonylated proteins levels in dry-cured ham

The effect of polyphenols on ham protein oxidation was assessed by evaluating carbonyl concentrations. Protein oxidation typically occurs in dry-cured ham and other meat products during processing, and protein carbonyls are generally used as a marker, representing the most common deleterious changes in muscle proteins (Davies et al. 2004). In particular, several findings evidenced that, during ripening and salting processes, protein carbonylation (Ventanas et al. 2006; Wang et al. 2011; Koutina et al. 2012; Li et al. 2020), reveals an impact on protein oxidation. Furthermore, protein carbonylation can affect meat quality traits including water holding capacity, color, sensory

parameters (e.g. texture and tenderness) and nutritional value (Rowe et al. 2004; Estévez and Cava 2004). A protective strategy against carbonylation may consist in the use of dietary supplementations that are known to improve the oxidative state of animal tissues (Estévez and Cava 2004). In the present study, the hams of the OMWW polyphenols-treated groups presented lower content of carbonylated proteins, compared to the C group. Similarly, in a study of Jongberg et al. (2013), protein carbonylation was reduced in Bologna type pork sausages after the addition of phenolic-rich plant extracts (Jongberg et al. 2013).

4.12 Lipid oxidation in dry-cured ham

Lipid oxidation is the main process responsible for the worsening of meat and meat products' shelf-life and affecting their nutritional and sensory quality (color, texture, aroma and taste) (Amaral et al., 2018). The degradation of lipids (i.e. unsaturated fatty acids) spontaneously occurs during meat and meat products' storage, with the development of oxidative rancidity products (Colonna et al., 2021; Amaral et al., 2018). The control of these processes is fundamental for consumers' acceptability, as they are responsible for off-flavors and bad taste (Amaral et al. 2018). One of the most important functions of polyphenols consists in the protection from lipid oxidation by chelating transition metal ions (Visioli et al. 1998). Several olive oil by-products rich in polyphenols recently demonstrated their antioxidant action against lipid peroxidation also in raw and cured meat products (Serra et al. 2018; Barbieri et al. 2021; Vasilopoulos et al. 2022; Bolletta et al. 2024; Iftikhar et al. 2024; Jovanovichs et al. 2025). The present study reports a significant difference in TBARS values between the experimental groups, with a decrease in the polyphenol-supplemented hams compared to the controls. This effect suggests a protective role from lipid peroxidation and, consequently, from an excessive accumulation of the volatile compounds generated during dry-cured ham processing.

4.13 Lipid oxidation after *in vitro* digestion of dry-cured ham

During the *in vitro* digestion of dry-cured ham, as expected, the levels of TBARS increased regardless of the treatment, because bile salts and iron, acting in the digestive phases stimulate the increase in lipid oxidation (Berton-Carabin et al. 2014). The findings of lower TBARS in the P-HIGH group during gastric digestive phase was confirmed by Martini et al. (2018) who, with the same *in vitro* stimulation, reported similar data after using other dietary antioxidant compounds.

4.14 Volatile compounds in dry-cured ham

During dry-cured ham processing, lipid oxidation occurs naturally and is responsible for the sensory characteristics typical of the final product e.g. odor, flavor and aroma. In particular, the oxidation and degradation of unsaturated free fatty acids results in the formation of volatile compounds including aldehydes and alcohols, which are crucial for the development of specific sensorial properties in dry-cured ham (Petrova et al. 2015). However, it is known that an excessive formation of volatile aldehydes can be detrimental for meat products' shelf life (Serra et al. 2018; Liu et al. 2020; Zhang et al. 2024). Among these aldehydes, hexanal is the first marker of rancid taste, odor and flavor in dry-cured ham (García-González et al. 2008; Karolyi et al. 2024). Nonanal and octanal are mainly responsible for the rancid odor, while 3-methylbutanal is frequently associated with rancid taste. The reduced levels of hexanal, nonanal, octanal, and 3-methylbutanal in dry-cured hams of the P-LOW and P-HIGH groups, when compared to C, suggest a protective role from OMWW polyphenols against the peroxidation of unsaturated fatty acids (i.e., rancidity). Similarly, Serra et al. (2018) reported a decrease in hexanal values in sausages from pigs supplemented with olive pomace.

Concerning 3-methyl-1-butanol, it is one of the most representative alcohols detected in dry-cured hams. In the present study, this volatile compound had a marked increase after supplementation

with the highest phenolic dosage compared to the other two diets, but without reaching a significance. This could be considered an additional indication of the protective action against oxidation, as shown by Comi et al. (2014) in San Daniele hams. These authors reported that the unspoiled hams had higher concentrations of alcohols (including 3-methyl-1-butanol) and lower concentrations of aldehydes. Other studies reported that 3-methyl-1-butanol is responsible for the typical flavor and odor (specifically, the so-called “green aroma”) of many dry-cured hams (Sánchez-Peña et al. 2005; Ramírez and Cava 2007; Zhu et al. 2018).

4.15 Sensory analysis

In both blind and informed tests, hams from P-LOW and P-HIGH groups were more appreciated in terms of appearance, taste, texture and overall liking. This could be related to the presence of polyphenols in pigs’ diet, which may enhance the overall quality and shelf-life of meat products (Zhang et al. 2010). Similarly, a study of Rossi et al. (2020) demonstrated that the dry-cured ham Speck from pigs fed with an antioxidant mixture (vitamin E and verbascoside) was more appreciated than controls (Rossi et al. 2021). Furthermore, in both tests, consumers showed a higher intention to consume and purchase hams from pigs supplemented with OMWW polyphenols and preferred the intermediate price over the control hams. Interestingly, when participants were informed about the dietary supplementation (informed test), their preference for the treated hams increased further, while a lower price was assigned to the control hams. Finally, hams of the polyphenols-supplemented groups were rated as less salty than those from the control group. This can be considered a favourable result, assuming that modern consumers’ preferences have recently shifted towards the use of reduced salt levels in cured products, due to health concerns (Şevkat Uğuz, Ayla Soyer, Ülkü Dalmış 2010).

5. Conclusion

Dietary supplementation with two different dosages (74 ppm and 225 ppm) of polyphenols from OMWW in finishing pigs’ diet did not negatively affect animal productive performances. Phenolic compounds (particularly at 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. OMWW polyphenols were shown to increase water retention capacity in meat and dry-cured ham, preserve redness stability and protect from lipid oxidation in the latter, as shown by the decrease in yellowness values. The protective effects on ham lipid oxidation were confirmed by the decrease in TBARS levels (even after the stimulation of an *in vitro* gastric digestion) and in volatile compounds. Furthermore, a reduction in protein carbonylation was detected in the hams from the polyphenols-supplemented pigs. The use of OMWW polyphenols in the diet positively influenced consumers’ sensory acceptability of the dry-cured ham. The potential effects of polyphenols on the color parameters of meat and dry-cured ham can be related to the increase in moisture as well as to other factors (e.g. the presence and nature of the pigments) that need further investigations. Overall, the influence of polyphenols from olive oil industry on the oxidation processes typically occurring in dry-cured ham is an interesting development of research, potentially following the modern consumer demand.

References

FAOSTAT. [accessed 2024 Oct 24]. <https://www.fao.org/faostat/en/#data/QCL>

Alimenti C, Lianza M, Antognoni F, Giusti L, Bistoni O, Liotta L, Angeloni C, Lupidi G, Beghelli D. 2023. Characterization and Biological Activities of In Vitro Digested Olive Pomace Polyphenols Evaluated on Ex Vivo Human Immune Blood Cells. *Molecules*. 28(5):2122. <https://doi.org/10.3390/molecules28052122>

Altissimi C, Roila R, Ranucci D, Branciarri R, Cai D, Paulsen P. 2024. Preventing Microbial Growth in Game Meat by Applying Polyphenolic Extracts from Olive Mill Vegetation Water. *Foods*. 13(5):658. <https://doi.org/10.3390/foods13050658>

Amaral AB, Silva MVD, Lannes SCDS. 2018. Lipid oxidation in meat: mechanisms and protective factors – a review. *Food Sci Technol*. 38(suppl 1):1–15. <https://doi.org/10.1590/fst.32518>

AOAC: Official Methods of Analysis (Volume 1).

Apak R, Güçlü K, Ozyürek M, Karademir SE. 2004. Novel total antioxidant capacity index for dietary polyphenols and vitamins C and E, using their cupric ion reducing capability in the presence of neocuproine: CUPRAC method. *J Agric Food Chem*. 52(26):7970–7981. <https://doi.org/10.1021/jf048741x>

Baldioli M, Servili M, Perretti G, Montedoro GF. 1996. Antioxidant activity of tocopherols and phenolic compounds of virgin olive oil. *Journal of the American Oil Chemists' Society*. 73(11):1589–1593. <https://doi.org/10.1007/BF02523530>

Barbieri S, Mercatante D, Balzan S, Esposto S, Cardenia V, Servili M, Novelli E, Taticchi A, Rodriguez-Estrada MT. 2021. Improved Oxidative Stability and Sensory Quality of Beef Hamburgers Enriched with a Phenolic Extract from Olive Vegetation Water. *Antioxidants (Basel)*. 10(12):1969. <https://doi.org/10.3390/antiox10121969>

Benzie IF, Strain JJ. 1996. The ferric reducing ability of plasma (FRAP) as a measure of “antioxidant power”: the FRAP assay. *Anal Biochem*. 239(1):70–76. <https://doi.org/10.1006/abio.1996.0292>

Bertin L, Ferri F, Scoma A, Marchetti L, Fava F. 2011. Recovery of high added value natural polyphenols from actual olive mill wastewater through solid phase extraction. *Chemical Engineering Journal*. 171(3):1287–1293. <https://doi.org/10.1016/j.cej.2011.05.056>

Berton-Carabin CC, Ropers M-H, Genot C. 2014. Lipid Oxidation in Oil-in-Water Emulsions: Involvement of the Interfacial Layer. *Comprehensive Reviews in Food Science and Food Safety*. 13(5):945–977. <https://doi.org/10.1111/1541-4337.12097>

Bionaz M, Trevisi E, Calamari L, Librandi F, Ferrari A, Bertoni G. 2007. Plasma paraoxonase, health, inflammatory conditions, and liver function in transition dairy cows. *J Dairy Sci*. 90(4):1740–1750. <https://doi.org/10.3168/jds.2006-445>

Bolletta V, Menci R, Valenti B, Morbidini L, Servili M, Taticchi A, Lilli E, Pauselli M. 2024. Feeding pigs with hazelnut skin and addition of a concentrated phenolic extract from olive-milling wastewaters during pork processing: Effects on salami quality traits and acceptance by the consumers. *Meat Science*. 213:109479. <https://doi.org/10.1016/j.meatsci.2024.109479>

- Branciarri R, Galarini R, Giusepponi D, Trabalza-Marinucci M, Forte C, Roila R, Miraglia D, Servili M, Acuti G, Valiani A. 2017. Oxidative Status and Presence of Bioactive Compounds in Meat from Chickens Fed Polyphenols Extracted from Olive Oil Industry Waste. *Sustainability*. 9(9):1566. <https://doi.org/10.3390/su9091566>
- Branciarri R, Ranucci D, Miraglia D, Urbani S, Esposto S, Servili M. 2015. Effect of Dietary Treatment with Olive Oil By-Product (Olive Cake) on Physicochemical, Sensory and Microbial Characteristics of Beef During Storage. *Italian Journal of Food Safety*. 4(4):5496. <https://doi.org/10.4081/ijfs.2015.5496>
- Branciarri R, Ranucci D, Ortenzi R, Roila R, Trabalza-Marinucci M, Servili M, Papa P, Galarini R, Valiani A. 2016. Dietary Administration of Olive Mill Wastewater Extract Reduces *Campylobacter* spp. Prevalence in Broiler Chickens. *Sustainability*. 8(9):837. <https://doi.org/10.3390/su8090837>
- Buege JA, Aust SD. 1978. [30] Microsomal lipid peroxidation. In: Fleischer S, Packer L, editors. *Methods in Enzymology* [Internet]. Vol. 52. [place unknown]: Academic Press; [accessed 2025 Mar 13]; p. 302–310. [https://doi.org/10.1016/S0076-6879\(78\)52032-6](https://doi.org/10.1016/S0076-6879(78)52032-6)
- Caballero-Guerrero B, Garrido-Fernández A, Feroso FG, Rodríguez-Gutierrez G, Fernández-Prior MÁ, Reinhard C, Nyström L, Benítez-Cabello A, Arroyo-López FN. 2022. Antimicrobial effects of treated olive mill waste on foodborne pathogens. *LWT*. 164:113628. <https://doi.org/10.1016/j.lwt.2022.113628>
- Caparra P, Chies L, Scerra M, Foti F, Bognanno M, Cilione C, De Caria P, Claps S, Cifuni GF. 2023. Effect of Dietary Ensiled Olive Cake Supplementation on Performance and Meat Quality of Apulo-Calabrese Pigs. *Animals*. 13(12):2022. <https://doi.org/10.3390/ani13122022>
- Chaves-López C, Serio A, Mazzarrino G, Martuscelli M, Scarpone E, Paparella A. 2015. Control of household mycoflora in fermented sausages using phenolic fractions from olive mill wastewaters. *International Journal of Food Microbiology*. 207:49–56. <https://doi.org/10.1016/j.ijfoodmicro.2015.04.040>
- Chiaradia E, Sansone A, Ferreri C, Tancini B, Latella R, Tognoloni A, Gambelunghe A, dell’Omo M, Urbanelli L, Giovagnoli S, et al. 2023. Phospholipid fatty acid remodeling and carbonylated protein increase in extracellular vesicles released by airway epithelial cells exposed to cigarette smoke extract. *Eur J Cell Biol*. 102(1):151285. <https://doi.org/10.1016/j.ejcb.2022.151285>
- Chizzolini R, Novelli E, Campanini G, Dazzi G, Madarena G, Zanardi E, Pacchioli MT, Rossi A. 1996. Lean colour of green and matured Parma hams: Comparative evaluation and technological relevance of sensory and objective data. *Meat Science*. 44(3):159–172. [https://doi.org/10.1016/S0309-1740\(96\)00034-4](https://doi.org/10.1016/S0309-1740(96)00034-4)
- Cisneros F, Ellis M, Miller KD, Novakofski J, Wilson ER, McKeith FK. 1996. Comparison of transverse and longitudinal real-time ultrasound scans for prediction of lean cut yields and fat-free lean content in live pigs. *Journal of Animal Science*. 74(11):2566. <https://doi.org/10.2527/1996.74112566x>
- Comi G, Manzano M, Bricchese R, Iacumin L. 2014. New Cause of Spoilage in S an D aniele Dry Cured Ham. *Journal of Food Safety*. 34(4):263–269. <https://doi.org/10.1111/jfs.12122>

- Dal Bosco A, Mourvaki E, Cardinali R, Servili M, Sebastiani B, Ruggeri S, Mattioli S, Taticchi A, Esposito S, Castellini C. 2012. Effect of dietary supplementation with olive pomaces on the performance and meat quality of growing rabbits. *Meat Science*. 92(4):783–788. <https://doi.org/10.1016/j.meatsci.2012.07.001>
- Davies LC, Vilhena AM, Novais JM, Martins-Dias S. 2004. Olive mill wastewater characteristics: modelling and statistical analysis. *Grasas y Aceites*. 55(3):233–241. <https://doi.org/10.3989/gya.2004.v55.i3.171>
- Dedousi A, Kritsa M-Z, Sossidou EN. 2023. Thermal Comfort, Growth Performance and Welfare of Olive Pulp Fed Broilers during Hot Season. *Sustainability*. 15(14):10932. <https://doi.org/10.3390/su151410932>
- Duque-Acevedo M, Belmonte-Ureña LJ, Cortés-García FJ, Camacho-Ferre F. 2020. Agricultural waste: Review of the evolution, approaches and perspectives on alternative uses. *Global Ecology and Conservation*. 22:e00902. <https://doi.org/10.1016/j.gecco.2020.e00902>
- Echegaray N, Munekata PES, Centeno JA, Domínguez R, Pateiro M, Carballo J, Lorenzo JM. 2020. Total Phenol Content and Antioxidant Activity of Different Celta Pig Carcass Locations as Affected by the Finishing Diet (Chestnuts or Commercial Feed). *Antioxidants*. 10(1):5. <https://doi.org/10.3390/antiox10010005>
- Estévez M, Cava R. 2004. Lipid and protein oxidation, release of iron from heme molecule and colour deterioration during refrigerated storage of liver pâté. *Meat Sci*. 68(4):551–558. <https://doi.org/10.1016/j.meatsci.2004.05.007>
- Ferlisi F, De Ciucis CG, Trabalza-Marinucci M, Fruscione F, Mecocci S, Franzoni G, Zinellu S, Galarini R, Razzuoli E, Cappelli K. 2024. Olive Mill Waste-Water Extract Enriched in Hydroxytyrosol and Tyrosol Modulates Host–Pathogen Interaction in IPEC-J2 Cells. *Animals*. 14(4):564. <https://doi.org/10.3390/ani14040564>
- Fernández-López J, Sevilla L, Sayas-Barberá E, Navarro C, Marín F, Pérez-Alvarez J a. 2003. Evaluation of the Antioxidant Potential of Hyssop (*Hyssopus officinalis* L.) and Rosemary (*Rosmarinus officinalis* L.) Extracts in Cooked Pork Meat. *Journal of Food Science*. 68(2):660–664. <https://doi.org/10.1111/j.1365-2621.2003.tb05727.x>
- Ferré N, Camps J, Prats E, Vilella E, Paul A, Figuera L, Joven J. 2002. Serum paraoxonase activity: a new additional test for the improved evaluation of chronic liver damage. *Clin Chem*. 48(2):261–268.
- Ferreira I, Leite A, Vasconcelos L, Rodrigues S, Mateo J, Munekata PES, Teixeira A. 2022. Sodium Reduction in Traditional Dry-Cured Pork Belly Using Glasswort Powder (*Salicornia herbacea*) as a Partial NaCl Replacer. *Foods*. 11(23):3816. <https://doi.org/10.3390/foods11233816>
- Ferreira VCDS, Martins TDD, Batista EDS, Santos EPD, Silva FAPD, Araújo ÍBDS, Nascimento MCO. 2013. Physicochemical and microbiological parameters of dried salted pork meat with different sodium chloride levels. *Food Sci Technol*. 33(2):382–386. <https://doi.org/10.1590/S0101-20612013005000055>
- Ferrer P, Calvet S, García-Rebollar P, De Blas C, Jiménez-Belenguer AI, Hernández P, Piquer O, Cerisuelo A. 2020. Partially defatted olive cake in finishing pig diets: implications on performance,

faecal microbiota, carcass quality, slurry composition and gas emission. *Animal*. 14(2):426–434. <https://doi.org/10.1017/S1751731119002040>

Forte C, Ranucci D, Beghelli D, Branciaro R, Acuti G, Todini L, Cavallucci C, Trabalza-Marinucci M. 2017. Dietary integration with oregano (*Origanum vulgare* L.) essential oil improves growth rate and oxidative status in outdoor-reared, but not indoor-reared, pigs. *J Anim Physiol Anim Nutr (Berl)*. 101(5):e352–e361. <https://doi.org/10.1111/jpn.12612>

Fuhrman B, Aviram M. 2002. Preservation of Paraoxonase Activity by Wine Flavonoids. *Annals of the New York Academy of Sciences*. 957(1):321–324. <https://doi.org/10.1111/j.1749-6632.2002.tb02933.x>

Gallego M, Mora L, Concepción Aristoy M, Toldrá F. 2016. The use of label-free mass spectrometry for relative quantification of sarcoplasmic proteins during the processing of dry-cured ham. *Food Chem*. 196:437–444. <https://doi.org/10.1016/j.foodchem.2015.09.062>

García Casco JM, Muñoz M, Martínez Torres JM, García López A, González Barroso MÁ, Gonzáles-Sánchez E. 2017. Alternative Feeding in Iberian Pigs during Growth Period: Incorporation of Olive Cake in a Dry or Wet (silage) Form. *Agriculturae Conspectus Scientificus*. 82(2):147–150.

García-González DL, Tena N, Aparicio-Ruiz R, Morales MT. 2008. Relationship between sensory attributes and volatile compounds qualifying dry-cured hams. *Meat Sci*. 80(2):315–325. <https://doi.org/10.1016/j.meatsci.2007.12.015>

García-Esteban M, Ansorena D, Gimeno O, Astiasarán I. 2003. Optimization of instrumental colour analysis in dry-cured ham. *Meat Science*. 63(3):287–292. [https://doi.org/10.1016/S0309-1740\(02\)00084-0](https://doi.org/10.1016/S0309-1740(02)00084-0)

Garrido MD, Auqui M, Martí N, Linares MB. 2011. Effect of two different red grape pomace extracts obtained under different extraction systems on meat quality of pork burgers. *LWT - Food Science and Technology*. 44(10):2238–2243. <https://doi.org/10.1016/j.lwt.2011.07.003>

Gibson-Corley KN, Olivier AK, Meyerholz DK. 2013. Principles for valid histopathologic scoring in research. *Vet Pathol*. 50(6):1007–1015. <https://doi.org/10.1177/0300985813485099>

Gilles G. 2009. Dry cured ham quality as related to lipid quality of raw material and lipid changes during processing: a review. *Grasas y Aceites*. 60(3):297–307. <https://doi.org/10.3989/gya.130908>

Gimeno O, Astiasarán I, Bello J. 2001. Calcium ascorbate as a potential partial substitute for NaCl in dry fermented sausages: effect on colour, texture and hygienic quality at different concentrations. *Meat Sci*. 57(1):23–29. [https://doi.org/10.1016/s0309-1740\(00\)00070-x](https://doi.org/10.1016/s0309-1740(00)00070-x)

Giovanelli G, Buratti S, Laureati M, Pagliarini E. 2016. Evolution of physicochemical, morphological and aromatic characteristics of Italian PDO dry-cured hams during processing. *Eur Food Res Technol*. 242(7):1117–1127. <https://doi.org/10.1007/s00217-015-2616-6>

Hashem NM, Gonzalez-Bulnes A, Simal-Gandara J. 2020. Polyphenols in Farm Animals: Source of Reproductive Gain or Waste? *Antioxidants (Basel)*. 9(10):1023. <https://doi.org/10.3390/antiox9101023>

- He P, Lei Y, Zhang K, Zhang R, Bai Y, Li Z, Jia L, Shi J, Cheng Q, Ma Y, et al. 2023. Dietary oregano essential oil supplementation alters meat quality, oxidative stability, and fatty acid profiles of beef cattle. *Meat Science*. 205:109317. <https://doi.org/10.1016/j.meatsci.2023.109317>
- Hernández-Matamoros A, Breña M, Cebrián M, Sereno J, Sánchez EG. 2011. Use of olive cake and tomato peel in the Iberian pig feed. [Internet]. In: [place unknown]; [accessed 2024 Nov 12]. <https://www.semanticscholar.org/paper/Use-of-olive-cake-and-tomato-peel-in-the-Iberian-Hern%C3%A1ndez-Matamoros-Bre%C3%B1a/a6982e59942d1ec71f352743b15a9c7c1057fd7b>
- Holmer SF, McKeith RO, Boler DD, Dilger AC, Eggert JM, Petry DB, McKeith FK, Jones KL, Killefer J. 2009. The effect of pH on shelf-life of pork during aging and simulated retail display. *Meat Sci*. 82(1):86–93. <https://doi.org/10.1016/j.meatsci.2008.12.008>
- Honikel KO. 1998. Reference methods for the assessment of physical characteristics of meat. *Meat Science*. 49(4):447–457. [https://doi.org/10.1016/S0309-1740\(98\)00034-5](https://doi.org/10.1016/S0309-1740(98)00034-5)
- Hume DA, Whitelaw CBA, Archibald AL. 2011. The future of animal production: improving productivity and sustainability. *J Agric Sci*. 149(S1):9–16. <https://doi.org/10.1017/S0021859610001188>
- Iftikhar A, Dupas-Farrugia C, Leonardis AD, Macciola V, Moiz A, Martin D. 2024. Antioxidant potential of olive leaf (*Olea europaea* L.) sustainable extracts evaluated in vitro and minced beef meat. *Italian Journal of Food Science*. 36(4):305–316. <https://doi.org/10.15586/ijfs.v36i4.2697>
- Israr M, Chand N, Khan RU, Alhidary IA, Abdelrahman MM, Al-Baddani HH, Laudadio V, Tufarelli V. 2021. Dietary Grape (*Vitis vinifera*) Seed Powder and Zn–Gly Chelate Complex for Mitigating Heat Stress in Broiler Chickens: Growth Parameters, Malondialdehyde, Paraoxonase-1, and Antibody Titer. *Agriculture*. 11(11):1087. <https://doi.org/10.3390/agriculture11111087>
- Jaiswal N, Rizvi SI. 2014. Onion extract (*Allium cepa* L.), quercetin and catechin up-regulate paraoxonase 1 activity with concomitant protection against low-density lipoprotein oxidation in male Wistar rats subjected to oxidative stress. *J Sci Food Agric*. 94(13):2752–2757. <https://doi.org/10.1002/jsfa.6620>
- Jang A, Liu X-D, Shin M-H, Lee B-D, Lee S-K, Lee J-H, Jo C. 2008. Antioxidative Potential of Raw Breast Meat from Broiler Chicks Fed a Dietary Medicinal Herb Extract Mix. *Poultry Science*. 87(11):2382–2389. <https://doi.org/10.3382/ps.2007-00506>
- Jongberg S, Tørngren MA, Gunvig A, Skibsted LH, Lund MN. 2013. Effect of green tea or rosemary extract on protein oxidation in Bologna type sausages prepared from oxidatively stressed pork. *Meat Sci*. 93(3):538–546. <https://doi.org/10.1016/j.meatsci.2012.11.005>
- Jovanovichs MRC, Dos Santos BA, Sant’Anna Monteiro C, Pedro D, Correa LP, Cordeiro MWS, Pinton MB, Cichoski AJ, Mallmann CA, Wagner R, et al. 2025. Micronized olive pomace: A sustainable and innovative strategy to improve the oxidative stability of omega-3 enriched salamis. *Meat Sci*. 221:109715. <https://doi.org/10.1016/j.meatsci.2024.109715>
- Joven M, Pintos E, Latorre MA, Suárez-Belloch J, Guada JA, Fondevila M. 2014. Effect of replacing barley by increasing levels of olive cake in the diet of finishing pigs: Growth performances, digestibility, carcass, meat and fat quality. *Animal Feed Science and Technology*. 197:185–193. <https://doi.org/10.1016/j.anifeedsci.2014.08.007>

- Karolyi D, Škrlep M, Marušić Radovčić N, Luković Z, Škorput D, Salajpal K, Kljak K, Čandek-Potokar M. 2024. Effects of Animal Diet and Processing Methods on the Quality Traits of Dry-Cured Ham Produced from Turopolje Pigs. *Animals*. 14(2):286. <https://doi.org/10.3390/ani14020286>
- Khemakhem M, Papadimitriou V, Sotiroudis G, Zoumpoulakis P, Arbez-Gindre C, Bouzouita N, Sotiroudis TG. 2016. Melanin and humic acid-like polymer complex from olive mill waste waters. Part I. Isolation and characterization. *Food Chem*. 203:540–547. <https://doi.org/10.1016/j.foodchem.2016.01.110>
- King AJ, Griffin JK, Roslan F. 2014. In Vivo and in Vitro Addition of Dried Olive Extract in Poultry. *J Agric Food Chem*. 62(31):7915–7919. <https://doi.org/10.1021/jf4050588>
- Koutina G, Jongberg S, Skibsted LH. 2012. Protein and lipid oxidation in Parma ham during production. *J Agric Food Chem*. 60(38):9737–9745. <https://doi.org/10.1021/jf3026887>
- Kyriakoudi A, Mourtzinis I, Tyśkiewicz K, Milovanovic S. 2024. An Eco-Friendly Supercritical CO₂ Recovery of Value-Added Extracts from *Olea europaea* Leaves. *Foods*. 13(12):1836. <https://doi.org/10.3390/foods13121836>
- Lawrie's Meat Science. 2022. [place unknown]; [accessed 2025 Mar 3]. <https://shop.elsevier.com/books/lawries-meat-science/toldra/978-0-323-85408-5>
- Leite A, Domínguez R, Vasconcelos L, Ferreira I, Pereira E, Pinheiro V, Outor-Monteiro D, Rodrigues S, Lorenzo JM, Santos EM, et al. 2022. Can the Introduction of Different Olive Cakes Affect the Carcass, Meat and Fat Quality of Bísaro Pork? *Foods*. 11(11):1650. <https://doi.org/10.3390/foods11111650>
- Leite A, Vasconcelos L, Lopez S, Outor-Monteiro D, Pinheiro V, Rodrigues S, Teixeira A. 2024. Incorporating Olive By-Products in Bísaro Pig Diets: Effect on Dry-Cured Product Quality. *Foods*. 13(16):2579. <https://doi.org/10.3390/foods13162579>
- Leite A, Vasconcelos L, Rodrigues S, Pereira E, Domínguez-Valencia R, Lorenzo JM, Teixeira A. 2024. Effect of Olive Cake in Bísaro Pig Feed on Physicochemical Composition and Fatty Acid Profile of Three Different Muscles of Dry-Cured Shoulder. *Animals (Basel)*. 14(11):1697. <https://doi.org/10.3390/ani14111697>
- Li C, Mora L, Gallego M, Aristoy M-C, Toldrá F. 2020. Evaluation of main post-translational modifications occurring in naturally generated peptides during the ripening of Spanish dry-cured ham. *Food Chemistry*. 332:127388. <https://doi.org/10.1016/j.foodchem.2020.127388>
- Liotta L, Chiofalo V, Lo Presti V, Chiofalo B. 2019. In Vivo Performances, Carcass Traits, and Meat Quality of Pigs Fed Olive Cake Processing Waste. *Animals*. 9(12):1155. <https://doi.org/10.3390/ani9121155>
- Lipiński K, Mazur M, Antoszkiewicz Z, Purwin C. 2017. Polyphenols in Monogastric Nutrition – A Review. *Annals of Animal Science*. 17(1):41–58. <https://doi.org/10.1515/aoas-2016-0042>
- Liu D, Bai L, Feng X, Chen YP, Zhang D, Yao W, Zhang H, Chen G, Liu Y. 2020. Characterization of *Jinhua ham* aroma profiles in specific to aging time by gas chromatography-ion mobility spectrometry (GC-IMS). *Meat Science*. 168:108178. <https://doi.org/10.1016/j.meatsci.2020.108178>

- Mahfuz S, Shang Q, Piao X. 2021. Phenolic compounds as natural feed additives in poultry and swine diets: a review. *J Animal Sci Biotechnol.* 12(1):48. <https://doi.org/10.1186/s40104-021-00565-3>
- Martini S, Cavalchi M, Conte A, Tagliazucchi D. 2018. The paradoxical effect of extra-virgin olive oil on oxidative phenomena during *in vitro* co-digestion with meat. *Food Research International.* 109:82–90. <https://doi.org/10.1016/j.foodres.2018.04.031>
- Marušić Radovčić N, Poljanec I, Petričević S, Mora L, Medić H. 2021. Influence of Muscle Type on Physicochemical Parameters, Lipolysis, Proteolysis, and Volatile Compounds throughout the Processing of Smoked Dry-Cured Ham. *Foods.* 10(6):1228. <https://doi.org/10.3390/foods10061228>
- Masuda T, Inai M, Miura Y, Masuda A, Yamauchi S. 2013. Effect of polyphenols on oxymyoglobin oxidation: prooxidant activity of polyphenols *in vitro* and inhibition by amino acids. *J Agric Food Chem.* 61(5):1097–1104. <https://doi.org/10.1021/jf304775x>
- Minekus M, Alminger M, Alvito P, Ballance S, Bohn T, Bourlieu C, Carrière F, Boutrou R, Corredig M, Dupont D, et al. 2014. A standardised static *in vitro* digestion method suitable for food – an international consensus. *Food & Function.* 5(6):1113–1124. <https://doi.org/10.1039/C3FO60702J>
- Møller JKS, Adamsen CE, Skibsted LH. 2003. Spectral characterisation of red pigment in Italian-type dry-cured ham. Increasing lipophilicity during processing and maturation. *Eur Food Res Technol.* 216(4):290–296. <https://doi.org/10.1007/s00217-003-0668-5>
- Morales R, Guerrero L, Aguiar APS, Guàrdia MD, Gou P. 2013. Factors affecting dry-cured ham consumer acceptability. *Meat Science.* 95(3):652–657. <https://doi.org/10.1016/j.meatsci.2013.05.012>
- Moroney NC, O’Grady MN, O’Doherty JV, Kerry JP. 2013. Effect of a brown seaweed (*Laminaria digitata*) extract containing laminarin and fucoidan on the quality and shelf-life of fresh and cooked minced pork patties. *Meat Science.* 94(3):304–311. <https://doi.org/10.1016/j.meatsci.2013.02.010>
- Muzolf-Panek M, Waśkiewicz A, Kowalski R, Konieczny P. 2016. The Effect of Blueberries on the Oxidative Stability of Pork Meatloaf During Chilled Storage. *Journal of Food Processing and Preservation.* 40(5):899–909. <https://doi.org/10.1111/jfpp.12668>
- Nenadis N, Kyriakoudi A, Tsimidou MZ. 2013. Impact of alkaline or acid digestion to antioxidant activity, phenolic content and composition of rice hull extracts. *LWT - Food Science and Technology.* 54(1):207–215. <https://doi.org/10.1016/j.lwt.2013.05.005>
- Obied HK, Bedgood DR, Prenzler PD, Robards K. 2007. Bioscreening of Australian olive mill waste extracts: biophenol content, antioxidant, antimicrobial and molluscicidal activities. *Food Chem Toxicol.* 45(7):1238–1248. <https://doi.org/10.1016/j.fct.2007.01.004>
- Paiva-Martins F, Barbosa S, Pinheiro V, Mourão JL, Outor-Monteiro D. 2009. The effect of olive leaves supplementation on the feed digestibility, growth performances of pigs and quality of pork meat. *Meat Sci.* 82(4):438–443. <https://doi.org/10.1016/j.meatsci.2009.02.014>
- Palma-Granados P, García-Casco JM, Fernandez-Barroso MA, López-García A, Martínez-Torres JM, Muñoz M, González-Sánchez E. 2022. Inclusion of olive by-products in growing diets causes

minor effects on meat quality of Iberian pigs fattened in a traditional system. *Span j agric res*. 20(3):e0607. <https://doi.org/10.5424/sjar/2022203-19149>

Papakonstantinou GI, Meletis E, Petrotos K, Kostoulas P, Tsekouras N, Kantere MC, Voulgarakis N, Gougoulis D, Filippopoulos L, Christodouloupoulos G, et al. 2023. Effects of a Natural Polyphenolic Product from Olive Mill Wastewater on Oxidative Stress and Post-Weaning Diarrhea in Piglets. *Agriculture*. 13(7):1356. <https://doi.org/10.3390/agriculture13071356>

Parolari G, Aguzzoni A, Toscani T. 2016. Effects of Processing Temperature on Color Properties of Dry-Cured Hams Made without Nitrite. *Foods*. 5(2):33. <https://doi.org/10.3390/foods5020033>

Pérez-Alvarez J a., Sayas-Barberá M e., Fernández-López J, Gago-Gago M a., Pagán-Moreno M j., Aranda-Catalá V. 1999. Chemical and Color Characteristics of Spanish Dry-Cured Ham at the End of the Aging Process. *Journal of Muscle Foods*. 10(2):195–201. <https://doi.org/10.1111/j.1745-4573.1999.tb00395.x>

Petrova I, Aasen IM, Rustad T, Eikevik TM. 2015. Manufacture of dry-cured ham: a review. Part 1. Biochemical changes during the technological process. *Eur Food Res Technol*. 241(5):587–599. <https://doi.org/10.1007/s00217-015-2490-2>

Ramírez R, Cava R. 2007. Volatile Profiles of Dry-Cured Meat Products from Three Different Iberian X Duroc Genotypes. *J Agric Food Chem*. 55(5):1923–1931. <https://doi.org/10.1021/jf0628101>

Re R, Pellegrini N, Proteggente A, Pannala A, Yang M, Rice-Evans C. 1999. Antioxidant activity applying an improved ABTS radical cation decolorization assay. *Free Radic Biol Med*. 26(9–10):1231–1237. [https://doi.org/10.1016/s0891-5849\(98\)00315-3](https://doi.org/10.1016/s0891-5849(98)00315-3)

Rey AI, De Cara A, Segura JF, Martí P, Hechavarría T, Calvo L. 2021. Dietary oleuropein extract supplementation and its combination with α -tocopheryl acetate and selenium modifies the free fatty acid profile of pork and improves its stability. *J Sci Food Agric*. 101(6):2337–2344. <https://doi.org/10.1002/jsfa.10855>

Rossi R, Ratti S, Moretti VM, Vasconi M, Corino C. 2021. Sensory characteristics and volatile compounds of dry cured ham Speck are affected by pig dietary supplementation with antioxidant mixture. *Journal of the Science of Food and Agriculture*. 101(3):1134–1142. <https://doi.org/10.1002/jsfa.10724>

Rossi R, Tucci T, Giordano A, Moretti P, Corino C. 2022. Dietary brown seaweed and plant polyphenols in hyperprolific sows: Productive performance, blood parameters and antioxidant status. *Czech Journal of Animal Science*. 67(12):493–502. <https://doi.org/10.17221/98/2022-CJAS>

Rowe LJ, Maddock KR, Lonergan SM, Huff-Lonergan E. 2004. Influence of early postmortem protein oxidation on beef quality. *J Anim Sci*. 82(3):785–793. <https://doi.org/10.2527/2004.823785x>

Ruiz J, García C, Muriel E, Andrés AI, Ventanas J. 2002. Influence of sensory characteristics on the acceptability of dry-cured ham. *Meat Science*. 61(4):347–354. [https://doi.org/10.1016/S0309-1740\(01\)00204-2](https://doi.org/10.1016/S0309-1740(01)00204-2)

Sabino M, Cappelli K, Capomaccio S, Pascucci L, Biasato I, Verini-Supplizi A, Valiani A, Trabalza-Marinucci M. 2018. Dietary supplementation with olive mill wastewaters induces

modifications on chicken jejunum epithelial cell transcriptome and modulates jejunum morphology. *BMC Genomics*. 19(1):576. <https://doi.org/10.1186/s12864-018-4962-9>

Sánchez CJ, Barrero-Domínguez B, Martínez-Miró S, Madrid J, Baños A, Aguinaga MA, López S, Hernández F. 2022. Use of Olive Pulp for Gestating Iberian Sow Feeding: Influence on Performance, Health Status Indicators, and Fecal Microbiota. *Animals*. 12(22):3178. <https://doi.org/10.3390/ani12223178>

Sánchez-Peña CM, Luna G, García-González DL, Aparicio R. 2005. Characterization of French and Spanish dry-cured hams: influence of the volatiles from the muscles and the subcutaneous fat quantified by SPME-GC. *Meat Sci*. 69(4):635–645. <https://doi.org/10.1016/j.meatsci.2004.10.015>

Sar T, Akbas MY. 2023. Antimicrobial Activities of Olive Oil Mill Wastewater Extracts against Selected Microorganisms. *Sustainability*. 15(10):8179. <https://doi.org/10.3390/su15108179>

Scicchitano S, Vecchio E, Battaglia AM, Oliverio M, Nardi M, Procopio A, Costanzo F, Biamonte F, Faniello MC. 2023. The Double-Edged Sword of Oleuropein in Ovarian Cancer Cells: From Antioxidant Functions to Cytotoxic Effects. *International Journal of Molecular Sciences*. 24(1):842. <https://doi.org/10.3390/ijms24010842>

Serra A, Conte G, Giovannetti M, Casarosa L, Agnolucci M, Ciucci F, Palla M, Bulleri E, Cappucci A, Servili M, Mele M. 2018. Olive Pomace in Diet Limits Lipid Peroxidation of Sausages from Cinta Senese Swine. *Euro J Lipid Sci & Tech*. 120(1):1700236. <https://doi.org/10.1002/ejlt.201700236>

Servili M, Sordini B, Esposto S, Urbani S, Veneziani G, Di Maio I, Selvaggini R, Taticchi A. 2013. Biological Activities of Phenolic Compounds of Extra Virgin Olive Oil. *Antioxidants*. 3(1):1–23. <https://doi.org/10.3390/antiox3010001>

Şevkat Uğuz, Ayla Soyer, Ülkü Dalmiş. 2010. Effects of different salt contents on some quality characteristics during processing of dry-cured ham pastirma [Internet]. [accessed 2025 Mar 3]. <https://onlinelibrary.wiley.com/doi/10.1111/j.1745-4557.2011.00382.x>

Sharma P, Yadav S. 2020. Effect of Incorporation of Pomegranate Peel and Bagasse Powder and Their Extracts on Quality Characteristics of Chicken Meat Patties. *Food Sci Anim Resour*. 40(3):388–400. <https://doi.org/10.5851/kosfa.2020.e19>

Silvan JM, Martinez-Rodriguez AJ. 2019. Food By-products as Natural Source of Bioactive Compounds Against *Campylobacter*. In: *Encyclopedia of Food Security and Sustainability* [Internet]. [place unknown]: Elsevier; [accessed 2024 Jun 10]; p. 336–350. <https://doi.org/10.1016/B978-0-08-100596-5.22139-2>

Skinner JG, Brown RA, Roberts L. 1991. Bovine haptoglobin response in clinically defined field conditions. *Vet Rec*. 128(7):147–149. <https://doi.org/10.1136/vr.128.7.147>

Sunderman FW Jr, Nomoto S. 1970. Measurement of Human Serum Ceruloplasmin by Its p-Phenylenediamine Oxidase Activity. *Clinical Chemistry*. 16(11):903–910. <https://doi.org/10.1093/clinchem/16.11.903>

Tarladgis BG, Watts BM, Younathan MT, Dugan L. 1960. A distillation method for the quantitative determination of malonaldehyde in rancid foods. *J Am Oil Chem Soc*. 37(1):44–48. <https://doi.org/10.1007/BF02630824>

- Taticchi A, Esposito S, Veneziani G, Minnocci A, Urbani S, Selvaggini R, Sordini B, Daidone L, Sebastiani L, Servili M. 2021. High vacuum-assisted extraction affects virgin olive oil quality: Impact on phenolic and volatile compounds. *Food Chemistry*. 342:128369. <https://doi.org/10.1016/j.foodchem.2020.128369>
- Trevisi E, Jahan N, Bertoni G, Ferrari A, Minuti A. 2015. Pro-Inflammatory Cytokine Profile in Dairy Cows: Consequences for New Lactation. *Italian Journal of Animal Science*. 14(3):3862. <https://doi.org/10.4081/ijas.2015.3862>
- Tsala A, Mpekelis V, Karvelis G, Tsikakis P, Goliomytis M, Simitzis P. 2020. Effects of Dried Olive Pulp Dietary Supplementation on Quality Characteristics and Antioxidant Capacity of Pig Meat. *Foods*. 9(1):81. <https://doi.org/10.3390/foods9010081>
- Untea AE, Cornescu MG, Dumitra T, Olteanu M, Gavris T, Lupu A. 2018. Effect of dietary antioxidant plant mixture on the oxidative stability of liver in piglets.
- Van Soest PJ, Robertson JB, Lewis BA. 1991. Methods for Dietary Fiber, Neutral Detergent Fiber, and Nonstarch Polysaccharides in Relation to Animal Nutrition. *Journal of Dairy Science*. 74(10):3583–3597. [https://doi.org/10.3168/jds.S0022-0302\(91\)78551-2](https://doi.org/10.3168/jds.S0022-0302(91)78551-2)
- Vasilopoulos S, Dokou S, Papadopoulos GA, Savvidou S, Christaki S, Kyriakoudi A, Dotas V, Tsiouris V, Bonos E, Skoufos I, et al. 2022. Dietary Supplementation with Pomegranate and Onion Aqueous and Cyclodextrin Encapsulated Extracts Affects Broiler Performance Parameters, Welfare and Meat Characteristics. *Poultry*. 1(2):74–93. <https://doi.org/10.3390/poultry1020008>
- Vasilopoulou K, Papadopoulos GA, Lioliopoulou S, Pyrka I, Nenadis N, Savvidou S, Symeon G, Dotas V, Panitsidis I, Arsenos G, Giannenas I. 2023. Effects of Dietary Supplementation of a Resin-Purified Aqueous-Isopropanol Olive Leaf Extract on Meat and Liver Antioxidant Parameters in Broilers. *Antioxidants*. 12(9):1723. <https://doi.org/10.3390/antiox12091723>
- Vastolo A, Calabró S, Liotta L, Musco N, Di Rosa AR, Cutrignelli MI, Chiofalo B. 2019. In Vitro Fermentation and Chemical Characteristics of Mediterranean By-Products for Swine Nutrition. *Animals*. 9(8):556. <https://doi.org/10.3390/ani9080556>
- Vela A, Suárez-Usbeck A, Lafoz L, Mitjana O, Tejedor MT, Martín S, López M, Falceto MV. 2022. Determination of puberty in gilts: contrast of diagnostic methods. *Porc Health Manag*. 8(1):28. <https://doi.org/10.1186/s40813-022-00271-0>
- Veneziani G, Novelli E, Esposito S, Taticchi A, Servili M. 2017. Applications of recovered bioactive compounds in food products [Internet]. In: [place unknown]: Elsevier; [accessed 2024 Jun 10]; p. 231–253. <https://doi.org/10.1016/B978-0-12-805314-0.00011-X>
- Ventanas S, Estevez M, Tejada JF, Ruiz J. 2006. Protein and lipid oxidation in Longissimus dorsi and dry cured loin from Iberian pigs as affected by crossbreeding and diet. *Meat Sci*. 72(4):647–655. <https://doi.org/10.1016/j.meatsci.2005.09.011>
- Visioli F, Bellomo G, Galli C. 1998. Free radical-scavenging properties of olive oil polyphenols. *Biochem Biophys Res Commun*. 247(1):60–64. <https://doi.org/10.1006/bbrc.1998.8735>
- Wang Z, Xu Y, Zhang J, Li X, Lin Z, Ma C. 2011. Proteolysis, protein oxidation and protease activity in dry-cured Xuanwei ham during the salting stages. *International Journal of Food Science & Technology*. 46(7):1370–1377. <https://doi.org/10.1111/j.1365-2621.2011.02626.x>

- Wu H, Bak KH, Goran GV, Tatiyaborworntham N. 2024. Inhibitory mechanisms of polyphenols on heme protein-mediated lipid oxidation in muscle food: New insights and advances. *Crit Rev Food Sci Nutr.* 64(15):4921–4939. <https://doi.org/10.1080/10408398.2022.2146654>
- Xiao L, Lee J, Zhang G, Ebeler SE, Wickramasinghe N, Seiber J, Mitchell AE. 2014. HS-SPME GC/MS characterization of volatiles in raw and dry-roasted almonds (*Prunus dulcis*). *Food Chemistry.* 151:31–39. <https://doi.org/10.1016/j.foodchem.2013.11.052>
- Xu Q-D, Yu Z-L, Zeng W-C. 2021. Structural and functional modifications of myofibrillar protein by natural phenolic compounds and their application in pork meatball. *Food Research International.* 148:110593. <https://doi.org/10.1016/j.foodres.2021.110593>
- Zeng Z, Jiang J, Yu J, Mao X, Yu B, Chen D. 2019. Effect of dietary supplementation with mulberry (*Morus alba* L.) leaves on the growth performance, meat quality and antioxidative capacity of finishing pigs. *Journal of Integrative Agriculture.* 18(1):143–151. [https://doi.org/10.1016/S2095-3119\(18\)62072-6](https://doi.org/10.1016/S2095-3119(18)62072-6)
- Zhang J, Mora L, Toldrá F, Zhang W, Flores M. 2024. Effects of ultrasound pretreatment on flavor characteristics and physicochemical properties of dry-cured ham slices during refrigerated vacuum storage. *LWT.* 199:116132. <https://doi.org/10.1016/j.lwt.2024.116132>
- Zhang W, Xiao S, Samaraweera H, Lee EJ, Ahn DU. 2010. Improving functional value of meat products. *Meat Sci.* 86(1):15–31. <https://doi.org/10.1016/j.meatsci.2010.04.018>
- Zhou Y, Wang Q, Wang S. 2020. Effects of rosemary extract, grape seed extract and green tea polyphenol on the formation of N-nitrosamines and quality of western-style smoked sausage. *Journal of Food Processing and Preservation.* 44(6):e14459. <https://doi.org/10.1111/jfpp.14459>
- Zhu C-Z, Zhao J-L, Tian W, Liu Y-X, Li M-Y, Zhao G-M. 2018. Contribution of Histidine and Lysine to the Generation of Volatile Compounds in Jinhua Ham Exposed to Ripening Conditions Via Maillard Reaction. *J Food Sci.* 83(1):46–52. <https://doi.org/10.1111/1750-3841.13996>

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 *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. This finding was further demonstrated by the decrease in thiobarbituric acid reactive substances (TBARS), detected through an *in vitro* digestion method, and in the main markers of sensory quality (aldehydes and alcohols), with a positive final 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.

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