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RESEARCH ARTICLE

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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 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 caecum 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.

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.

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Gut health; amplicon-based metagenomics; olive polyphenols; swine

Introduction

The increasing global demand for pig production (FAOSTAT, 2024) 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, phytogenic feed additives (PFAs) are considered potential alternatives to antibiotics for their antimicrobial actions (Papakonstantinou et al. 2023),

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and their antioxidant activities have 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 utilisation (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, 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; 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; Fiesel et al. 2014; Verhelst et al. 2014; Silva-Guillen et al. 2020; Xu et al. 2020; Bonos et al. 2022; Sánchez 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; 2021; Sánchez et al. 2022; Belloumi et al. 2024). However, the effects of OMWW on swine gut health and microbiota composition have never been analysed 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.

Materials and methods

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. (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 was 9.39 ± 17.4 g/kg, 1.09 ± 3.2 g/kg, and 0.59 ± 0.4 g/kg, respectively.

RP-HPLC-DAD analysis of polyphenols

The hydroxytyrosol, tyrosol, and oleuropein content in OMWW extract was determined by reversed-phase high-performance liquid chromatography with diode array detector (RP-HPLC-DAD), according to 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-C184μm (150 × 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 analysed 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.

Animal welfare and ethical 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.

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 × 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 was 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.

	Diet
Items	% as fed basis
Grain corn flour	41.8
Grain barley flour	21.7
Wheat middlings	8.1
Proteins	2.5
Wheat flour middlings	9.0
Soybean meal	14.4
Mineral-vitamin supplement[a]	2.5
Analysed **nutrients**	g/100 g
Moisture	11.20
CP	15.10
Ether extracts	3.61
Ash	6.83
Crude fibre	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 fibre; ADF: acid detergent fibre; 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.

Feed analysis

Crude protein, crude fibre, 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 fibre (NDF), acid detergent fibre (ADF), and acid detergent lignin (ADL) was performed following the methods of Van Soest, Robertson, and Lewis (Van Soest et al. 1991).

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 days of the trial.

Sample collection

Faeces 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, faecal 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 with European Council Regulation (EC) N° 1099/2009 on the protection of animals at the time of killing. After slaughtering, the carcases were weighed and the stomach, jejunum, ileum, and colon were macroscopically inspected, sectioned, and fixed in formalin. For 16S amplicon sequencing, samples were collected by opening the 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.

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 the C group, 14 from the 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 aetiological 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 Haematoxylin 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 analysing 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 the ileum.

Sequencing of 16S rRNA amplicons

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 caecal 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.

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 analysed 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 an 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 normalised 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 generalised UniFrac metrics, and statistical significance was calculated by applying the PERMANOVA test with 9999 permutations. Differentially abundant (DA) taxa were retrieved normalising 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).

Statistical analysis

A two-way ANOVA model was applied, with the diet (C, P-LOW, and P-HIGH) and pens as fixed variables, to analyse 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 (2020.12.0 + 369 version). A chi-square test was 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 $p < 0.05$.

Results

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 faecal samples taken during the experiment were negative for intestinal parasites, and the consistency of the faeces was uniform across the experimental groups. Furthermore, with the exception of a single pig, no episodes of diarrhoea are recorded during the whole experimental period.

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 the 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 groups, 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

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.

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

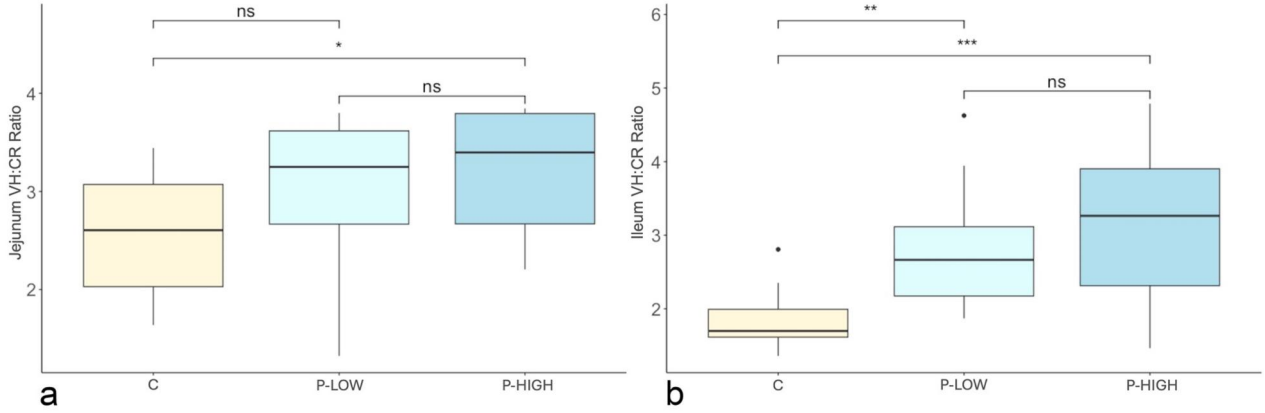

Figure 1. Boxplot summarising jejunum (a) and ileum (b) villous height to crypt depth (VH/CD) ratio according to dietary treatment. ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$. *Note*: ns = not significative; VH = villous height; CD = crypt depth.

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 significantly 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.

Sequencing 16S rRNA amplicons

Regarding the microbial content of the caecum, 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

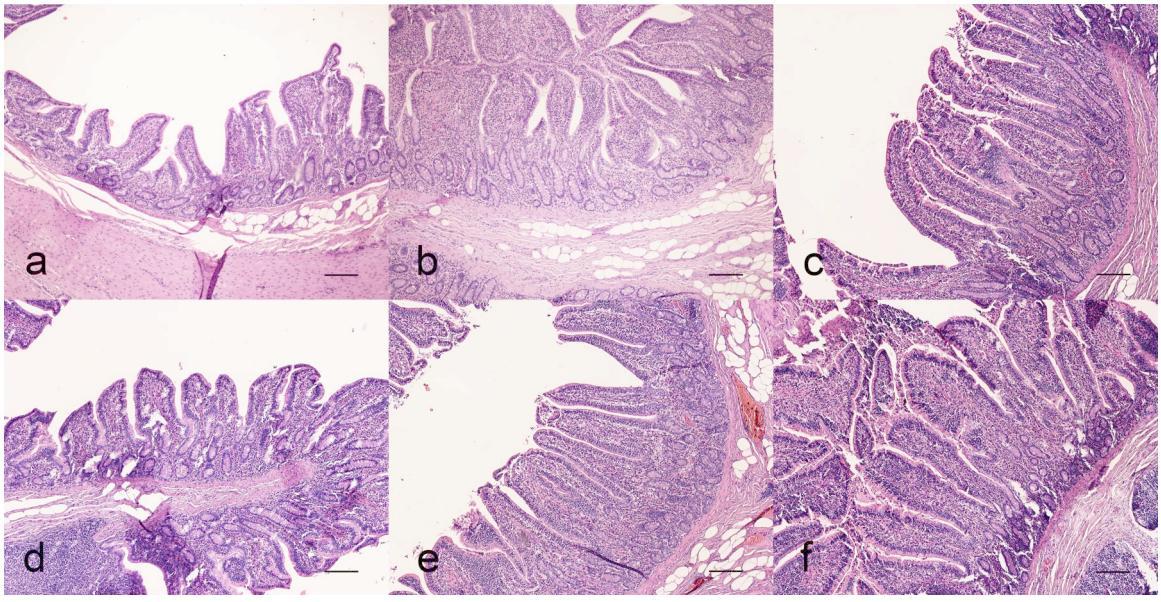

Figure 2. The panel presents histological sections (Haematoxylin and Eosin) of the jejunum (a–c) and ileum (d–f) (x100); scale bar = 100 μm. The jejunal mucosal architecture of sections from group C (a) shows short, intact villi and crypts. In group P-LOW (b) and P-HIGH (c), the villi and crypts are 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.

significant differences between the experimental groups at the genus and family level (Figure 3A).

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 caecum was similar across the three experimental groups. The results of the PERMANOVA test confirmed the lack of any statistically significant differences (Table 3).

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 (2 T, 4 T and 9 T; 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 phylogenic tree used to calculate UniFrac distances is shown in Supplementary Figure 3C.

Finally, the differentially abundant (DA) genera and species in the caecal and jejunal contents are reported in Figures 5 and 6, respectively. Details for the caecum 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 caecal and jejunal substrates, respectively.

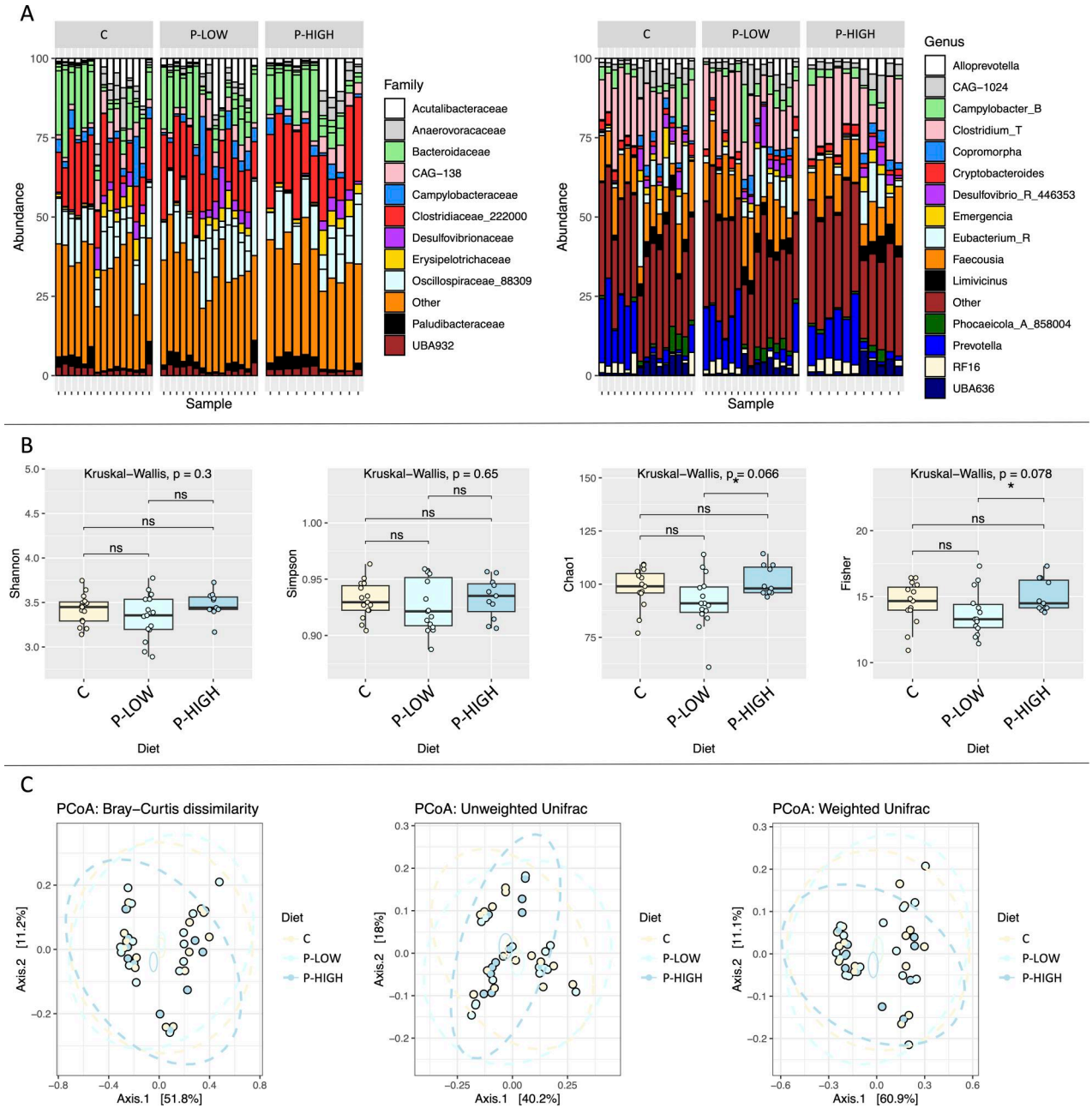

Figure 3. Caecal content characterisation and differences between experimental groups (control C, olive mill waste water phenolic diets P-LOW and P-HIGH). A) relative abundances of the 15 families and genera. B) Alpha diversity indices (Shannon, Simpson, Chao1, and fisher), testing relative statistical 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 significative; * $p < 0.05$.

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

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

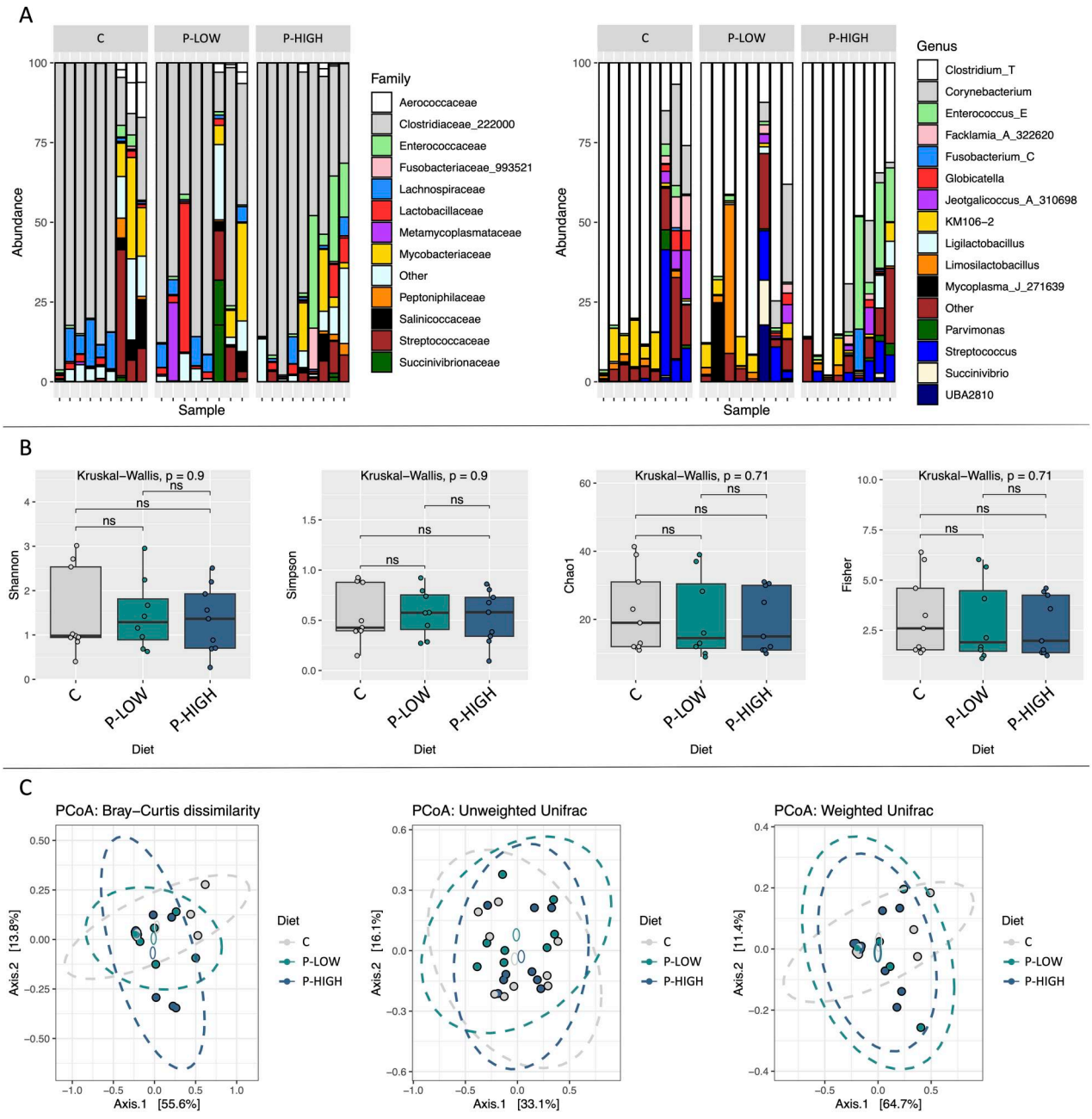

Figure 4. Jejunum content characterisation and differences between experimental groups (control C, olive mill wastes water phenolic diets P-LOW and P-HIGH). A) relative abundances of the 15 most common families and genera. B) Alpha diversity indices (Shannon, Simpson, Chao1 and Fisher)), testing relative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

Table 4. Statistical 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 normalised data)	0.05	0.57	0.80
Unweighted UniFrac distance (rarefaction normalised data)	0.08	1.03	0.40
Weighted UniFrac distance (rarefaction normalised data)	0.02	0.26	0.99

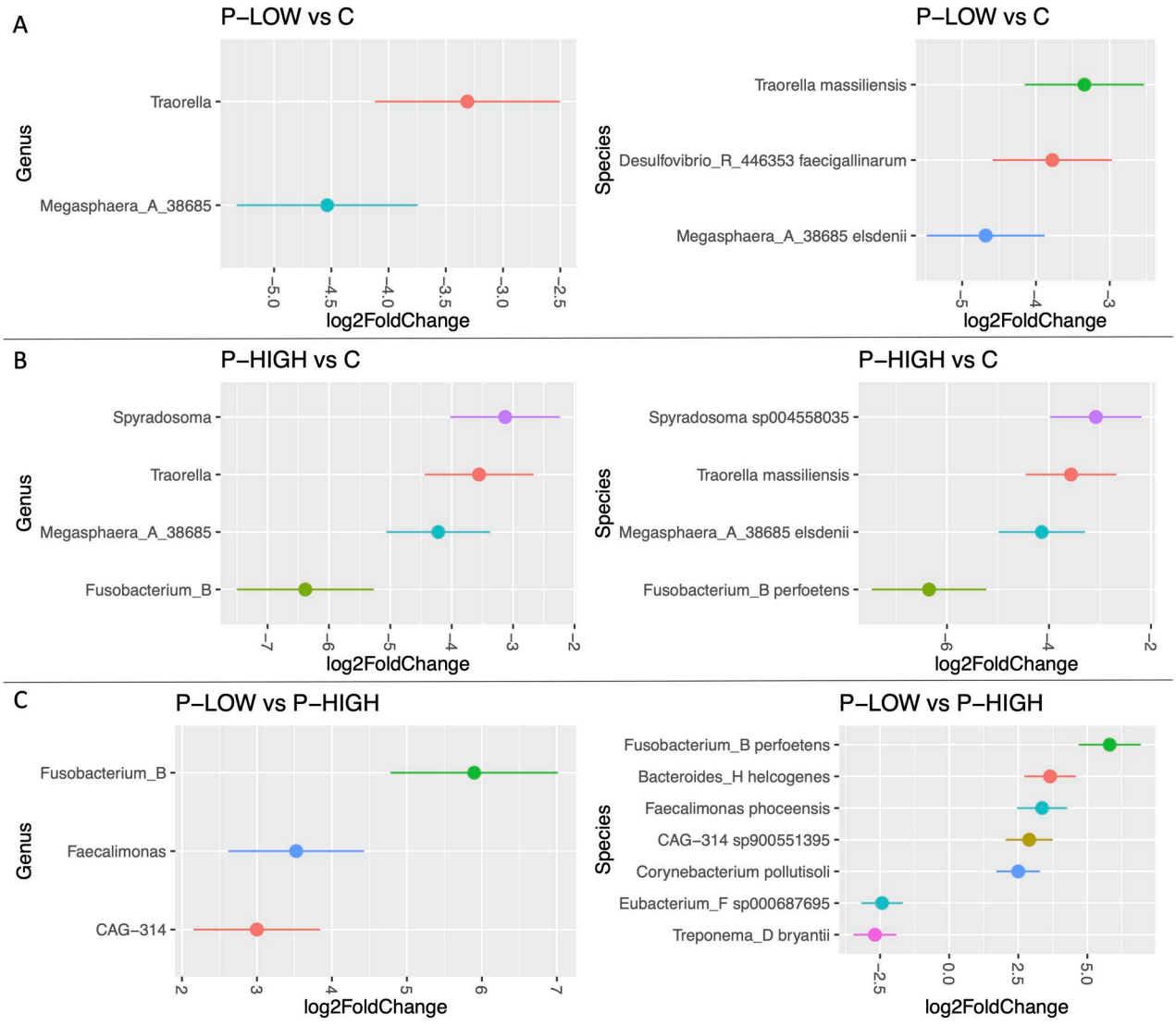

Figure 5. Differentially abundant (DA) genus and species identified in caecum contents from the three experimental groups (control C, olive mill waste water phenolic diets P-LOW and P-HIGH). For each comparison, genera (left panel) and species (right panel), are shown with a multiple testing corrected *p*-value ≤ 0.05. Statistically significant features (false discovery rate – FDR < 0.05; and $\log_2$ fold change - $|\log_2 FC| > 1.5$) are reported for A) P-LOW vs control; taxa with a fold change greater than 0 are more abundant in 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 colours of the dots indicate the same family, and bars show the standard error of the log fold change.

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.

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

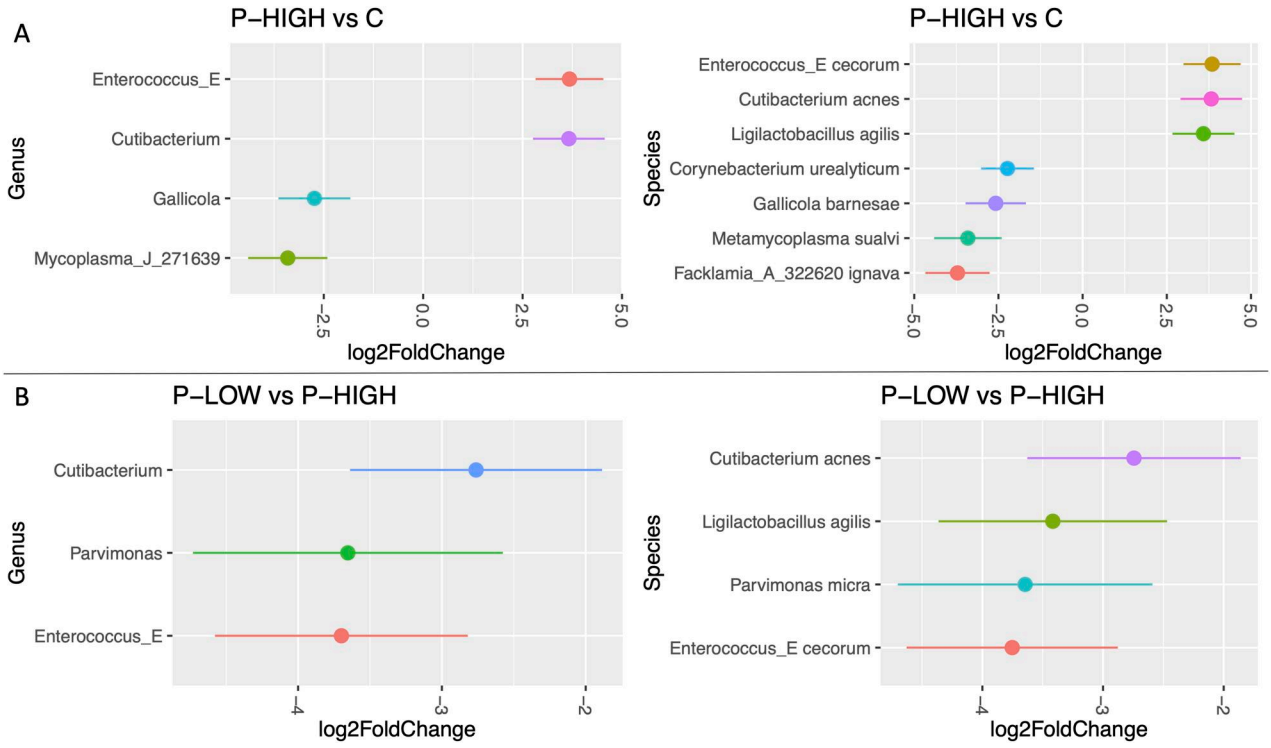

Figure 6. Differentially abundant (DA) genus and species identified in jejunum contents from the three experimental groups (control C, olive mill waste water phenolic diets P-LOW and P-HIGH). Statistically significant features (false discovery rate – FDR $<$ 0.05; and $\log_2$ fold change - $|\log_2 FC| > 1.5$) 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 colours of the dots indicate the same family, and bars show the standard error of the log fold change.

have limited positive effects on product quality (Branciari et al. 2017, 2020, 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 diarrhoea).

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 the experimental group and the presence of a 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 favourable 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).

Relative abundances and microbial diversity

Concerning the caecal 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 caecum 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 caecum 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 faecal 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 caecum and jejunum.

Differential abundances in the caecum

Analysis of caecum 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, log2FC $<$ 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. 2021). Thus, the greater abundance of *Eubacterium* F sp000687695 in the P-HIGH group suggests that the beneficial effects of polyphenols on the pig caecum may be dose-dependent. *Treponema* is a fibre-degrading bacterium playing a significant role in the digestion of complex carbohydrates in the pig caecum (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 caecum 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 aetiology of diarrhoea 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 faeces and 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 sulphate-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 sulphide (the product of sulphate reduction), which is associated with intestinal damage (Deplancke et al. 2000). Overall, the diversity of sulphate-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. *Megasphera* belongs to the phylum Firmicutes and is one of the anaerobic bacteria dominating the pig large intestine (Grosu et al. 2019) it 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 frequently 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 though dietary supplementation with grape seed meal, due to its high level of polyphenol flavonoids (Grosu et al. 2019, 2020). These findings contrast with our results, potentially due to the different chemical nature of the olive polyphenols.

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 faeces of diarrhoeic piglets (Sun et al. 2019). However, the pigs in the present trial did not present any episodes of diarrhoea (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 (Gil Sánchez et al. 2017; 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 sualvi* was significantly lower in the P-HIGH group compared with C, however, *M. sualvi* 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 et al. 2003; Eiamsam-Ang et al. 2023) and may be associated with urinary tract infections *via* the ascending route (Carlton et al. 2010).

Conclusions

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, an increase in beneficial intestinal bacteria and a reduction in harmful ones). These two outcomes may be linked to the 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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Authors' contributions

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

Disclosure statement

No potential conflict of interest was reported by the authors.

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Data availability statement

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

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