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DIET ADDITION WITH POMEGRANATE AND ONION EXTRACT, EITHER IN AN AQUEOUS OR CYCLODEXTRIN-ENCAPSULATED FORM, ALTERS INTESTINAL MICROBIOTA, MORPHOMETRY, AND TIGHT JUNCTION GENE EXPRESSION IN BROILERS

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Abstract

This study investigated the effects of dietary supplementation with pomegranate peel and onion leaf extracts, administered either as an aqueous solution or in cyclodextrin-encapsulated form, on broiler chickens. 120 day-old Ross 308 broiler chicks were allocated to three groups: non-supplemented (CONTROL), supplemented with aqueous (AQPOMON) and cyclodextrin (CDPOMON) extracts (0.1%/kg DM). Intestinal morphometry was assessed by measuring crypt depth (CD), villus length (VL), and VL:CD ratio in the mid-duodenum, mid-jejunum, and mid-ileum. Tight junction (TJ) protein expression in the mid-duodenum, mid-ileum and mid-jejunum, especially claudin-3, was assessed through immunohistochemistry (IHC), and gene expression of TJ proteins (zonula occludens 1 (*TJP1*), claudin-1 (*CLDN1*), claudin-3 (*CLDN3*), and occludin (*OCLDN*)) via qPCR in the ileum and cecum. Additionally, 16S rRNA gene sequencing was used to study bacterial diversity in the ileum and cecum. Results showed that VL and CD were variably affected by extract supplementation resulting in decreased VL:CD in all intestinal segments. by the aqueous extract (P<0.05). The AQPOMON diet increased TJ protein gene expression and enhanced the intestinal barrier. *Firmicutes*, *Proteobacteria*, and *Bacteroidota* were the dominant phyla across all groups, with differentially abundant taxa like *Campylobacteria* (ileum) and *Clostridia* (cecum) in AQPOMON, and *Lactobacillaceae* (ileum) and *Methanobacteriaceae* (cecum) in CDPOMON. The AQPOMON diet proved more effective than the CDPOMON in improving aspects of broiler intestinal health.

Key words: nutrition, intestinal health, sequencing, 16S rRNA, VL:CD ratio

Poultry industry is one of the most important industries in the world for ensuring food security, especially with regard to the supply of meat. The health and welfare of broilers is important in not only maximizing the economic returns, but also in tackling social and environmental issues (Mottet and Tempo, 2017; Liao et al., 2020). Along with other factors which determine the productivity of a broiler, intestinal health significantly impacts the growth, feed utilization, and productivity of the chicken (Teng and Kim, 2018). Considering the increasing concerns of consumer's shifting towards the use of no antibiotics in poultry production and growing worries of antibiotic resistance, there is a recent shift towards the use natural dietary supplements over conventional antibiotics. They serve as a pragmatic solution in providing intestinal health while meeting the needs of both the industry and public health (Yarmohammadi Barbarestani et al., 2020).

Plant-derived compounds have garnered increasing interest as natural supplements in broiler diets due to their improving health properties, though they have been widely studied as feed additives (Giannenas et al., 2020). Pomegranate (*Punica granatum*) and onion (*Allium cepa*) stand out because of their rich content of bioactive compounds which include polyphenols and flavonoids with antioxidant, antimicrobial, and anti-inflammatory effects (Vasilopoulos et al., 2022; Saparbekova et al., 2023). Notably, peels of pomegranate and onion, which are byproducts of the fruit juice and ready to eat fresh salad preparation industry, are bioactive compounds rich. Using them to produce functional feed additives fits well within circular economy concepts. They possess distinct and additionally complementary phytochemical profile; onion peels are considered to be rich in bioactive compounds such as flavonoids, phenolic acids, and dietary fiber (Ristivojević et

al., 2024). On the contrary, pomegranate peels are rich in polyphenols like ellagitannins, ellagic acid, and flavonoids (Saparbekova et al., 2023). These metabolites from plants are likely to have synergistic effects because some studies proved that combining onion peels' flavonoids with pomegranate peels' polyphenols could bolster antioxidant, anti-inflammatory, and antimicrobial activities (Savvidou et al., 2023). Although there is growing interest in adding these extracts to broiler diets, little is known about how they specifically affect intestinal morphometry and microbiota. Understanding the interactions between these natural compounds interact with the avian gut ecosystem is crucial to developing long-term, successful plans to enhance broiler health while upholding strict guidelines for environmental responsibility and animal welfare (Wickramasuriya et al., 2022; Savvidou et al., 2023).

The methodology applied for extraction and delivery of plant bioactive compounds is crucial in determining additive efficacy. Over the past few decades, a lot of research has been done on encapsulating active ingredients in order to prevent oxidation, improve their solubility, and boost their bioactivity when taken orally (Mourtzinos et al., 2007). Microencapsulation technology, in particular, allows the controlled release of additives at specific locations, particularly in the intestine's distal sections. This focused distribution can decrease the quantity of product needed in the diet and enhance nutrient absorption, providing a more effective and economical option. (Galli et al., 2021; Almassri et al., 2024; Contreras-López et al., 2024). Amongst microencapsulation technologies, cyclodextrins are widely utilized as a result of their ability to form inclusion complexes with bioactive compounds, protecting them from degradation while enhancing their stability, bioavailability, and targeted delivery in the gastrointestinal tract (Sarabia-Vallejo et al., 2023). This makes cyclodextrins a promising tool for improving nutrient delivery in functional foods and animal feed applications (Gonzalez Pereira et al., 2021). Aqueous cyclodextrin solutions present a highly effective, sustainable and eco-friendly solvent option, for extracting and encapsulating polyphenols, as the hydrophobic cavities form complexes with polyphenols, thereby improving extraction yields. In contrast, aqueous solutions typically facilitate the solubility of hydrophilic compounds, limiting their use for polyphenol encapsulation. Cyclodextrins have been employed in a comprehensive manner for utilizing both the edible and non-edible components of pomegranate. It has been reported that the inclusion of cyclodextrin during the extraction process increases the overall phenolic yield and radical scavenging activity of pomegranate extracts as compared to aqueous solutions (Diamanti et al., 2017; Vasilopoulos et al., 2022; Vhangani et al., 2022). Likewise, the use of aqueous cyclodextrin solutions as a medium for extracting polyphenols from onion solid waste enhances their solubility while preserving the characteristic phenolic composition (Mourtzinos et al., 2007; Vasilopoulos et al., 2022). A recent study demonstrated that both aqueous and cyclodextrin-encapsulated onion and pomegranate extracts differentially reduced protein and lipid oxidation while improving the fatty acid profile in poultry meat. Also, dietary intervention influenced cellular stress protein responses, suggesting a protective effect against oxidative stress in the tissues (Savvidou et al., 2023). However, despite these biochemical benefits, no significant changes were observed in broiler welfare or performance parameters (Vasilopoulos et al., 2022).

The aim of this study is to build upon the findings of our previous research and evaluate the effects of pomegranate (*Punica granatum*) and onion (*Allium cepa*) peel extracts on broiler chickens' intestinal health and function. Specifically, we examined how intestinal morphometry, gut barrier function, and microbiota composition were affected by the two approaches – aqueous and cyclodextrin-encapsulated formulations. We hypothesized that cyclodextrin-encapsulated extracts would outperform aqueous extracts in improving gut morphometry, strengthening gut barrier integrity, and positively influencing microbiota composition, ultimately enhancing broiler intestinal health. This hypothesis is supported by the fact that cyclodextrin encapsulation, as opposed to aqueous extracts, improves the bioavailability, stability, and targeted distribution of bioactive compounds in broiler diets (Christaki et al., 2023; Bist et al., 2024). The results of this study complement our previous findings and contribute to a deeper understanding of whether encapsulation with psychology increases biological reactions compared to traditional extraction technologies. Despite the increasing interest in natural feed additives, a significant knowledge about mechanisms remains, through which cyclodextrin encapsulation affects bioavailability, intestinal health, and broiler performance. Additionally, concerns such as the fluctuations, cost-effectiveness, and availability or potential for commercial application have still not been adequately addressed. This study provides critical insights into the application of the most effective methodologies for extracting plant bioactive compounds, assisting in decision-making to maximize effectiveness in enhancing the health of broilers. Furthermore, these findings are expected to provide insight into the molecular mechanisms through which such extracts influence gut health, and their potential long-term impacts on broiler performance, establishing a connection between the practical uses of poultry nutrition and scientific developments.

Material and methods

Ethics and procedures

Husbandry, euthanasia, experimental procedures, and biosecurity precautions were conducted in accordance with Greek legislation governing experimental animals and were approved by the local Public Veterinary Service (Reg. 489181(3254)/07.02.2018) in research experimental facilities. All institutional and national guidelines for the care and use of laboratory animals were followed.

Table 1. Ingredients and chemical composition of the basal starter (days 1–10), grower (days 11–24) and finisher diets (days 25–35) offered to chickens in mash form

Ingredients (%)	Basal diet		
	Starter (1–10)	Grower (11–24)	Finisher (25–35)
Maize	30.00	20.00	20.00
Wheat, soft	30.13	46.53	47.81
Soybean meal	32.41	26.34	23.65
Soybean oil	2.54	2.53	3.43
Palm fat	-	-	1
Calcium phosphate	1.56	1.38	1.28
Limestone (calcium carbonate)	1.50	1.38	1.23
Salt	0.31	0.31	0.31
Sodium carbonate (27%)	0.09	0.11	0.11
L-Lysine	0.44	0.44	0.37
DL-Methionine	0.43	0.39	0.33
L-Threonine	0.20	0.20	0.14
L-Valine	0.14	0.14	0.09
Vitamin, enzymes and mineral premix*	0.25	0.25	0.25
Calculated chemical composition			
Metabolizable energy (Kcal/kg)	2.95	3.02	3.1
Moisture (%)	11.45	11.37	11.30
Protein (%)	22.12	21.20	20.12
Crude fiber (%)	3.38	3.20	3.05
NFD (%)	7.67	7.89	7.67
ADF (%)	2.68	2.57	2.44
Crude fat (%)	4.71	4.50	5.42
Ash (%)	6.37	6.08	5.90
Starch (%)	35.94	38.45	39.58
Lysine (%)	1.44	1.29	1.17
Methionine+Cystine (%)	1.11	1.02	0.94
Methionine (%)	0.73	0.67	0.60
Threonine (%)	0.97	0.88	0.78
Tryptophan (%)	0.25	0.25	0.23
Valine (%)	1.14	1.03	0.93
digLysine (%)	1.30	1.16	1.05
digMethionine (%)	0.69	0.63	0.56
digThreonine (%)	0.86	0.77	0.68
digrp (%)	0.23	0.22	0.20
digValine (%)	0.97	0.88	0.79
Total NSPs (%)	9.8	7.7	6.9
Calcium (%)	1.01	1.01	1.01
Total phosphorus (%)	0.76	0.71	0.66
Sodium (%)	0.17	0.18	0.19
Chloride (%)	0.25	0.25	0.25

*Supplying per kg feed: 13,000 IU vitamin A, 4,000 IU vitamin D_3, 40 mg vitamin E, 9 mg vitamin K, 3 mg thiamin, 7 mg riboflavin, 6 mg pyridoxine, 0.035 mg vitamin B_{12}, 40 mg niacin, 13 mg pantothenic acid, 1.5 mg folic acid, 0.13 biotin, 340 mg choline chloride, 55 mg Zn, 155 mg Mn, 20 mg Fe, 12 mg Cu, 0.2 mg Co, 1 mg I, 0.2 mg Se, and phytase 0.01 g.

Animals, diet and experimental design

A total of 120 one-day-old male Ross-308 broiler chicks were randomly assigned to three groups, each with four replicates of 10 birds. Each replicate was housed in individual floor pens equipped with infrared heating lamps in a controlled experimental facility at

the Research Institute of Animal Science, Hellenic Agricultural Organization-DEMETER, Paralimni, Giannitsa, Greece (latitude 40.45°, longitude 22.27°). Environmental conditions, including temperature, humidity, and lighting, were maintained according to the breeder's recommendations (Aviagen®, ROSS Nutrition Specifications; Aviagen, Huntsville, AL, USA). Animal health was monitored twice daily by a veterinarian. The control group (CONTROL) was fed basal diets, including starter (days 1–10), grower (days 11–24), and finisher (days 25–35) phases, formulated with maize and soybean meal and offered in mash form (Table 1). The other two groups received the same basal diets supplemented with 0.1%/kg of dry matter (DM) from an extract of pomegranate (*Punica granatum*) peels and onion (*Allium cepa*) peels, either in aqueous (AQPOMON group) or cyclodextrin-encapsulated form (CDPOMON group). Specifically, the raw material used for extraction consisted of dried pomegranate peels and onion peels. Pomegranate peels were dried for 48 hours at 40°C. Both pomegranate and onion peels were then ground in a mill (Janke and Kunkel, IKA Labortechnik, Germany), with the particle size of the ground plant material standardized to 0.1 mm. For the extraction, 10 g of each dried plant material was used to prepare 100 mL of extract, based on a solid/liquid ratio of 1:10 (w/v). The preparation of both the aqueous and cyclodextrin extracts followed the methodology described by Vasilopoulos et al. (2022). Also, the characterization of the total phenolic content and the antiradical activity of the extracts is presented in Vasilopoulos et al. (2022). Briefly, the β-cyclodextrin extract exhibited a slightly higher total phenolic content compared to the aqueous extract, highlighting its effectiveness in stabilizing polyphenols. However, the aqueous extract demonstrated greater antiradical activity, suggesting that water extraction yields more components with DPPH scavenging properties.

Euthanasia and bird sampling

Prior to slaughter, broiler chickens were euthanized by electrical stunning procedures with the aid of a VE Memory stunner (FAF, Saint-Sernin-Sur-Rance, France). Two birds per replicate, close to mean pen weight, were randomly selected for sampling, for intestinal morphometric measurements, immunohistochemical protein expression, and RNA extraction.

Intestinal histomorphometry and immunohistochemistry of tight junction (TJ) proteins

For assessment of intestinal histomorphometry and immunohistochemistry tissue samples from the mid-jejunum, mid-duodenum and mid-ileum were carefully preserved to maintain their structural integrity for subsequent histological analysis. Immediately after euthanasia (within 2–5 minutes), the samples were placed in 10% neutral buffered formalin, a standard fixative that ensures the preservation of cellular morphology. This fixation process was followed by cutting the samples into thin sections, approximately 3–4 μm thick. These sections were then stained with hematoxylin and eosin, a widely used method that enhances the visualization of tissue structures under the microscope (Sakkas et al., 2019). Morphometrical analysis of the small intestine images was conducted under light microscopy, with a Nikon microscope coupled with a Microcomp integrated digital imaging analysis system (Nikon Eclipse 200, Tokyo, Japan). Images were viewed (5×) to measure morphometric parameters of intestinal architecture. For this purpose, Image-Pro Plus analysis software was used. Three favorably orientated sections cut perpendicularly from villus enterocytes to the muscularis mucosa were selected from each bird and measurements were carried as follows: villus length (VL), from intact villus for measurements, was estimated by measuring the vertical distance from the villus tip to villus-crypt junction level for 10 villi per section; crypt depth (CD) (the vertical distance from the villus-crypt junction to the lower limit of the crypt) was estimated for 10 corresponding crypts per section (Gava et al., 2015).

Deparaffinization was performed, and endogenous peroxidases were blocked using a 0.3% H_2O_2 solution for 30 minutes. Antigen retrieval was achieved by incubation in Tris-EDTA buffer (pH 9.0) at 98°C for 30 minutes. The primary monoclonal antibody targeting claudin-3 (rabbit polyclonal antibody specific to claudin 3, ab15102, Abcam, Cambridge, UK) was applied and left to incubate at 4°C overnight. Staining was carried out using a universal secondary antibody (BioGenex Super Sensitive™ (SS) Link-Label IHC Detection System). Evaluation of *CLDN3* imnunoexpression levels was performed using a quantitative comparison of mean pixel intensity, as outlined by Tomaszewska et al. (2023).

Table 2. Primers used in qPCR analysis

Genes	Reverse (5′-′3)	Forward (5′-′3)
β-actin	TCTGGCACACACTTTCTACA	CACAGGACTCCATACCCAA
GAPDH	TATCTTCCAGGAGCGTGACC	TCTCCATGGTGGTGAAGACA
TJPN[1]	CAACTGGTGTGGGTTTCTGAA	TCACTACCAGGAGCTGAGAGGTA
CLDN1[1]	CCAGGTGAAGAAGATGCGGA	GGTGTGAAAGGGTCATAGAAC
CLDN3[1]	CCAGGTGAAGAAGATGC GA	GGTGTGAAAGGGTCATAGAAGGC
OCLDN[1]	CAGCACCTACCTCAACCAGTACAT	AGGCAGAGCAGGATGACGAT

[1]*TJPN:* zonula occludens 1*; CLDN1:* claudin-1; *CLDN3:* claudin-3; *OCLDN:* occludin.

RNA isolation and RT-PCR analysis

Cecum samples were collected and immediately placed in *RNAlater* solution (Qiagen, UK). The samples were stored at –80°C to maintain RNA integrity before further processing. Total RNA was extracted from the cecum and ileum tissues of broiler chickens using the TRI reagent (Ambion). One microgram (μg) of this RNA was converted into complementary DNA (cDNA) with the SuperScript II Reverse Transcriptase kit (Invitrogen). Quantitative expression of tight junction proteins, including zonula occludens 1 (*TJP1*), claudin-1 (*CLDN1*), claudin-3 (*CLDN3*), and occludin (*OCLN*), was evaluated using real-time PCR on a LightCycler machine (Roche Molecular Biochemicals). The PCR reactions utilized the KAPA SYBR FAST qPCR kit (KapaBiosystems). Gene expression was normalized using β-actin and GAPDH as reference genes, and all reactions were carried out in triplicate. The reactions were carried out with an initial denaturation at 95°C for 5 minutes, followed by 40 cycles of amplification. Each cycle consisted of three steps: denaturation at 95°C for 10 seconds, annealing at 60°C for 30 seconds, and extension at 72°C for 30 seconds. The relative expression data were analyzed using the 2-ΔΔCt method (Livak and Schmittgen, 2001). Primer sequences are provided in Table 2, as described by Metzler-Zebeli et al. (2019) and Daza-Leon et al. (2022).

DNA extraction and amplicon sequencing analysis

For DNA extraction, approximately 250 mg (wet weight) of homogenized pooled samples of ileal and cecal digesta, were derived from 16 broilers in each experimental group. The pooling process was performed within each replicate pen to secure that samples remained representative of the microbial composition within a single experimental unit. DNA was extracted from 12 separate samples of control group (6 ileal and 6 cecal) and 16 separate samples of AQPOMON and CDPOMON groups (8 ileal and 8 cecal) using the Qiagen DNeasy PowerSoil Pro Kit (Qiagen, Carlsbad, CA, USA). The DNA concentration and quality were assessed using a NanoDrop™ spectrophotometer (Thermo Fisher Scientific, Waltham, MA, USA) and agarose gel electrophoresis, respectively, to ensure it was suitable for sequencing. Samples were stored at −80°C until further analysis. The V3–V4 hypervariable region of the bacterial 16S rRNA gene was amplified using the 341F/806R primer pair (341F: 5′-CCTAYGGGRBGCASCAG-3′, 806R: 5′-GGACTACCVGGGTATCTAAT-3′) following the PCR protocol outlined by Cui et al. (2019). The primers, containing specific barcodes, were used to amplify the targeted region, and the resulting 16S rRNA amplicons were paired-end sequenced (2 × 250) using the Illumina NovaSeq 6000 platform.

Sequencing data processing and operational tax units analysis

Species annotation for each representative sequence was performed using the Mothur pipeline and the SILVA138 SSU rRNA database, with a confidence threshold of 0.8–1 at each taxonomic level (Quast et al., 2013). Before conducting alpha- and beta-diversity analyses, the OTU abundance data were normalized by adjusting the sequence count to match the sample with the fewest sequences. By accounting for the correlation between multiple measurements from a single bird, these statistical methods help ensure the results are valid and account for intra-subject variability.

Statistical analysis

Power analysis was conducted prior to the experiment to ensure sufficient replication for detecting biologically relevant differences in histological measurements. Based on a desired power of 0.95 and an alpha level of 0.05, a minimum of 12 replicate pens (4 per treatment) was determined to be necessary for achieving statistical robustness. The actual power achieved for the histological parameters was calculated to be 0.993. This is regarded by standard statistical rules as a large impact size. The specific power analysis was done according to Charan and Kantharia (2013). One-way ANOVA was used to evaluate the possible significant effects of the groups on the expression of the selected genes, the expression of *CLDN3* through IHC and in the histomorphometric measurements. In case of significance, differences between mean values of specific groups were estimated using Duncan's New Multiple Range Test. In case where assumptions about either variability or the form of the population distribution were violated, the nonparametric Kruskal-Wallis test was applied to evaluate differences between groups, while differences between mean values of specific groups were estimated using the nonparametric Mann-Whitney U test. Results were expressed as the mean ± SEM. In all applications differences were considered significant at $P<0.05$.

Also, the "phyloseq" package (McMurdie and Holmes, 2013) in R software (version 2.15.3) was used to display the alpha-diversity (Chao1 and Shannon) indices, which were computed using QIIME software (version 1.7.0). The QIIME software package's alpha_rarefaction.py script was utilized to calculate rarefaction measures (Kuczynski et al., 2012). R software was used to create a heatmap based on the relative abundance of OTUs (Ling et al., 2014). Kruskal-Wallis non-parametric method, followed by Dunn's post-hot test with Bonferroni correction, was performed to identify the differences in alpha-diversity indices across different diets. Additionally, beta-diversity was evaluated with QIIME using principal coordinates analysis (PCoA) based on weighted and unweighted UniFrac distance matrices (Lozupone and Knight, 2005), which assess community structure by taking OTU abundances into account and membership by considering the presence or absence of OTUs. Using analysis of similarities (ANOSIM), the relevance of spatial separation in PCoA was tested in order to determine the general similarity between the various groups.

Results

Intestinal histomorphometry and immunohistochemistry of tight junction (TJ) proteins

In the duodenum, the CD was significantly higher in the AQPOMON than the CDPOMON and the control group (P<0.001) and was higher in the CDPOMON than the control (P<0.001) (Figure 1 A). In terms of VL, no difference was noted between groups (Figure 1 B). The VL:CD ratio in the duodenum varied across the diets, with the control group displaying a higher ratio compared to both the CDPOMON and AQPOMON groups (P<0.0001), while it was also lower in the AQPOMON than the control and CDPOMON (P<0.0001 and P<0.01, respectively) (Figure 1 C).

In the ileum, CD was significantly higher in the AQPOMON than the control group and the CDPOMON (P<0.001 and P<0.01, respectively) (Figure 1 D). Conversely, VL was notably higher in the control diet than in the supplemented diets (P<0.001 and P<0.01, respectively) (Figure 1 E). Likewise, the VL:CD ratio was higher in the control diet compared to both AQPOMON or CDPOMON diets (P<0.0001 and P<0.01, respectively) (Figure 1 F).

In the jejunum, the CD was greater in the CDPOMON diet group than the control and the AQPOMON group (P<0.0001 and P<0.01, respectively) (Figure 1 G). No difference occurred between treatment for jejunum VL (Figure 1 H). The VL:CD ratio was higher in the control diet compared to both AQPOMON or CDPOMON diets (P<0.05 and P<0.0001, respectively) (Figure 1 I).

Regarding *CLDN3* immunohistochemical expression, no significant differences between treated groups and control were detected (Table 3). The *CLDN3* through IHC in the jejunum is shown in Figure 2.

Gene expression

Figure 3 A illustrates the results of relative gene expression of *TJP1* gene in the cecum. Notably, there were no significant differences in *TJP1* gene expression between the AQPOMON and CDPOMON diets; however, the AQPOMON group exhibited increased *TJP1* gene expression compared to the control group (P<0.05). The relative gene expression of *CLDN1* showed no significant changes across the groups (Figure 3 B). In contrast, *OCLN* gene expression was significantly higher in the AQPOMON group compared to the other diets (Figure 3 C). Additionally, the expression levels of *CLDN3* were significantly elevated in the AQPOMON group compared to both the control and CDPOMON groups (P<0.05) (Figure 3 D).

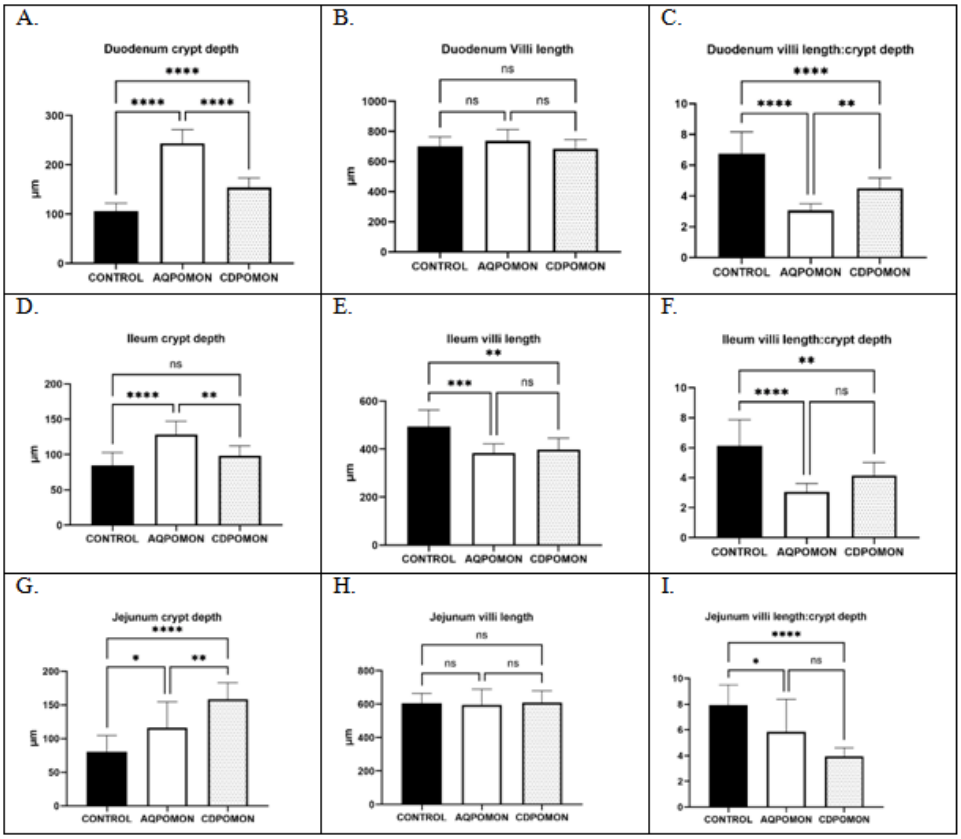

Figure 1. The crypt depth (CD), villi length (VL) and VL:CD ratio in three diet groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM in duodenum (A, B, C), in ileum (D, E, F) and in jejunum. (G, H, I), respectively. Asterisks indicate significant differences (*P<0.05; **P<0.01, ***P<0.001, ****P<0.0001); "ns" indicates non-significant differences (P>0.05) (n=4 per treatment)

Table 3. Mean pixel intensity values (MPV) in each group's *CLDN3* immunohistochemical reaction. Different letters indicate significant differences

Diet groups[1]	Duodenum	Jejunum	Ileum
Control	122±1.50 a	123 a±1.45 a	119 a±1.70 a
AQPOMON	129±1.75 a	126±1.80 a	124±1.50 a
CDPOMON	149±1.20 a	142±1.70 a	145±1.80 a

[1]Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM.

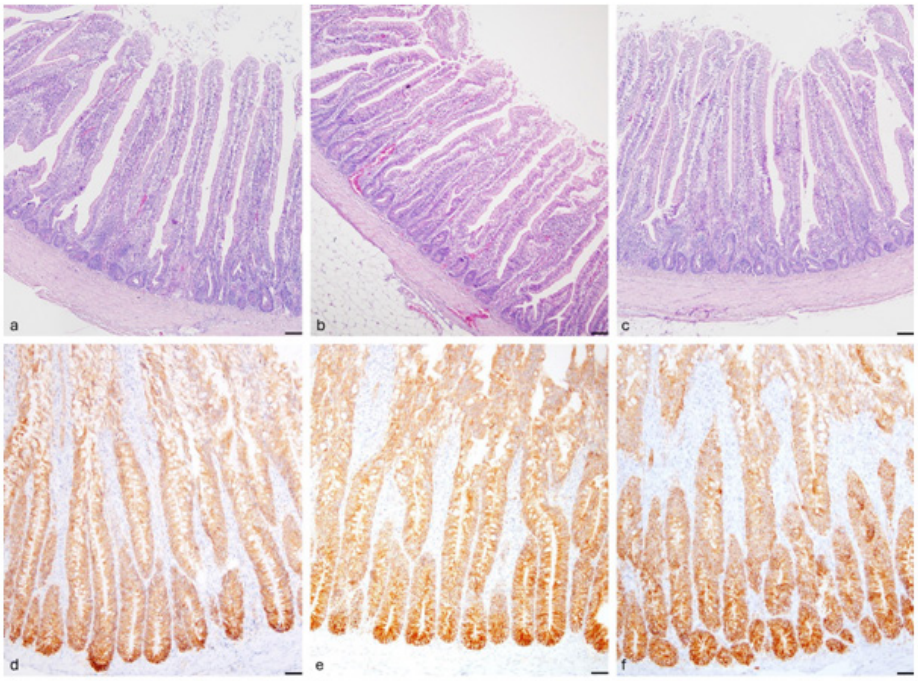

Figure 2. Jejunum: Main histological appearance of three diet groups (a–c), i.e., a) Control: basal diet; b) AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; c) CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM. The immunohistochemical expression of *CLDN3* (on apical and basal regions and the pericellular borders of the epithelial cells) showed continuity or differences in the spatial distribution resulting in epithelial gaps (d–f) Bar=100 µm

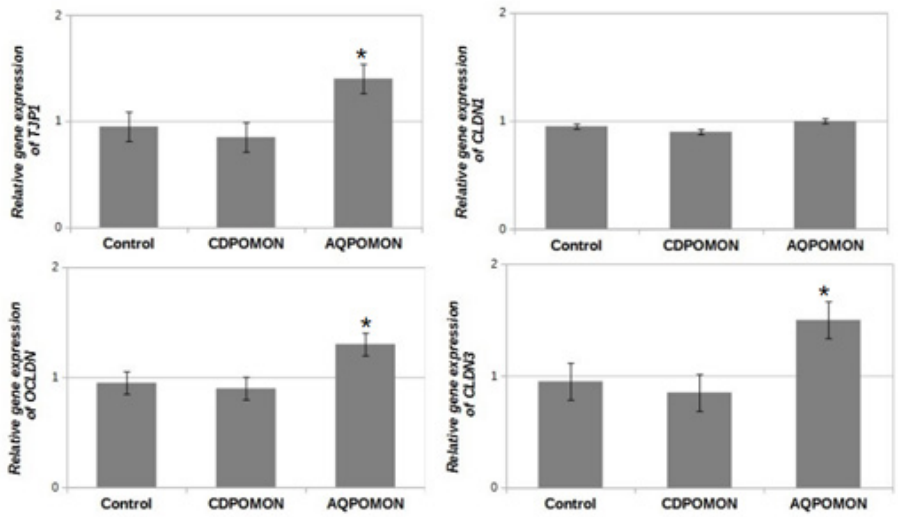

Figure 3. Relative gene expression of *TJP1* (A), *CLDN1* (B), *OCDLN* (C) and *CLDN3* (D) in cecum tissue in three diet groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM. Asterisks indicate significant differences (*P<0.05)

Microbiota analysis

A total of 2,688,014 paired-end raw sequences were generated from both ileal and cecal tissue samples with an average of 96,001 per sample. Following data trimming and chimeric sequences removal, the final number of 1,643,447 high-quality paired-end reads, with an average of 58,695 per sample, were obtained. The reads were assigned to 2538 OTUs. The cecal samples exhibited a greater number of OTUs compared to the ileal samples, with average counts of 612 and 550, respectively (P = 0.049). However, no statistically significant distinctions were detected among the various diet groups. The most abundant phyla were *Firmicutes* and *Bacteroidota* in both ileum and cecum samples. The *Firmicutes* to *Bacteroidota* ratio exhibited an elevation in the ileum sample in contrast to the cecum. In addition, there was a higher prevalence of *Campylobacterota*, *Actinobacteriota* and *Proteobacteria* in the ileum while *Euryarchaeota* were higher in cecum. Significant variations in microbial composition attributed to dietary differences were also observed. In the ceca of broilers, the supplementation of herbal extracts resulted in an increase of WPS-2 populations, whereas in birds administered with the aqueous herbal mixture, the *Proteobacteria* and *Cyanobacteria* communities exhibited a greater abundance. *Proteobacteria* showed greater populations in the ileum of herbal supplemented birds while *Euryarchaeota* increased only in the aqueous supplemented birds. At the genus level, a significant dominance of *Lactobacillus* was observed consistently across all experimental groups in the ileal samples. Conversely, in the cecum, the microbial population distribution exhibited notable differences, with more dominant genera such as *Lactobacillus*, *Methanobrevibacter*, *Faecalibacterium*, and *Alistipes* observed in all samples. In the cecal samples of broilers supplemented with herbal mixture, there was an elevated presence of *Staphylococcus*. Conversely, in the ileal samples, the pattern was reversed, with *Staphylococcus* exhibiting higher levels in the control group birds. Broilers administered the herbal mixture in aqueous form demonstrated a reduction in *Methanobrevibacter* levels in their cecum, alongside an increase in *Campylobacter* abundance in their ileum (Figure 4).

Alpha diversity, assessed using the Chao1 index, Shannon index, and rarefaction analysis of observed species, revealed higher species richness and diversity in the cecal microbiota compared to the ileal microbiota (Figure 5). Chao1 index did not show significant changes. Notably, the Shannon index was higher in the CDPOMON diet than in the AQPOMON diet within the ileal tissue, while no significant differences were observed in the cecal tissue (Figure 5 B). Additionally, the ACE index showed similar trends across both intestinal tissues (Figure 5 C).

Principal component analysis (PCA) showed the distinct clustering of the cecal and ileal microbiota (Figure 6). To investigate beta-diversity one-way analysis of similarity (ANOSIM) was completed (Figure 7) and the results confirmed the significant difference present among the ileal and cecal microbiota. According to R-value both herbal supplemented groups showed significant differences in community structure in contrast to the control broilers regarding ileum and cecum tissues.

Figure 8 A represents the LDA score analysis between the 3 diet groups. The data revealed that there were 26 species that were most prevalent in the cecal microbiota, compared to 7 species in the ileal microbiota. Specifically, in CDPOMON diet *Bacteroidaceae*, *Bacteroidia* and *Bacteroides* dominate and the other genera have smaller score in cecum tissue. On the other hand, in AQPOMON diet *Firmicutes*, *Bacteria* and *Clostridia* show the highest score. In ileal tissue, in AQPOMON diet only *Ruminococcaceae* has a score higher than 4 and in control diet *Actinobacteria* and *Actinobacteriota* have a score higher than 4. Besides, in CDPOMON diet *Bacteroidota*, *Bacteroidia*, and *Bacteroides* have a score of 4.5 (P<0,05) (Figure 8 B).

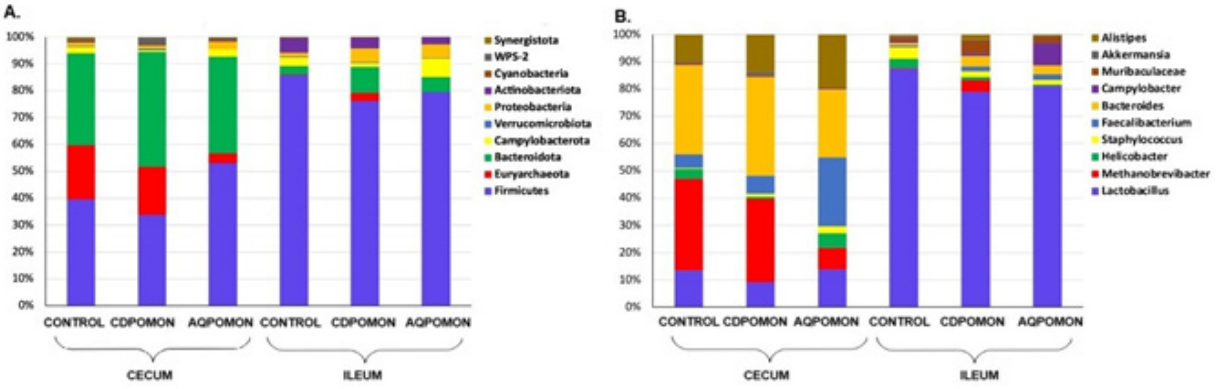

Figure 4. Distribution of the top 10 most abundant taxa of intestinal microbiota in ileum and cecum digesta at the levels of phylum (A) and genus (B) in three diet groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM

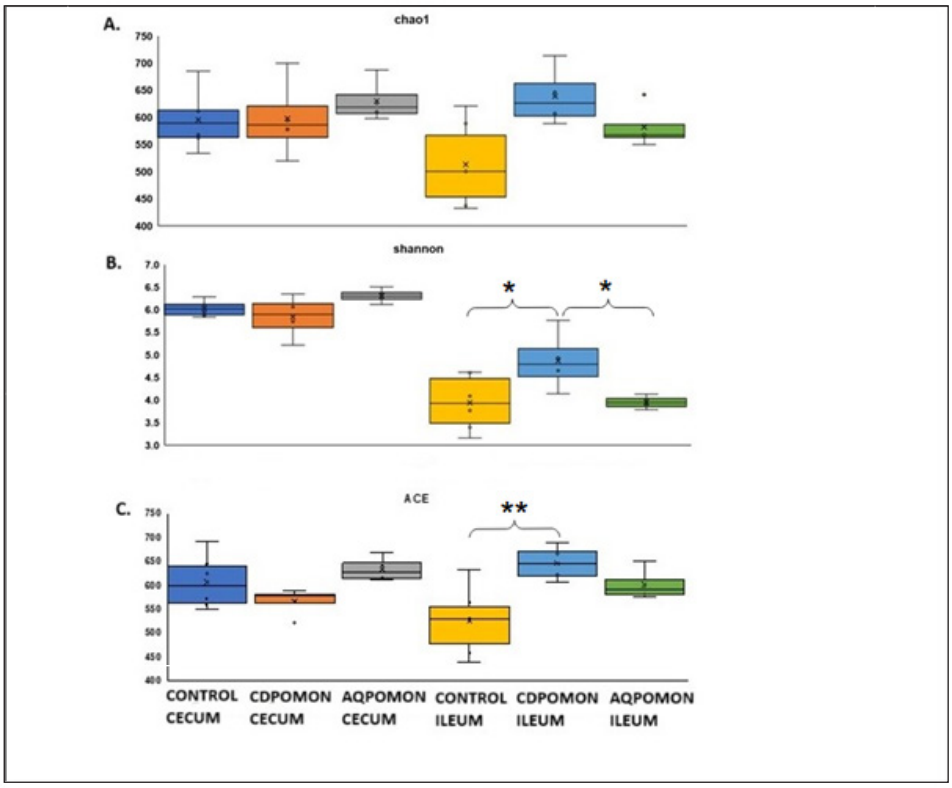

Figure 5. Box plots of alpha-diversity of microbiota residing in the ileum and cecum of broiler chickens in the three different dietary groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM. Richness (Chao-1), and evenness (Shannon evenness) and Abundance-based Coverage Estimator (ACE) indexes observed in the different samples (A, B and C). Asterisks indicate significant differences (*P<0.05; **P<0.01, ***P<0.001) based on Kruskal-Wallis test followed by Dunn-tests for multiple comparisons among the three diets in cecum and ileum, separately

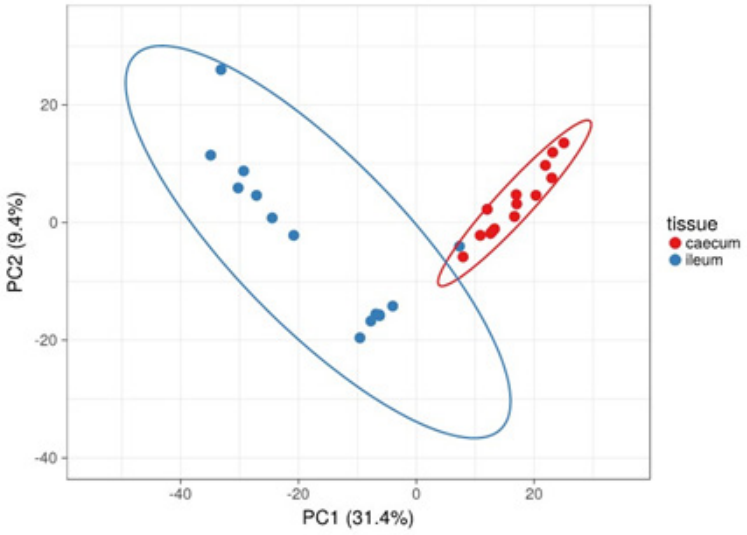

Figure 6. Principal component analysis (PCA) based on OTUs level microbiota composition of samples (P<0.05)

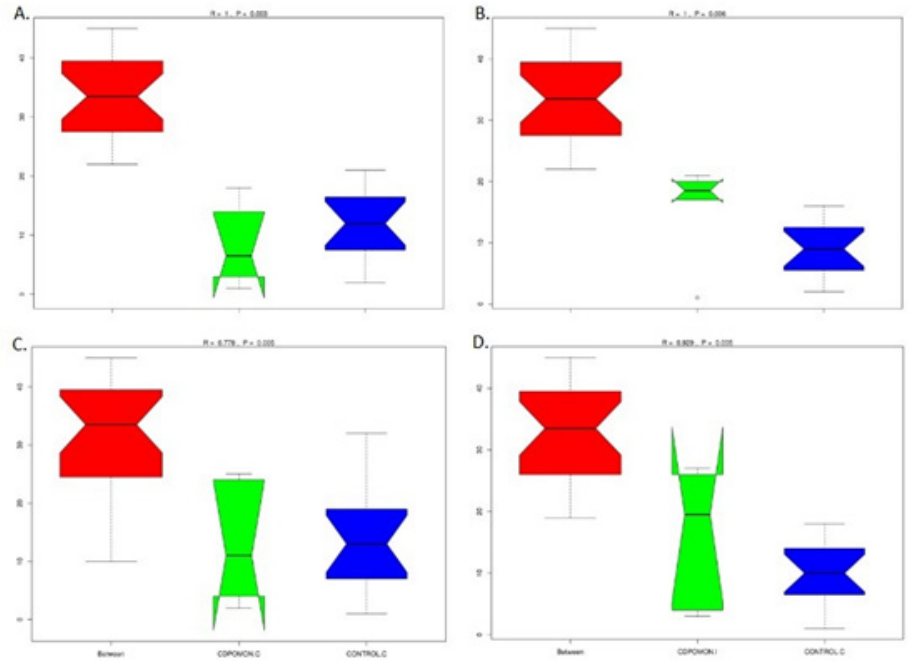

Figure 7. ANOSIM analysis of beta distance based on unweighted UniFrac distances of microbial communities in the three different dietary groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM, i.e., (A) beta distance of AQPOMON and control, (B) beta distance of AQPOMON and CONTROL, (C) beta distance of CDPOMON and control, and (D) beta distance of CDPOMON and control

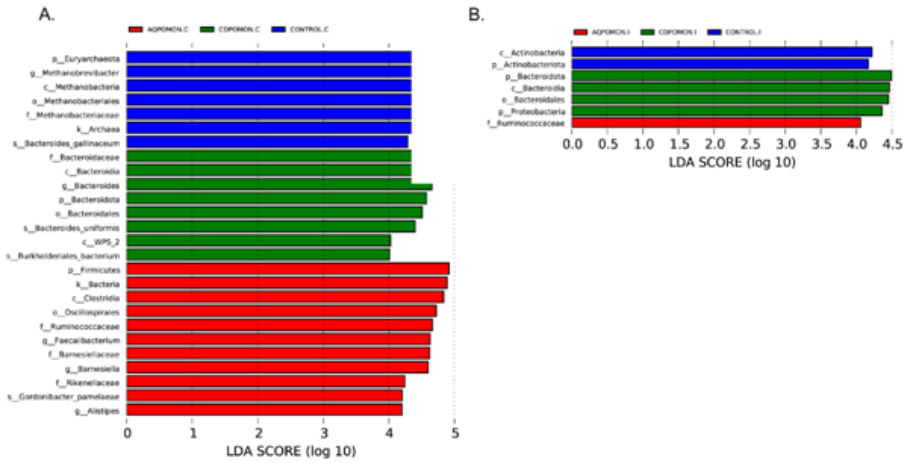

Figure 8. Lefse analysis in three diet groups in two examined intestinal tissues (A) cecum (B) ileum in three diet groups, i.e., Control: basal diet; AQPOMON: diet supplemented with aqueous pomegranate and onion extract at 0.1% per kg of DM; CDPOMON: diet supplemented with pomegranate and onion in cyclodextrin at 0.1% per kg of DM

Discussion

The effects of incorporating pomegranate and onion extracts into broiler chicken diets, whether in aqueous or cyclodextrin-encapsulated forms, remain inadequately explored. The present study aimed to increase our knowledge of how well the extraction procedure might change intestinal health, with the hypothesis that cyclodextrin solutions will outperform aqueous solutions because of their higher bioavailability.

In contrast to our expectations, intestinal histomorphometric features were adversely affected by offering the two extract effects being more pronounced in the CDPOMON group. Supplementation with extracts and contained plant secondary metabolites has been shown to overall improve intestinal architecture due to reduced inflammation (Liu et al., 2023). Increases in CD have been previously observed, suggesting increased epithelial cell turnover (Cloft et al., 2023). However, the parallel decrease in VL may suggest that although selected levels

of dietary extract supplementation improved antioxidant (Savvidou et al., 2023) and fatty acid profile in breast (Vasilopoulos et al., 2022), they adversely affected morphometric features as indicated by reduced VL:CD ratio in supplemented groups. The previous results may suggest that selected doses were significantly above effective levels of supplementation for improving intestinal health. The changes in effects across different sections of the intestinal tract and between extraction methods could be due to variations in the absorption dynamics of the bioactive compounds in each extract, indicating that the bioavailability and impact of plant bioactives are influenced by both the extraction procedure and the intestinal site of absorption. Therefore, a more targeted method in selecting extract concentrations and methods may be necessary to optimize the benefits of plant-derived bioactive compounds without exceeding thresholds that negatively affect gut health (Grgić et al., 2020). The method of encapsulation increases the absorption of bioactive compounds by intestinal epithelial cells (Pérez-Pérez et al., 2024), which may describe why the adverse impacts were more noticeable in the jejunum for the CDPOMON group compared to the aqueous extract. Increased concentrations in the jejunum due to the increased absorption of encapsulated substances may surpass ideal levels and adversely impact intestinal morphology (Grgić et al., 2020). In contrast, the aqueous extract, with lower absorption, showed less severe negative consequences in this region. This suggests that the encapsulation method and dose must be carefully optimized to avoid harmful effects (Domínguez et al., 2021), particularly in the jejunum. Increased VL and decreased CD, which indicate a bigger surface area for nutrient absorption, are mostly linked to enhanced nutrient absorption and help broilers grow more (Basit et al., 2020). Future studies should concentrate on enhancing intestinal health by refining the dosage and methods of extracting plant-based bioactive compounds.

Another possible explanation could be related to the site of activity. It is possible that the aqueous extract exhibited higher bioactivity in the proximal part of the small intestine whereas the encapsulated form of herbal extracts may show stronger activity on the last section of the intestine (Dokou et al., 2023; Savvidou et al., 2023).

Contrary to the effects on intestinal architecture, the gene expression analysis showed notable differences in the expression of TJPs across the dietary treatments. The elevated expression of *OCLDN* in the AQPOMON diet compared to both the CONTROL and CDPOMON diets suggests enhanced intestinal barrier function, as occludin is a crucial protein in TJs that maintain the integrity of the intestinal epithelial barrier (Daza-Leon et al., 2022; Citi, 2020). Regarding antioxidant effects, the AQPOMON diet appears to have a different effect on the intestinal tract compared to the CDPOMON diet, particularly regarding its antioxidant effects. AQPOMON has been associated with better regulation of the intestinal barrier, likely due to the increased bioavailability of its bioactive compounds. This may also relate to the accumulation of metabolites in the cecum. In contrast to the CDPOMON diet, which is known for its enhanced absorption kinetics, the AQPOMON diet might limit the absorption of certain compounds. This limitation might result in higher bioactive metabolites concentration in the cecum, which could affect the microbiota as well as antioxidant activity in the region. The disparities in metabolite profiles associated with the two diets may account to a larger extent for the difference in antioxidant activities and their contribution to the intestinal health through different mechanism of absorption. Thus, AQPOMON may reduce the concentrative systemic absorption, but may enhance the local retention, especially in the cecum, which could increase localized antioxidant activity without causing injuries to the intestinal epithelial cells in other regions. Also, the elevation of MUFA, PUFA, and DHA in the thigh and breast tissues of the broilers fed with these diets can inset the intestinal obstruction by less oxidative injury to epithelial cells and anti-inflammatory response (Vasilopoulos et al., 2022). The integrity of the intestinal obstruction can be maintained by enhancing antioxidant defense and fatty acid structure in tissues. Intestinal obstruction is maintained by cecum epithelial cells, which can be protected from harm by low oxidative stress (Chelakkot et al., 2018). In addition, the increased levels of beneficial fatty acids including DHA can have anti-inflammatory effects that support the expression of TJ proteins and enhance intestine health. In the cecum, the *CLDN3* gene expression is upregulated by gut microbiota alteration, SCFA (butyrate) production, and activation of AMPK signaling pathways that support epithelial tissue integrity (Liu et al., 2021; Stein and Riber, 2023). Likewise, the emission of anti-inflammatory cytokines, lower oxidative stress, and mTOR- and FXR-like nutrient sensing TJs regulating pathways assist to maintain balance of cecal health and energy expenditure in broilers (Li and Li, 2017; Mihaylova and Shaw, 2011). It should be pointed out that although the level of expressed genes is indicative of transcriptional activity, it does not always convert into protein in the presence of post transcriptional processes of regulation and degradation (Wang et al., 2008). However, high mRNA expression usually suggests attempts by the cells to promote intestinal barrier function, or in other words, is an indicator of functional responses to stress or inflammation (Akram et al., 2024).

Also, the analysis of the microbial communities in the examined digesta content revealed that the most abundant phyla were *Firmicutes*, *Eyriarchaiota* and *Bacteroidota*. In accordance to previous studies *Firmicutes* and *Bacteroidota* were two most common bacterial phyla (Wen et al., 2019; Dokou et al., 2023). As a prominent phylum in the intestinal microbiota, the *Firmicutes* are able to produce resistant endospores that germinate in the colon to aid in transmission and environmental survival (Browne et al., 2021). On the other hand, *Campilobactereota* were abundant in the ileum of chicken fed the AQPOMON diet including species such as *Campylobac-*

ter which can impact human health. Although there is a high prevalence of *Campylobacter* in poultry microbiota, they are generally considered non-pathogenic to birds (Clavijo and Flórez, 2018).

Notably, the AQPOMON diet's ability to increase beneficial bacteria, such as *Lactobacillus* and *Clostridium* in the cecum, aligns with the findings of Yan et al. (2017), highlighting the potential of aqueous herbal extracts to support gut health. Similarly, Chinese phytogenics contain plant secondary metabolites that have the potential to promote the increase of prevalence of *Lactobacillus* and *Clostridium*, two bacteria that are known to improve intestinal mucosa integrity and stimulate the intestinal immune system (Bai and Li, 2022; Liu et al., 2024). It is well known that pomegranate includes phenolic components, such as hydrolysable tannins, flavonoids (both anthocyanins and catechins) that display potent anti-oxidant activities (Gessner et al., 2017), favorably modify microbiota composition (Oteiza et al., 2018).

The results indicated a decrease in microbial populations in the ileum for both examined diets compared to the control diet. Intestinal health is positively correlated with a reduction in *Bacteroides* and an increase in *Lactobacilli* (Józefiak et al., 2020). *Bacteroidetes* are among the most prevalent bacteria in the intestines of broiler chickens. Previous studies have shown that a one-week treatment with lincomycin in mice led to a reduction in *Bacteroidetes* numbers (Zhang et al., 2020). In addition, chicks treated with the aqueous extract of the herbal mixture displayed greater species richness and diversity in the cecum than in the ileum, as indicated by alpha-diversity indices. Notably, the Simpson index increased in the presence of oregano aqueous extract in cecal tissue. Moreover, broilers fed an aqueous extract of oregano demonstrated an increase in beneficial microbiomes (Zhang, 2022).

Staphylococcus was detected in higher concentrations in the cecal digesta of broiler chickens that were treated with the AQPOMON diet. In contrast, the pattern in the ileal samples was reversed, with control group birds showing higher levels of *Staphylococcus*. Additionally, the administration of the herbal mixture in aqueous form, resulted in increased abundance of *Campylobacter* in the ileum, whereas their cecum's *Methanobrevibacter* levels decreased. According to earlier research, staphylococci are frequently found in the intestinal tract of broilers (Salanitro et al., 1978; Syed et al., 2020). However, considering the dual roles that staphylococci (commensals and pathogens) can play in the gut, their presence may not be entirely beneficial (Rosenstein and Götz, 2013; Syed et al., 2020).

It is worth noticing that *Ruminococcaceae* has been only found in the ileal digesta of AQPOMON diet in Lefse analysis. The only carbohydrates that are not digested in the gut are fibers, being present in plants; known to regularly boost lactic acid bacteria like *Ruminococcus.* Additionally polyphenols, prevalent in plant-based diets, boost the growth of *Bifidobacterium* and *Lactobacillus*, which possess anti-inflammatory and anti-pathogenic properties (Tomova et al., 2019). It is possible that the increase of members of the *Lachnospiraceae* and *Ruminococcaceae* families observed in the intestinal microflora of tannins-fed chickens could further alter the SCFAs profile in the cecum towards butyrate production (Díaz Carrasco et al., 2018). Moreover, the data of Lefse analysis revealed that *Firmicutes, Bacteria* and *Clostridia* are present in AQPOMON diet in cecal tissue. It has been discovered that the number of certain *Clostridiales* species is associated with improved intestinal health and increased energy harvesting efficiency in broilers (Díaz Carrasco et al., 2018). However, the presence of *Bacteroides* in CDPOMON diet in ileum tissue indicates the saccharolytic and proteolytic role of these microbes (Cardona et al., 2013). Also, while *Actinobacteria* and *Actinobacteriota* make up a very minor portion of the control diet, they are essential for maintaining gut homeostasis.

Conclusions

This research highlights the effectiveness of dietary supplementation with aqueous (AQPOMON) and cyclodextrin-encapsulated (CDPOMON) formulations derived from *Allium cepa* and *Punica granatum* peels. The AQPOMON diet was found to increase beneficial bacteria such as *Lactobacillus* and *Clostridium* in the cecum. In contrast, while AQPOMON enhanced the abundance of *Helicobacter* and decreased *Lactobacillus* in the ileum, the cyclodextrin formulation effectively reduced the prevalence of pathogenic *Campylobacterota*. On the other hand, the cyclodextrin formulation successfully decreased the prevalence of pathogenic *Campylobacterota*, whereas AQPOMON increased the amount of *Helicobacter* and decreased *Lactobacillus* in the ileum. The AQPOMON diet increases TJ expression, however it has a detrimental effect on intestinal morphometry, which would indicate oversupplementation with the plant bioactive chemicals. Despite the fact that both diets encouraged good bacteria, the AQPOMON diet worked better overall.

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Disclosure statement

Authors have no financial interest or conflicts.

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