Impact of Dietary Supplementation of Flaxseed Meal on Intestinal Morphology, Specific Enzymatic Activity, and Cecal Microbiome in Broiler Chickens

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Abstract: The use of natural feed additives could be a beneficial approach to maintaining the health of chickens and a way to improve food digestion. Flaxseed is a rich source of omega-3 fatty acid, alpha linolenic acid, oleic acid, and fiber. The purpose of this study was to evaluate the effects of dietary inclusion of 4% flaxseed on the intestinal morphology, specific enzymatic activity, and cecal microbiome in broiler chickens. The 4-week feeding trial was conducted on 100 Cobb 500 (14 days of age) unsexed broiler chickens divided into two groups: a control group (C) and an experimental group (E). The broilers were housed in boxes of size 3 m² (each group was housed in a single box with 10 replicates, 5 chickens per replicate) and reared on permanent wood shaves litter (10–12 cm thick). At the end of the experiment, chickens (n = 10) were sacrificed and tissue samples were harvested from the duodenum, jejunum, and cecum for histological, enzymatic, and microbiome analyses. In group E, histological analysis revealed a significant increase in villus height (p < 0.001) possibly leading to enhanced intestinal nutrient absorption. An increase in the specific activities of α-amylase (p < 0.05), invertase (p < 0.01), and endo-β-1,4-glucanase (p < 0.001) was noticed in the E group for the duodenum and jejunum compared to the control group. In contrast, maltase activity decreased in the duodenum and increased in the jejunum in the E group. The trypsin and lipase specific activities did not vary in a significant way. In addition, the cecal microbiome of the E group was characterized by an increase in Lactobacilli (p < 0.01) and Clostridium cocoides and a decrease in Bacteroides, Ruminococcus, Enterobacteriaceae, and Clostridium leptum. In conclusion, our results suggest that dietary supplementation of flaxseed meal may boost intestinal health status in poultry.

Keywords: flaxseed meal; poultry; digestive enzyme activities; nutrition; microbiome

1. Introduction

At a global level, poultry meat is a mass consumer product. Among the industrialized Western countries, the United States and the members of the European Union are among the highest poultry consumers [1]. Poultry are commonly the most prevalent livestock in resource-poor areas and contribute to food security in world meat production through the supply of nutrient-rich products [2]. Cost efficiency is pivotal in poultry production; therefore, it is desirable not to include antibiotics in the diet because their use in both
humans and animals contributes to the increasing resistance of microorganisms to these therapeutic agents [3,4].

The Cobb 500 hybrid is the most widely used in industry and is also the broiler with the earliest growing time and the best feed conversion rate. It is appreciated due to its superior productive performance in conditions with low nutrient density [5].

Macronutrients in the diet of birds are represented by monosaccharides, monoglycerides, amino acids, free fatty acids, and dipeptides, all of which can be absorbed [6]. High growth rate and feed efficiency are crucial aims to be achieved in poultry production [7]. Several aspects should be considered for the optimum performance of birds, including weight gain, genetic potential, diet quality, feed intake, feed conversion ratio, disease status, and environmental conditions [8,9]. Apart from these mentioned factors, a well-functioning and healthy gut is essential in poultry production, improving nutrient digestion, absorption, metabolism, and overall health and growth performance [10]. When gut homeostasis is disturbed, digestion and nutrient absorption are impaired, impairing the health and performance of birds [9,11].

Recent studies indicate that the nutritional quality of feed used in poultry farming is correlated with the activity of digestive enzymes [12]. In birds, the proventriculus/gizzard participates in grinding and mixing ingested feed to facilitate the activity of endogenous enzymes; therefore, the gastrointestinal tract of poultry produces enzymes to aid in the digestion of nutrients [13,14]. Nutrients from the diet are absorbed along the gastrointestinal tract through the intestinal mucosa, folded into villi and polarized epithelial cells that are present on the apical domain of the microvilli forming the brush border. This enhances the small intestinal surface by around 600-fold, leading to higher capacity for nutrient absorption [12,15].

Among the determinants that may be involved in the gut health and performance of chickens, the commensal microbiome is of paramount importance due to its multiple roles in host physiology, including the maturation of the immune system and protection against pathogenic microorganisms [10,16]. Previously, it was suggested that the gut microbial profile can be shaped by changes in the host diet [17]. In addition to these, some feed or feed ingredients, referred to as nutraceuticals, have been shown to generate protective effects on the intestinal tract of chickens by modulating the microbiome and the immune system [9,18].

In broiler nutrition, probiotic species such as Bifidobacterium, Lactobacillus, Streptococcus, Enterococcus, Bacillus, Candida, Saccharomyces, and Aspergillus are used to improve broilers’ growth performance and counteract infections [19–21].

Phytogenic feed additives (PFA) are plant-derived natural bioactive compounds that are incorporated into animal feed to enhance productivity [22,23]. In recent years, PFAs have been employed as natural growth promoters in the poultry and pig industries [24]. Flaxseeds contain about 34% oil and 50% α-linolenic acid, and they are a common feed ingredient in poultry diets. Due to their rich content in omega-3 fatty acids, they represent an increasingly popular additive to the diets of many consumers [25,26]. Also, they contain some anti-nutritional factors, such as cyanogenic glucosides and mucilage, which can reduce the enzymatic activity of the pancreas, leading to decreased digestion and lower bird performance [27].

Flaxseed and flaxseed meal have been shown to promote several health benefits, including anticancer effects, antimicrobial activity, immunomodulation, laxative effects, and reduction of atherogenic risks [28,29]. In brown layer hens’ diets, the inclusion of flaxseed meal reduced crude protein digestibility and apparent metabolizable energy corrected for nitrogen (AMEn), while egg production and hen performance increased [30,31]. Compared to laying hens, broiler chickens fed a flaxseed diet exhibited negative effects on production performance due to anti-nutritional factors (ANFs) that interfered with nutrients used, with an impact on the birds’ performance parameters, including final body weight, average daily gain, yield of cut-up parts, and carcass weight [32]. However, poultry fed a 10%
flaxseed diet for 24 h, 4–5 days before slaughter, showed polyunsaturated fatty acid (PUFA) enrichment of the carcass, without any effect on body weight [32,33].

In poultry, the site of nutrient absorption is in the small intestine, especially the duodenum and jejunum segments. Recent studies have shown that non-starch polysaccharides from flaxseed have decreased nutrient digestibility and have a negative impact on productive performance [34], whereas carbohydrate hydrolyzing enzymes improve the digestibility of poultry diets [35]. The capacity for nutrient absorption at the intestinal level depends on the gastrointestinal tract’s mucosal surface area and the functional properties of the epithelium [36].

Previously, it was shown that flaxseed meal administration decreased chicken meat cholesterol [37] and improved the egg nutritional quality by increasing the yolk PUFA content [38].

The aim of this study was to evaluate the effects of the presence of flaxseed meal as a source of PUFAs in the conventional diet of Cobb 500 broiler chickens on digestive health by histologic and morphometric analyses of the intestinal mucosa, analysis of the cecal microbiome, and specific enzymatic activity measurements of lipase, trypsin, alpha-amylase, invertase, maltase, and endo-β-1,4-glucanase.

2. Materials and Methods

2.1. Plant Material and Proximate Chemical Composition and Fatty Acid Profiles

Phytochemical characterization of plant material samples of flaxseed meal (Linum usitatissimum L.) was performed by a commercial company producing cold-pressed oils on the local market in Alexandria (Teleorman, Romania). The proximate chemical composition determinations were performed using methods from Regulation (CE) no. 152/2009 (Sampling and analytical methods for the official inspection of feeds): the Kjeldahl method (SR EN ISO 5983-2:2009) for crude protein (CP); the gravimetric method (SR ISO 6496:2001) for dry matter (DM); extraction in organic solvents (SR ISO 6492:2001) for ether extractives (EE); the gravimetric method (SR EN ISO 2171:2010) for ash (Ash); and acid hydrolysis followed by alkaline hydrolysis (SR EN ISO 6865:2002) for crude fiber (CF).

The fatty acid content was assessed via fatty acid methyl ester (FAME) gas chromatography according to ISO/TS 17764-2 (2008), as described by Panaite et al. [39]. In brief, fatty acids from the total lipid extracts were converted to methyl esters by transesterification (in methanol containing 3% concentrated H₂SO₄, for 4 h at 80 °C). Methyl esters of fatty acids were evaluated in a Perkin Elmer-Clarus 500 chromatograph equipped with a flame ionization detector (FID) fitted with a BPX70 capillary column (60 m × 0.25 mm i.d., 0.25 µm film thickness). The column temperature was programmed at 5 °C/min from 180 °C to 220 °C. The carrier gas was hydrogen (35 cm/s linear velocity at 180 °C) and the splitting ratio was 1:100. The injector and detector temperatures used were 250 °C and 260 °C, respectively. FAME identification was performed by comparison with the retention times of the standards, and the results are expressed as grams of fatty acid per 100 g of total fatty acids.

2.2. Poultry and Experimental Treatments

The 4-week feeding trial was conducted on 100 Cobb 500 broilers (14 days of age), assigned to 2 groups, housed in boxes of size 3 m² (50 chickens per group, 10 replicates, with 5 chickens per replicate) and housed in an experimental hall on permanent wood shaves litter in a controlled microclimate according to the Cobb 500 Management Breeding Guide. The broilers were procured from a commercial hatchery at 1 day old. During the starter stage (1–14 days) all chicks received a conventional diet formulation (based on corn, gluten, and soybean meal) with 23% CP and 3039.79 kcal/kg ME. After 14 days, the broilers were individually weighed and assigned to one of two groups: a control group (C), fed the conventional diet, and an experimental group (E), fed the conventional diet supplemented with 4% flaxseed meal (experimental diet structure published previously by Turcu et al. [40]). Diet formulations were calculated to meet the feeding requirements of
broiler chickens as given by the NRC [41]. Throughout the experimental period (14–42 days, broiler age) the following parameters were monitored: body weight (grams); average daily feed intake (grams of feed per broiler per day); average daily weight gain (grams per broiler per day); and feed conversion ratio. They had free access to feed and water. At the end of the experimental period (42 days), 10 broilers per group (randomly selected) were electrically stunned and subsequently slaughtered by cervical dislocation, according to the Romanian Law 43/2014 for the handling and protection of animals used for experimental purposes (EU Council Directive 98/58/EC and Directive 2010/63/EU). After slaughtering the broiler chickens, samples from intestinal contents from the distal portion of the duodenum and jejunum were collected in order to perform enzymatic analysis (alpha-amylase, trypsin, and endo-β-1,4-glucanase); samples from the intestinal wall of the distal portion of the duodenum and jejunum were collected in order to perform enzymatic analysis (invertase and maltase) and histologic analyses; and samples from the cecal intestinal content were collected in order to perform microbiome characterization, according to Popescu et al. [12].

2.3. Histology

The fragments of the duodenum and jejunum were fixed in 4% paraformaldehyde solution in PBS for 24 h, followed by dehydration in a graded series of alcohols, clarification in toluene, and embedding in paraffin. Sections of 4 µm thickness were adhered on slides and stained with hematoxylin-eosin (H&E) and Alcian Blue pH 2.5 (AB). Histomorphometry of the intestinal segments was performed on ten villi and crypts per individual using an optical microscope (Olympus BX43, Tokyo, Japan), a camera (30 XC Olympus, Tokyo, Japan), and the image analysis software Olympus Cell Dimension. The following variables (randomly selected) were measured in each segment: villus height (villus tip to base of villus) and crypt depth (based of villus to bottom of crypt). An average value for each variable was calculated for each intestinal segment of each bird.

2.4. Enzymatic Analysis

Alpha-Amylase (EC 3.2.1.1) activity was quantified using a modified version of the protocol by Bernfeld [42] in a reaction mixture with a final volume of 405 µL. Trypsin (EC 3.4.21.4) activity was assayed according to the method described by Hummel [43] and modified according to Popescu et al. [12]. Lipase (EC 3.1.1.3) activity was determined using the Lipase Activity Colorimetric Assay Kit instructions (code K 722; Bio Vision, Milpitas, CA, USA). Maltase (EC 3.2.1.20) activity was measured using a commercial kit, following the manufacturer’s instructions (Maltase assay kit, My BioSource, San Diego, CA, USA). The enzymatic activity of invertase (EC 3.2.1.26) was determined according to the Invertase assay kit instructions (code MAK 118; Sigma-Aldrich, Darmstadt, Germany). Endo-β-1,4-glucanase (EC 3.2.1.4) activity was assayed according to the method described by Ahmed et al. [44] and Lokapirnasari et al. [45] using DNS reagent and carboxymethyl cellulose (CMC) as the substrate. Specific activities for amylase are expressed as micromoles of maltose released in one minute at 25 °C per milligram of protein; those for trypsin are expressed as micromoles of N-p-Tosyl-L-arginine methyl ester hydrochloride (TAME) hydrolyzed in one minute at 25 °C per milligram of protein; those for lipase are expressed as the amount of enzyme that hydrolyzes triglyceride to yield 1 µmol of glycerol per minute at 37 °C per gram of protein; those for maltase are expressed as micrograms of glucose released per minute at 37 °C per milligram of protein; those for invertase are expressed as nanomoles of glucose released per minute per milligram of protein; and those for endo-β-1,4-glucanase are expressed as one micromole of glucose released in one minute per gram of protein at 50 °C. The protein concentration of the samples was calculated by interpolating in a bovine serum albumin (BSA) standard curve using the Bradford method [46].
2.5. Microbiome Characterization

After chickens were sacrificed, samples of cecal contents were collected from each experimental batch (n = 10 animals per group). The changes in the microbiome were assessed by the qRT-PCR technique as described by Popescu et al. [12]. The primer sequences used are shown in Table 1, and *Eubacteria* primers were used as internal control.

**Table 1.** Primers used in this study (Hayashi, Matsuki).

| Taxonomic Target          | Primer Sequence       |
|---------------------------|-----------------------|
| *Eubacteria*              | UniF340 ACT CCT ACG GGA GGC AGC AGT |
|                           | UniR514 ATT ACC GCG GCT GCT GGC     |
| *Ruminococcus*            | Fwd ACTGAGAGTTGAACGGCCA |
|                           | Rev CCTTACACCCAGTAATTCCGGCA     |
| *Enterobacteriaceae*      | Uni515F GCC TCA AGG GCA CAA CCT CCA AG |
|                           | Ent826R GCC TCA AGG GCA CAA CCT CCA AG     |
| *Clostridium leptum*      | Fwd GCACAAGCAGTGGAGT |
|                           | Rev CTTCCTCCGTTTTTGCA     |
| *Clostridium coccoides*   | Fwd GAC GCC GCG TGA AGG A |
|                           | Rev AGC CCC AGC CTT TCA CAT C     |
| *Bacteroides*             | Fwd CCT ACG ATG GAT AGG GGT T |
|                           | Rev CAC GCT ACT TGG CTG GTT CAG     |
| *Lactobacilli*            | LabF362 ACG AGT AGG GAA ATC TTC CA |
|                           | LabR677 CAC CGC TAC ACA TGG AG     |

2.6. Statistical Analysis

All the data are expressed as mean ± standard deviation (n = 10). Unpaired t-test, followed by Bonferroni’s post hoc test, was performed (GraphPad Prism software, version 6) to investigate the statistical differences between the control and E groups for all parameters analyzed. *p*-values of <0.05 were considered statistically significant.

3. Results

3.1. Composition Analysis of Flaxseed Meal

Analyzing Table 2, it can be observed that the flaxseed meal used in broiler diets was a rich source of crude protein (33.29%) and polyunsaturated fatty acids (73.65%), especially alpha-linolenic acid (55.93%), with an ideal PUFA n6/n3 ratio of 0.31 [47]. The results obtained in this study are similar to those reported by Panaite et al. [48], Akhtar et al. [49], and Vlaicu et al. [50] regarding the flaxseed meal’s chemical composition.

3.2. Performance Parameters

The results regarding the production parameters recorded during the entire experimental period (1–42 days) are shown in Table 3. The inclusion of flaxseed meal in the broilers’ diets did not influence their feed conversion ratio, average daily weight gain, or, consequently, general body weight. The results of the current study are in agreement with those of Konieczka et al. [51] and Turcu et al. [40]. They reported statistically insignificant results in the use of flaxseed meal in broiler diets regarding effects on rearing parameters (feed intake, body weight gain, and feed conversion ratio) during the overall feeding trials.

3.3. Histology of the Duodenum

The villi and intestinal crypts of the duodenal segments of chickens fed with flaxseed meal had a histological appearance similar to that for the control group, with normal columnar epithelium and mucous cells (colored in blue in AB), producing a protective layer that lines the surface of the epithelium (Figure 1). The histomorphometric analysis of the duodenum showed that the flaxseed meal supplementation to the basal diet of broiler chickens increased (*p* < 0.001, Table 4) the villus height, crypt depth, and villus height/crypt depth ratio compared with those of the control.
Table 2. Nutrient contents of the flaxseed meal.

| Specification | Flaxseed Meal |
|---------------|--------------|
| Proximate chemical composition (%) |         |
| Dry matter, % | 91.06 ± 0.438 |
| Organic matter, % | 85.92 ± 0.354 |
| Crude protein, % | 33.29 ± 1.570 |
| Gross fat, % | 13.90 ± 0.438 |
| Fiber, % | 9.62 ± 0.028 |
| Ash, % | 5.15 ± 0.092 |
| Fatty acid profiles (g % of total fat) |         |
| Miristic acid, (C14:0) | 0.08 ± 0.007 |
| Pentadecanoic acid, (C15:0) | 0.17 ± 0.028 |
| Palmitic acid, (C16:0) | 4.69 ± 0.403 |
| Palmitoleic acid, (C16:1) | 0.10 ± 0.021 |
| Heptadecanoic acid, (C17:0) | 0.36 ± 0.035 |
| Stearic acid, (C18:0) | 2.49 ± 0.265 |
| Oleic acid, (C18:1) | 18.29 ± 0.537 |
| Linoleic acid, (C18:2n6) | 16.83 ± 0.576 |
| Arachidonic acid, (C20:4n6) | 0.14 ± 0.028 |
| Linolenic acid, (C18:3n3) | 0.21 ± 0.014 |
| Octadecatetraenoic acid, (C18:4n3) | 55.93 ± 0.539 |
| Eicosatrienioic acid, (C20:4n3) | 0.14 ± 0.010 |
| Eicosatrienioic acid, (C20:3n6) | 0.13 ± 0.027 |
| Eicosatrienioic acid, (C20:3n9) | 0.06 ± 0.021 |
| Eicosatrienioic acid, (C20:3n3) | 0.05 ± 0.014 |
| Arachidonic acid, (C20:4n6) | 0.11 ± 0.010 |
| Docosadienioic acid, (C22:2n6) | 0.09 ± 0.014 |
| Docosatrienioic acid, (C22:4n6) | 0.10 ± 0.014 |
| Other fatty acids | 0.24 ± 0.069 |
| SFAs | 7.92 ± 0.120 |
| MUFAs | 18.39 ± 0.516 |
| PUFAs | 73.65 ± 1.089 |
| n3 | 56.12 ± 0.559 |
| n6 | 17.53 ± 0.940 |
| n6/n3 | 0.31 ± 0.028 |

SFAs—sum of saturated fatty acids; PUFAs—sum of polyunsaturated fatty acids; n3—sum of omega 3 polyunsaturated fatty acids (C18:3n3 + C18:4n3 + C20:3n6); n6—sum of omega 6 polyunsaturated fatty acids (C18:2n6 + C18:3n6 + C20:3n6 + C20:4n6 + C22:2n6 + C22:4n6). All data are reported as mean ± standard deviation of three replicate analysis.

Table 3. Production parameters throughout the experimental period (1–42 days).

| Parameter | Experimental Diet 1 | p-Value |
|-----------|---------------------|---------|
| Initial body weight (14 days), g | 506.71 ± 29.70 | 506.53 ± 51.73 | 0.9852 |
| Final body weight (42 day), g | 3163.45 ± 315.84 | 3136.67 ± 346.47 | 0.7534 |
| Body weight gain, g | 2656.75 ± 309.96 | 2630.12 ± 363.60 | 0.7166 |
| Average daily gain, g | 91.61 ± 10.69 | 90.69 ± 12.54 | 0.7189 |
| Average daily feed intake, g | 150.96 ± 34.19 | 148.54 ± 30.37 | 0.7767 |
| Feed/gain ratio | 1.65 ± 0.44 | 1.64 ± 0.40 | 0.9680 |

1 C—Control diet; E—diet supplemented with flaxseed meal (4%); n = 50.

Table 4. Measurements of the duodenal villus height and crypt depth for the control and experimental groups.

| Parameter | Control | Dietary Treatment | p-Value |
|-----------|---------|------------------|---------|
| Villus height (µm) | $848 \pm 33.2$ | $1148 \pm 29.1$ *** | $1.01 \times 10^{-12}$ |
| Crypt depth (µm) | $145 \pm 7.4$ | $164 \pm 4.6$ *** | $1.90 \times 10^{-5}$ |
| Villus height/crypt depth | $5.9 \pm 0.4$ | $7.0 \pm 0.3$ *** | $1.18 \times 10^{-5}$ |

C group: basal diet/control group; E group: flaxseed meal supplementation to the basal diet of broiler chickens. All data are reported as mean ± standard deviation (SD) and statistical significance, where *** $p < 0.001$. 
3.4. Histology of the Jejunum

The histological aspect of the jejunum for the experimental group harbored a normal aspect similar to that for the control group in terms of epithelial and mucous cells in the villi, as well as crypt cells (Figure 2). The histomorphometric analysis of the jejunum showed that...
the flaxseed meal supplementation to the basal diet of broiler chickens increased ($p < 0.001$, Table 5) the villus height, crypt depth, and villus height/crypt depth ratio compared with those of the control.

![Histological aspects of the jejunal villi and crypts for the control and experimental (E) groups via hematoxylin and eosin (H&E) and Alcian blue (AB) staining. Scale bar = 20 μm.](image)

**Figure 2.** Histological aspects of the jejunal villi and crypts for the control and experimental (E) groups via hematoxylin and eosin (H&E) and Alcian blue (AB) staining. Scale bar = 20 μm.
Table 5. Measurements of the jejunal villus height and crypt depth for the control and experimental groups.

| Parameter                  | Control        | Dietary Treatment E | p-Value       |
|----------------------------|----------------|--------------------|---------------|
| Villus height (µm)         | 935 ± 25.6     | 1251 ± 14.4 ***    | 1.72 × 10⁻¹³ |
| Crypt depth (µm)           | 152 ± 7.4      | 174 ± 3.5 ***      | 5.45 × 10⁻⁶  |
| Villus height/crypt depth  | 6.2 ± 0.3      | 7.2 ± 0.2 ***      | 3.95 × 10⁻⁶  |

C group: basal diet/control group; E group: flaxseed meal supplementation to the basal diet of broiler chickens. All data are reported as mean values ± standard deviation (SD) and statistical significance, where *** p < 0.001.

3.5. Intestinal Enzyme Activities

In the duodenum, maltase activity decreased significantly by 42% for the E group (133 U/mg) compared to the control (232 U/mg). In the jejunum, maltase enzymatic activity increased significantly by 52% in the case of the E group (494 U/mg) compared to the control (325 U/mg) (Figure 3). The specific activity of duodenal invertase was significantly increased by 46% in the E group (14 U/mg), whereas in the jejunum, it increased significantly by almost 227% (24 U/mg) compared to the control (7.28 U/mg).

Our experimental data revealed an insignificant increase by 2.3 times in the specific activity of alpha-amylase in the duodenum intestinal contents of the E group (0.094 U/mg) as compared to the control (0.041 U/mg). In the jejunum, the same enzymatic activity increased significantly by 38% in the case of the E group (0.7 U/mg) compared to the control (0.5 U/mg) (Figure 4).

The specific activity of trypsin in the duodenum was insignificantly decreased by 30% in the E group (0.048 U/mg) compared to the control (0.069 U/mg). In contrast, the administration of the basal diet enriched with 4% flaxseed meal increased trypsin activity insignificantly by 71% in the jejunum (0.08 U/mg). The enzymatic activity of lipase in the duodenum was decreased by 19% in the E group (3.15 U/mg) compared to the control level (3.88 U/mg). Moreover, the enzymatic activity of endo-β-1,4-glucanase from both intestinal segments was increased by 83% in the duodenum (0.0062 U/mg) and significantly increased in the jejunum (0.001 U/mg) by 357% of the control group level in the experimental group (Figure 4).
3.5. Intestinal Enzyme Activities

In the duodenum, maltase activity decreased significantly by 42% for the E group (0.078 U/mg) compared to the control (0.131 U/mg). The enzymatic activity of lipase in the duodenum increased by 38% in the case of the E group (0.071 U/mg) compared to the control (0.052 U/mg). In the jejunum, the same enzymatic activity decreased significantly by 38% in the case of the E group (0.001 U/mg) compared to the control (0.008 U/mg). The enzymatic activity of trypsin in the duodenum was insignificantly decreased by 30% in the E group (0.048 U/mg) compared to the control (0.069 U/mg). In contrast, the specific activity of trypsin in the jejunum was insignificantly decreased by 19% in the E group (0.083 U/mg) compared to the control (0.101 U/mg). The specific activity of lipase in the jejunum was insignificantly decreased by 71% in the E group (0.007 U/mg) compared to the control (0.022 U/mg). The specific activity of α-amylase in the duodenum intestinal contents of the E group (0.094 U/mg) was insignificantly increased in the jejunal mucosa (0.147 U/mg) compared to the control (0.069 U/mg).

3.6. Intestinal Microbiome

Poultry diet exerts a crucial effect on the gut microbiome in terms of diversity and composition. Compared to the conventional diet, the one enriched with flaxseed meal did not produce significant microbiome changes in terms of the relative abundance of taxa such as Bacteroides spp., Ruminococcus spp., Enterobacteriaceae, or Clostridium leptum (Figure 5).

Flaxseed meal administration led to a low abundance of the Clostridium leptum group by 87% (0.147 relative abundance), but this was not statistically significant. Nevertheless, flaxseed meal administration led to a significant enrichment in Lactobacillus spp. by 5.6 times (5.310 relative abundance, p value = 0.0043, Mann-Whitney test, data point distribution...
for normalization was analyzed using the Kolmogorov-Smirnov test), a group of bacteria known to be probiotics.

4. Discussion

The intestinal nutrient uptake is highly dependent upon the interaction between digestive secretions and the condition of the absorptive surface [52]. We show here that flaxseed meal supplementation had beneficial effects in the duodenum and the jejunum morphology, characterised by a significant increase in villus height, probably leading to increased nutrient adsorption. Absorptive efficiency is controlled by the characteristics of the intestinal absorptive surface [53]. Morphologically, crypt depth and intestinal villus length are indicative of the intestinal absorptive capacity. High intestinal villus length triggers an increase in the mucosal surface area, which, in our study, may be due to the PUFA composition of flaxseed meal, as previously reported [54].

Digestion mediates the relation between animals and their environment [55]. Indeed, the digestive system’s efficiency in extracting energy from nutrients is influenced by hydrolysis rates, fermentation and absorption digesta retention times, and the gastrointestinal tract’s surface area and volume [56].

The enterocyte membrane-bound enzymes hydrolyze food molecules that arrive in the small intestine [57] from the upper digestive segments [56]. Increased effectiveness of birds’ endogenous enzymes allows for the gel forms characteristic of soluble fibers to be broken down more efficiently, hence improving starch, protein, fat, and amino acid digestibility [35,58].

Amylase acts on starch, catalyzing hydrolysis of the α1–4 glycosidic bond, generating maltose as an end product and thereby improving its digestibility. In our study, the activity of alpha-amylase was increased in the duodenum and jejunum when compared to that for the control group. This could be due to the important content of linoleic acid in the flaxseeds, a potent activator of amylase secretion by pancreatic acinar cells [59]. On the other hand, oleic and linoleic acids showed an important inhibitory action against α-glucosidase activity [60]. This could explain the decrease in maltase activity in the duodenum, even as the villus length increased. In the jejunum, this enzymatic activity was higher in the E group as compared to control one, this being correlated with the greater villus length. Probably due to the high secretion of amylase, its activity in the duodenum was also inhibited to a certain extent, but it remained a little bit higher than that in the control group. The other brush border disaccharidase, i.e., invertase, which catalyzes the hydrolysis of the α1–2 glycosidic bond between an α-glucose unit and a β-fructose one, showed increased activity in both analyzed intestinal segments, suggesting a direct correlation between the enzyme molecules and the increasing length of the villi in the E group compared to the control group.

Trypsin is a proteolytic enzyme that hydrolyses protein and peptides and accounts for most peptidase activity of the brush border membrane [56]. In our study, there was no significant variation of trypsin activity in the duodenum and jejunum in group E compared to the control group. Our results regarding this enzyme activity were in agreement with those of Attia et al. [61], showing that the free fatty acid content does not interfere with its pancreatic secretion and/or activity.

Lipids are the main energy source for broilers [62] and enter the duodenum intact. The presence of food in this part of the intestine stimulates the secretion of cholecystokinin, triggering the contraction of the gall bladder and exocrine pancreas secretion. In the presence of bile acids, micelles are formed, which are probably composed mainly of PUFAs. Lipase acts at the lipid-water interface of micelles, being stabilized by colipase [63]. According to our data, there was no significant difference between the lipase specific activity levels in the E and control groups. This could be due to the fact that colipase stabilizes the micelles and extremely diminishes the hydrolysis rate of triacylglycerols, such that the possible differences could no longer be observable [64]. This lack of variation in the lipase activity in group E could be the reason for the lack of weight gain. Our results
are different from those reported by Polycarpo et al. [65], who used soy oil as a source of PUFAs.

Gram-positive bacteria from the intestinal microbiome, such as *Lactobacillus* spp. and *Bifidobacterium* spp., are able to produce glycosyl hydrolases for digesting dietary nutrients and producing valuable metabolites [66]. The endo-β-1,4-glucanase catalyzes hydrolysis polysaccharides containing a 1,4-β-glucan backbone from a range of materials and is produced by both prokaryote and eukaryote organisms [67].

Our results showed that endo-β-1,4-glucanase activity from the duodenum and jejunum was higher in the E group as compared to the control group, which strongly correlates with the significant enrichment in *Lactobacillus* spp. from the cecal microbiome. Data regarding the intestinal microbiome indicated that flaxseed meal used as a feed additive in poultry may harbor prebiotic effects, triggering the expansion of beneficial members of the microbiome. Probiotics induce physiological changes in the intestinal tissue, culminating in immunological changes that augment the animal’s resistance to pathogens. Moreover, probiotics themselves produce short organic fatty acids (butyrate, acetate, propionate) and metabolites with antimicrobial activity (i.e., reactive oxygen species, lactic acid, bacteriocins, etc.) [68]. Previously, it was shown that oleic acid had a protective effect on the survival of lactobacilli [69], which could explain the important increase in the *Lactobacillus* spp. population in the cecum.

The abundance of *Clostridium coccoides* is positively associated with a high intake of MUFAs and PUFAs [70] and the composition of flaxseed meal used in this study (Table 3). Furthermore, flaxseed meal with high content of fiber and n-3 PUFAs [25] exerts a protective effect when included in the diet in the case of the development of inflammatory bowel disease. Previous research found that an appropriate n-6/n-3 PUFAs ratio in the diet is essential, the former being an anti-inflammatory molecule and the latter being pro-inflammatory [71]. Dietary flaxseed meal supplementation that contains a high level of n-3 PUFAs in chickens’ diets has an anti-inflammatory effect, significantly reducing spleen cytotoxic T-cell activity in comparison with diets containing 70 g/kg corn oil or lard [72].

Moreover, a high-fat diet can lead to a reduction in *Enterobacter* spp. and *Clostridium leptum* and an increase in *Lactobacillus* spp. [73], as shown in Figure 5 for the diet supplemented with flaxseed meal (group E).

Respondek at al. [74] showed that the presence of C18 hydroxy-monoenoic fatty acids was positively correlated with an increase in the *Clostridium coccoides* population and negatively with the *Clostridium leptum* population, which supports the microbiome changes (Figure 5) in accordance with the fatty acid profile from flaxseed meal (Table 3).

### 5. Conclusions

Our study shows that supplementation of the basal diet of Cobb 500 broiler chickens with flaxseed meal, rich in linolenic, linoleic, and oleic acids, modulated the enzymatic activities of α-amylase maltase and invertase. Moreover, there was an increase in the villus height and crypt depth as a result of the diet enriched with flaxseed oil, which can increase the surface area and absorption capacity of the small intestine. The end products of these enzymatic activities, as well as all PUFAs, sustained the increase in the *Lactobacillus* spp. population, which is a mark of a healthy microbiome. This was correlated with the endo-β-1,4-glucanase activity in the duodenum and jejunum. Even if this feed supplementation did not induce weight gain in the chickens, their health quality was probably improved.

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**Data Availability Statement:** The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy reasons.

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