Molecular Biology Reports (2024) 51:923
https://doi.org/10.1007/s11033-024-09858-y

ORIGINAL ARTICLE

Flaxseed promotes productive performance through regulating gut microbiome in ducks

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Received: 15 April 2024 / Accepted: 9 August 2024

Abstract

Background Flaxseed has been widely used in animal diets to increase the omega-3 polyunsaturated fatty acid content in animal products and promote overall animal health, but little known about its effects on the productive performance and the mictobita of gut of laying duck.

Methods and results Jinding duck, a Chinese indigenous breed, was used in the study. The corn-soybean basal diet supplemented with 0, 2%, 3% 4% and 5% flaxseed were provided to Control, 2% Fla, 3% Fla, 4% Fla and 5% Fla groups for 53 days, respectively. Compared with Control group, groups fed with flaxseed diets showed higher egg production, egg mass, ovary weight and more preovulatory follicles. The Docosahexaenoic Acid content of egg was extremely significantly elevated by flaxseed diets ($P < 0.01$), and the albumen height and haugh unit were elevated, especially in 4% Fla and/or 5% Fla group ($P < 0.05$). Groups 4% Fla and 5% Fla had highest ileal villus height, jejunal and ileal crypt depth. Moreover, Flaxseed diets significantly increased the levels of IgG and IgM in all Fla groups ($P < 0.01$), while increased IgA levels except for in 3% Fla group ($P < 0.05$). The results of 16s rDNA sequencing showed that flaxseed diet altered the microbial composition of gut and reduced the diversity and evenness of gut microbial communities except for 5% Fla. The correlation analysis identified *Blautia*, *Butyricicoccus* and *Subdoligranulum* positively associated with egg production. Genera *Fourinierella*, *Fusobacterium* and *Intestinimonas* positively associated with ovary weight, haught unit and album height. And *Mucispirillum* positively associated with haugh unit and album height.

Conclusion This study has suggested that flaxseed play a positive role in productive performance, the overall or intestinal health of laying ducks.

Keywords Laying duck · Flaxseed · Omega-3 Polyunsaturated Fatty Acids · Gut microbiome

Introduction

Flaxseed (*Linum usitatissimum*) is a globally cultivated oil-seed crop known for its high oil content, comprising 35–45% of the seed. This oil is rich in α-linolenic acid (ALA, 18:3n-3), which accounts for 45–54% of the total fatty acids. Except ALA, flaxseed oil also include linoleic acid, oleic acid, and saturated fatty acids. Additionally, flaxseed is a valuable source of proteins, lignans, soluble fiber, and phenolic compounds. ALA, a natural omega-3 polyunsaturated fatty acid (ω-3 PUFA), serves as the precursor to eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) [1]. EPA and DHA have demonstrated preventive and therapeutic properties in various human diseases, including hypertriglyceridemia [2], cardiovascular disease [3], and Alzheimer's disease [4]. Flaxseed and its extract are widely utilized as ingredients or food additives due to their

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Published online: 20 August 2024

health benefits, such as anti-inflammatory, antioxidant, and lipid-modulating properties [5].

The gastrointestinal tract of animals harbors a diverse microbial community, which plays essential roles in digestion, absorption, defense, and other physiological activities [6–8]. Diet composition affect the structure of the postnatal gut microbiota in animals and humans and entails complex interactions between the diet, microbiota, and the host [9]. Due to the prohibition of antibiotics in livestock and poultry farming, researchers have became more interested in the potential of herbal additives to improve the health and productivity of domesticated animals. Some beneficial effects of herbal additives may be attributed to their impact on the gut microbiota of these animals [10].

In recent years, flaxseed has been incorporated into the diets of domesticated animals to enhance the PUFA content in animal-derived products and promote animal health [11–13]. In ducks, addition of flaxseed in their diet has been shown to increase ALA, DHA, EPA, and total ω-3 PUFAs in meat or eggs [14, 15]. However, little is known about the effects of flaxseed supplementation on the gut microbiome of ducks. Therefore, this study aims to make a comprehensive knowledge of the effects of flaxseed on the growth, production and gut microbial communities of duck.

Materials and methods

Animal manipulation and sample collection

In the study, a total of 2250 healthy Jinding ducks, aged 140 days, were obtained from Hubei Chuda Duck Industry Co., Ltd (Hubei, China). Flaxseed was purchased from a biotechnology company (An You Biotechnology Inc.) and its composition was detected (Table S1). The ducks were randomly allotted into 15 replicates, each treatment had 3 replicates with 150 ducks in each replicate. These ducks were randomly reared in semi open rooms equipped with clean pool, feeders, defecation board and sufficient laying nests. The ducks were allowed to free access to feed and water during the experiment. A corn-soybean meal diet was formulated as the basal diet. Ducks were allotted to 5 dietary treatments, including the Control group (basal diet), 2% Fla group (basal diet supplemented with 2% flaxseed), 3% Fla group (basal diet supplemented with 3% flaxseed), 4% Fla group (basal diet supplemented with 4% flaxseed) and 5% Fla group (basal diet supplemented with 5% flaxseed). The four groups treated with flaxseed can be referred as Fla groups in the following description. The gross composition of the basal diet for the Control and Fla groups were formulated to meet the nutrient specifications recommended by the NRC (1994) (Tables S2 and S3).

The experiment lasted 53 days when the average daily laying rate of the five groups reached over 85%. During the experiment, daily feed intake (FI), egg weight (EW) and mass (EW), egg production (EP) and feed conversion ratio (FCR) of each group were assessed. The eeg production and FCR were calculated as rate of production per replicate per day and feed intake/egg mass. On the 53rd day, 30 ducks were randomly selected from five groups (two ducks in each replicate). The weight of body (BW), liver (LW), spleen (SW), pancreas (PW) and ovary (OW) of duck were recorded and the number of preovulatory follicles (NPF) were counted. The colon digest of 30 ducks (6 replicates of each group) was collected, transferred to sterile tubes, snap-frozen in liquid nitrogen, and stored at -80℃ for the 16s rDNA sequencing. The middle section of the duodenum, ileum and jejunum of three ducks of each group were randomly collected for morphological analysis. Meanwhile, three eggs of each group were collected and sent to the Institute of Oil Crops (Chinese Academy of Agricultural Sciences) to measure the concentrations of ALA, EPA, and DHA. Then ten eggs of each group were conducted quality determination.

Morphological analysis of gut

The segments derived from duodenum, jejunum and ileum of three ducks in each group were fixed using standard paraffin embedding method. Three cross sections of each intestinal segments were stained with hematoxylin and eosin (H&E). At least three well-oriented villi and the associated crypts of each segment were measured, and the villus/crypt ratio was calculated.

Serum protein profiling

Total protein (TP), albumin (ALB), globulin (GLOB) in serum were assessed using fully-automatic blood biochemical analyzer, and the concentrations of IgA, IgG and IgM (3 replicates of each group) were determined following the protocol of ELISA Kits (DRE-D5713b, DRE-D5712b, DRE-C5715b; Kamai Shu Biotechnology, China) under the sensitivities of 1.0 μg/mL,10 μg/mL and 10 μg/mL, respectively. The coefficients of variation inter-assay and intra-assay were all less than 15%.

DNA extraction and PCR amplification

Microbial DNA was extracted from the contents of the colon using the Magnetic Soil and Stool DNA Kit (TianGen, China, Catalog #: DP712). The 16S rRNA genes of the V3-V4 region were amplified using the specific primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 806R (5′-GGACTACNNGGGTATCTAAT-3′). The PCR reaction mixture

consisted of 15 μL of Phusion® High-Fidelity PCR Master Mix (New England Biolabs), 0.2 μL of forward and reverse primers, and approximately 10 ng of template DNA. The amplification protocol involved an initial denaturation step at 98 ℃ for 1 min, followed by 30 cycles of denaturation at 98 ℃ for 10 s, annealing at 50 ℃ for 30 s, and elongation at 72 ℃ for 30 s, and a final extension at 72 ℃ for 5 min. The quality of the amplified DNA was assessed using 2% agarose gel electrophoresis. The purified PCR products were then subjected to purification using the Universal DNA Purification Kit (Tianjin, China).

16s rDNA sequencing process and data analysis

Sequencing libraries were prepared using the NEB Next® UltraTM II FS DNA PCR-free Library Prep Kit (New England Biolabs, USA), following the manufacturer's recommendations. Indexes were incorporated during library preparation. The quality of the library was assessed using Qubit and real-time PCR. A bioanalyzer were applied to analyze the size distribution analysis. After quantification, the libraries were pooled and sequenced on the Illumina NovaSeq platform, generating 250 bp paired-end reads. Raw tags of each sample were obtained after removing barcodes and primers using FLASH software (V1.2.11, http://ccb.jhu.edu/software/FLASH/) [16]. Effective tags were finally obtained through filtering out tags with low quality and removing chimera sequences using fast software (Version 0.23.1) [17] and the UCHIME Algorithm (http://www.drive5.com/usearch/manual/uchime_algo.html) [18]. The final Amplicon Sequence Variants (ASVs) were obtained after denoising and then assigned to species annotation and phylogenetic analysis. Subsequent analysis were conducted based on the normalized data of each sample.

The alpha diversity indices, Goods coverage, Chao 1, Simpson and Pielou_e were generated and analyzed using wilcox test. The beta diversity were calculated based on unweighted unifrac distances and analyzed using wilcox test. Multiple Response Permutation Procedure (MRPP) based on Bray–Curtis distances and Principal Coordinates Analysis (PCoA) were performed in R. Differential microbiota based on the relative abundance at the phylum and genus levels were identified using MetaStat analysis in R.

Correlation analysis

Spearman's correlation analysis was conducted in R (R-4.3.2) to study the relationship between micorobial species and productive performance of duck. The results were presented by the heatmap plot and the *P*-value were also concerned.

Statistical analysis

The data related to the growth and production of duck, morphological analysis of gut and serum parameters were all checked for normality of variance and homogeneity of variance using the Shapiro–Wilk test and bartlett test in R, respectively. One-way analysis of variance (ANOVA) and Duncan's multiple range tests in R were carried out for the analysis of data that obeyed the conditions of ANOVA. The data that disobeyed the conditions were analyzed using the Kruskal–Wallis test in R. The *P*-value below 0.05 were considered statistically significant.

Result

Effects of flaxseed on the growth and productive performance of duck

As shown in Table 1, there were no statistically significant differences in the BW, LW, SW and PW among groups. Compared with control group, flaxseed did promote the OW and the NPF, which showed the highest levels in 4% Fla group.

Both production and quality of egg are the most important characteristics of laying duck. As shown in Table 2, the FI increased as the proportion of flaxssed in diet elevated even though there were no significant differences in FI among groups. And no significant difference in EW among groups were observed. However, the EP of laying ducks was extremely significantly elevated in 2% Fla, 3% Fla and 4% Fla groups ($P < 0.01$) compared to control group and 5% Fla group. Ducks fed with 3% flaxseed diet showed significantly higher average EM than other groups ($P < 0.05$). And the EM in 2% Fla and 4% Fla groups was significantly higher

Table 1 Effects of flaxseed on the growth of duck

Group	BW (g)	LW (g)	SW (g)	PW(g)	OW (g)	NPF
Control	1220 ± 110	41.68 ± 7.58	0.60 ± 0.30	4.83 ± 1.10	38.30 ± 3.28^{c}	5.17 ± 0.41^{b}
2%Fla	1290 ± 90	42.90 ± 4.74	0.68 ± 0.19	4.33 ± 0.43	45.94 ± 3.50^{b}	6.00 ± 0.63^{ab}
3%Fla	1260 ± 100	45.63 ± 8.24	0.70 ± 0.13	5.05 ± 0.50	49.71 ± 5.58^{b}	5.67 ± 0.52^{ab}
4%Fla	1400 ± 140	41.39 ± 7.92	0.71 ± 0.21	5.45 ± 1.17	56.98 ± 9.89^{a}	6.50 ± 1.05^{a}
5%Fla	1330 ± 110	41.10 ± 5.17	0.72 ± 0.12	5.04 ± 0.61	46.76 ± 4.91^{b}	5.67 ± 0.52^{ab}

Different superscript lowercase letter in one column indicated statistically difference at $P < 0.05$

Table 2 Effects of flaxseed on productive performance of laying duck

Group	FI (g/d/bird)	EP (%)	EW (g)	EM (g/d/bird)	FCR
Control	134.28 ± 7.22	51.55 ± 1.01^{D}	57.87 ± 2.86	29.84 ± 1.66^{c}	4.50 ± 0.08^{b}
2% Fla	134.38 ± 6.05	60.84 ± 1.39^{B}	57.70 ± 1.96	35.11 ± 1.72^{b}	3.83 ± 0.14^{c}
3% Fla	137.01 ± 5.00	65.11 ± 0.32^{A}	58.59 ± 2.49	38.15 ± 1.62^{a}	3.60 ± 0.24^{c}
4% Fla	138.54 ± 7.02	57.83 ± 1.22^{C}	58.60 ± 2.76	33.87 ± 1.25^{b}	4.10 ± 0.30^{bc}
5% Fla	158.07 ± 11.09	53.27 ± 1.31^{D}	57.34 ± 3.26	30.52 ± 1.00^{c}	5.18 ± 0.40^{a}

Different superscript lowercase letter and capital letter in one column indicated statistically difference at $P < 0.05$ and $P < 0.01$, respectively

than that in 5% Fla and control groups ($P < 0.05$). The FCR value of 5% Fla group was the highest, followed by Control and 4% Fla groups, and the FCR value of 3% Fla group was the lowest.

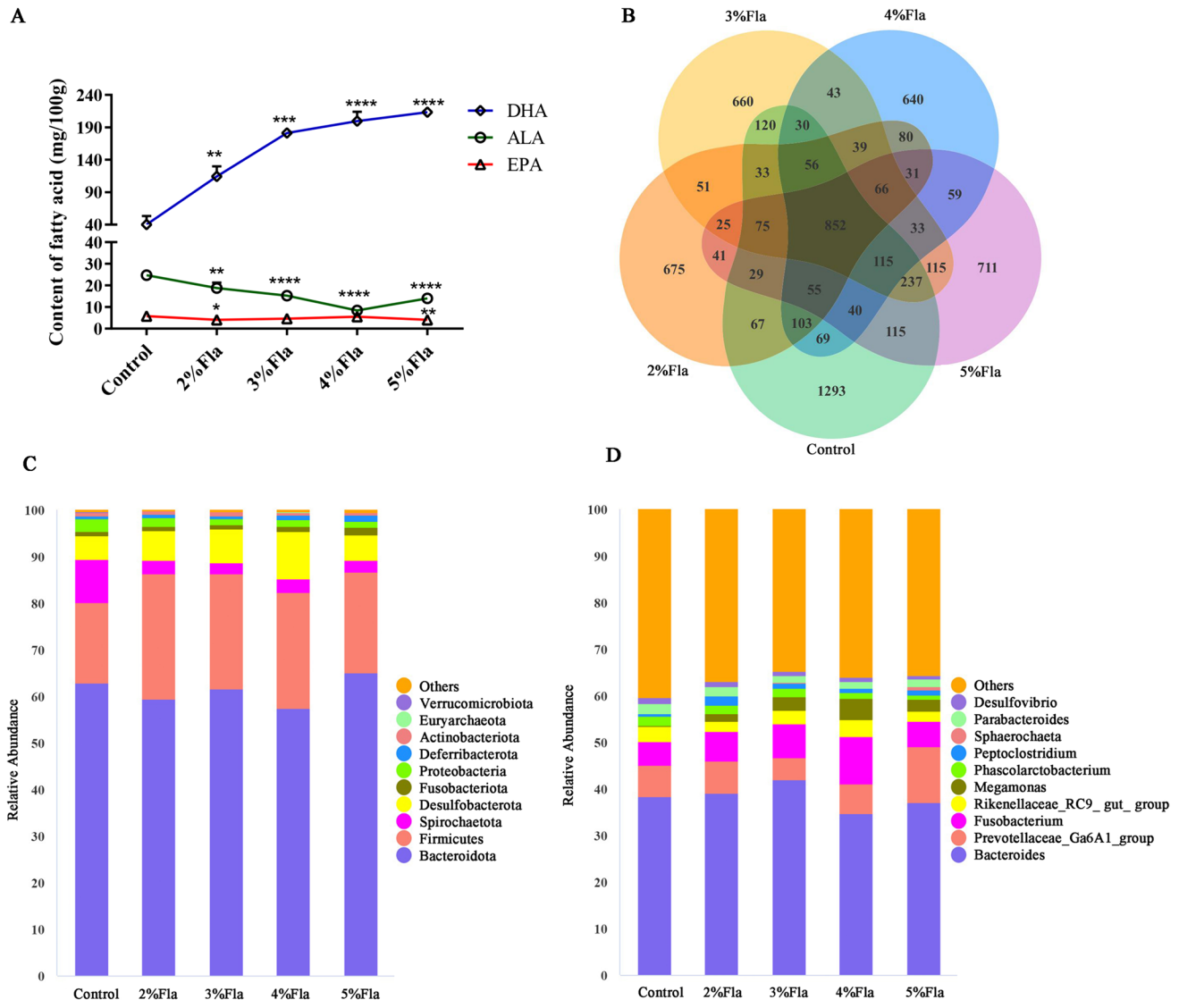


Fig. 1 Effects of flaxseed on the content of ω-3 PUFAs in egg and microbial compositon of gut. **A** The content of DHA, EPA and ALA in eggs; **B** Venn diagram of ASVs. Each pear-like plot represents a group; The top 10 abundant ASVs at phylum **C** and genus **D** level. Others represents the proportion of ASVs unannotated or with low abundance at the phylum and genus level, respectively. * represents the *P*-value is below 0.05; ** represents the *P*-value is below 0.01; *** represents the *P*-value is below 0.001; **** represents the *P*-value is below 0.0001

Table 3 Effects of flaxseed on the quality of egg

Group	AH	HU	STE
Control	4.28 ± 1.07^{b}	61.37 ± 10.70^{b}	0.44 ± 0.05
2% Fla	4.45 ± 0.94^{ab}	63.65 ± 7.97^{b}	0.49 ± 0.05
3% Fla	5.48 ± 1.80^{ab}	72.08 ± 13.51^{ab}	0.40 ± 0.04
4% Fla	6.57 ± 1.06^{ab}	81.13 ± 7.61^{a}	0.42 ± 0.06
5% Fla	7.68 ± 1.75^{a}	85.71 ± 10.97^{a}	0.45 ± 0.06

Different superscript lowercase letter in one column indicated statistically difference at $P < 0.05$

On the other hand, the unsaturated fatty acid content of the eggs and egg quality were also measured. Flaxseed diet significantly increased the content of DHA, but decreased the content of ALA (Fig. 1A; *P < 0.01*). The content of EPA remained a relatively low level although it significantly decreased in 2% and 5% Fla groups compared with Control group (*P < 0.05*). Moreover, The album height (AH) increased as the proportion of flaxseed elevated and it was significantly higher in 5% Fla group compared with Control group. Likewise, groups with 3–5% flaxseed had higher haugh unit (HU) of egg (*P < 0.05*). But flaxseed had no significant effects on the shell thickness (STE) (Table 3).

Effects of flaxseed on the gut morphology and serum parameters of duck

The crypt depth, villi height and the ratio of villi height to crypt depth are often used to value the absorption capacity and mucosal damage of intestine, respectively. In the study, morphological analysis for duodenum, jejunum and ileum were conducted (Table 4). Groups fed with diets containing 4% and 5% flaxeed had higher ileal villus height (VH) than other groups ($P < 0.05$). 4% Fla group had the highest jejunal and duodenal VH even though there was no significant difference between 4% Fla and Control groups. No significant differences in crypt depths (CD) between Control and Fla groups were observed, but they were upregulated in 4% and 5% Fla groups compared to Control group. As for the ratio of VH to CD (VCR), The dominant group varied in different parts of the gut and there were no significant differences between Control and Fla groups ($P > 0.05$).

Moreover, the content of ALB, TP, GLOB, IgA, IgG and IgM were measured to assess the effects of flaxseed on the health of duck. As shown in Table 5, addition of flaxseed extremely significantly elevated the concentrations of IgG and IgM in most Fla groups compared to Control group ($P < 0.01$). and except for 3% Fla group, the concentrations

Table 4 Effects of flaxseed on the gut morphology

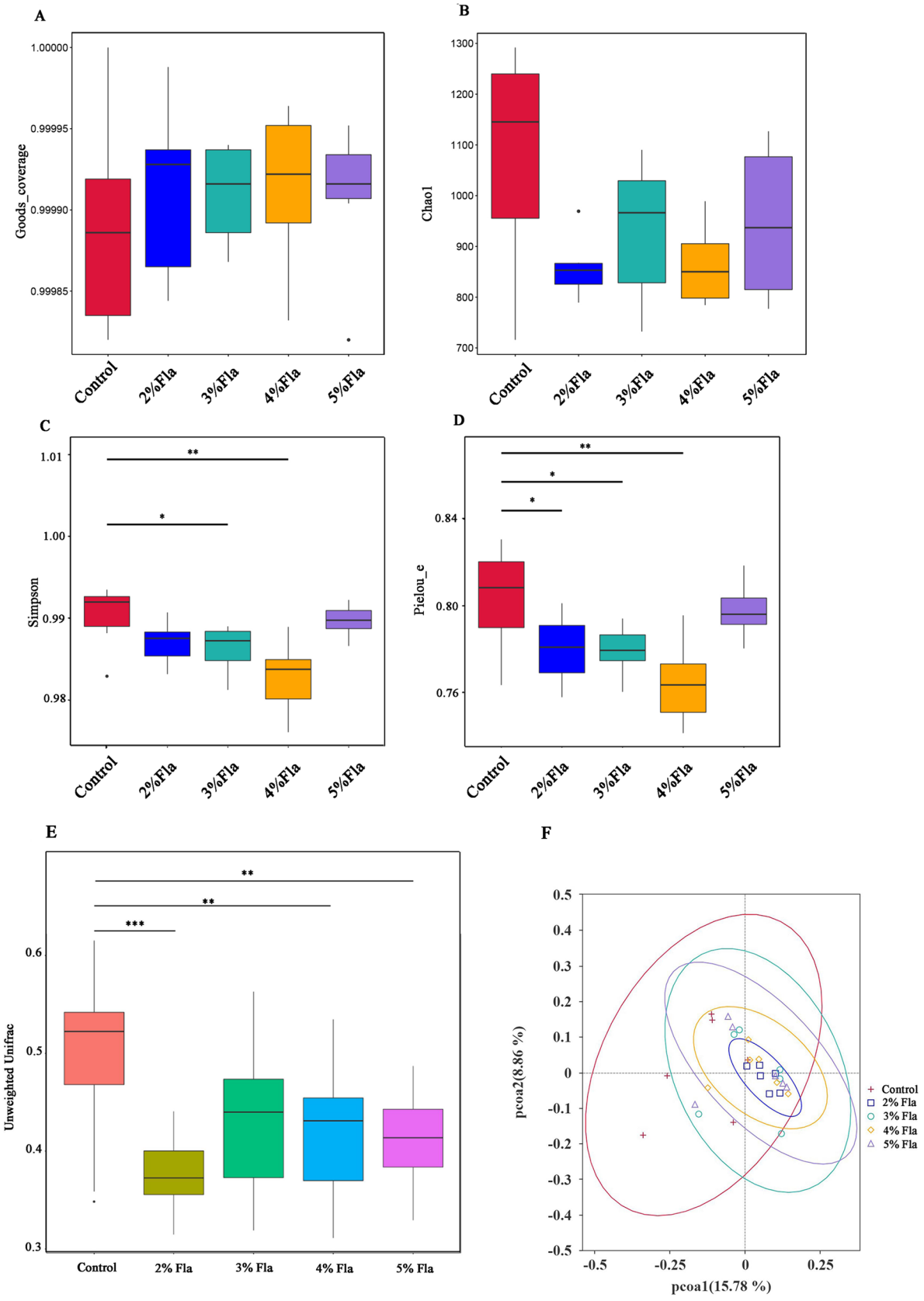
Item	Groups				
	Control	2%Fla	3%Fla	4%Fla	5%Fla
Duodenum					
VH(μm)	$788.32.92 \pm 21.73$	778.25 ± 37.00	768.87 ± 40.19	971.28 ± 94.88	762.09 ± 50.82
CD(μm)	357.38 ± 17.67^{ab}	320.74 ± 23.41^{ab}	283.65 ± 10.15^{b}	428.31 ± 20.20^{a}	326.69 ± 68.87^{ab}
VCR	2.21 ± 0.17^{ab}	2.44 ± 0.20^{ab}	2.72 ± 0.19^{a}	2.02 ± 0.18^{b}	2.39 ± 0.36^{ab}
Jejunum					
VH(μm)	935.24 ± 12.20^{ab}	890.22 ± 71.08^{ab}	836.13 ± 57.73^{b}	1204.77 ± 29.00^{a}	1074.26 ± 237.61^{ab}
CD(μm)	199.90 ± 9.47	195.89 ± 6.8	205.90 ± 11.39	210.51 ± 23.17	217.85 ± 15.03
VCR	4.69 ± 0.22^{ab}	4.56 ± 0.51^{ab}	4.08 ± 0.43^{b}	5.77 ± 0.60^{a}	4.91 ± 0.80^{ab}
ileum					
VH(μm)	713.61 ± 15.41^{b}	755.20 ± 30.71^{ab}	709.42 ± 45.86^{b}	836.51 ± 41.01^{a}	789.47 ± 23.54^{a}
CD(μm)	169.70 ± 38.60	144.25 ± 10.78	177.95 ± 16.06	185.46 ± 13.65	183.34 ± 25.33
VCR	4.4 ± 0.96^{ab}	5.26 ± 0.43^{a}	4.01 ± 0.44^{b}	4.54 ± 0.51^{ab}	4.36 ± 0.50^{ab}

Different superscript lowercase letter in one column indicated statistically difference at $P < 0.05$

Table 5 Effects of flaxseed on serum parameters of duck

Item	Control	2%Fla	3%Fla	4%Fla	5%Fla
ALB (g/L)	24.67 ± 1.15	24.33 ± 0.58	25.67 ± 2.08	26.67 ± 1.53	27.33 ± 0.58
TP (g/L)	61.67 ± 1.15	56.00 ± 4.36	57.67 ± 7.02	59.67 ± 4.51	65.67 ± 5.13
GLOB (g/L)	37.00 ± 2.00	31.67 ± 4.93	32.00 ± 5.00	33.00 ± 3.00	38.33 ± 4.73
IgA (μg/mL)	124.41 ± 3.98^{d}	257.56 ± 17.97^{a}	133.61 ± 19.34^{d}	172.98 ± 3.89^{b}	157.20 ± 8.78^{c}
IgG (μg/mL)	1191.34 ± 61.10^{C}	1740.88 ± 66.32^{B}	2068.18 ± 16.94^{A}	2135.82 ± 69.46^{A}	1691.36 ± 56.81^{B}
IgM (μg/mL)	1200.79 ± 95.31^{B}	1492.50 ± 80.36^{A}	1161.83 ± 36.18^{B}	1540.95 ± 63.44^{A}	1480.15 ± 45.57^{A}

Different superscript lowercase letter and capital letter in one column indicated statistically difference at $P < 0.05$ and $P < 0.01$, respectively


A
Goods_coverage
1.00000
0.99995
0.99990
0.99985
Control
2%Fla
3%Fla
4%Fla
5%Fla
B
Chao1
1300
1200
1100
1000
900
800
700
C
Simpson
1.01
1.00
0.99
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**
*
D
Pielou_e
0.84
0.80
0.76
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E
Unweighted Unifrac
0.6
0.5
0.4
0.3
**
**
***
Control
2% Fla
3% Fla
4% Fla
5% Fla
F
pcoa2(8.86 %)
pcoa1(15.78 %)
0.5
0.4
0.3
0.2
0.1
0
-0.1
-0.2
-0.3
-0.4
-0.5
-0.5
-0.25
0
0.25
Control
2% Fla
3% Fla
4% Fla
5% Fla

◂**Fig. 2** Effects of flaxseed on the compositon and diversities of intestinal microbiota. Analysis of alpha indices including Goods Coverage **A**, Chao1 **B**, Simpson **C** and Pielou_e **D**. Beta diversity analysis basing on unweighted UniFrac distances matrices **E**. PCoA analysis **F**.* represents the *P*-value is below 0.05; ** represents the *P*-value is below 0.01; *** represents the *P*-value is below 0.001

of IgA were significantly elevated in other Fla groups compared to Control group ($P < 0.05$). There were no significant differences in ALB, TP and GLOB.

Data summary of 16s rDNA sequencing

A total of 4,082,163 raw reads were obtained, and an average of 108,310 clean reads were generated (Table S4). After removing duplicate sequences, a total of 6558 unique ASVs were obtained. The Venn chart showed that there were 852 common ASVs among the five groups, accounting for 25.90% of Control group, 37.40% of 2% Fla group, 33.41% of 3% Fla group, 36.87% of 4% Fla group, and 32.78% of 5% Fla group. Additionally, each group also has unique ASVs, with 1293 ASVs in the Control group, 675 ASVs in the 2% Fla group, 660 ASVs in the 3% Fla group, 640 ASVs in the 4% Fla group, and 711 ASVs in the 5% Fla group (Fig. 1B).

Flaxseed altered the microbial composition and diversity of duck's colon

At the phylum level (Fig. 1C), the most abundant ASVs were Bacteroidota, Firmicutes, Proteobacteria, Fusobacteriota, Spirochaetota, Desulfobacterota, Deferribacterota, Actinobacteriota, Euryarchaeota, and Verrucomicrobiota. Bacteroidota accounted for more than 55% of the ASVs in each group, making it the most dominant group. The average percentage of Firmicutes of Fla groups was 1.4 times higher than that of Control group. There was a slight increase in the percentage of Fusobacteriota and Deferribacterota in each Fla group compared to Control group. However, there was a substantial decline of about 70% in the percentage of Proteobacteria in each Fla group. Moreover, the percentage of Verrucomicrobiota in Control group was one order of magnitude higher than that in each Fla group. At the genus level (Fig. 1D), the top 10 abundant ASVs were Bacteroides, Prevotellaceae_Ga6A1_group, Fusobacterium, Rikenellaceae_RC9_gut_group, Megamonas, Phascolarctobacterium, Peptoclostridium, Sphaerochaeta, Parabacteroides, and Desulfovibrio. Among them, the percentage of Megamonas increased from 0.24% in Control group to an average of 2.94% in Fla groups. The abundance of Peptoclostridium also increased by almost an order of magnitude in Fla groups. Prevotellaceae_Ga6A1_group and Fusobacterium showed significant increases in 5% Fla and 4% Fla group, respectively. Conversely, the percentages of Phascolarctobacterium and Desulfovibrio declined gradually with increased flaxseed intake.

Alpha diversities were assessed using several indices, including Good's coverage, Chao1, Simpson, and Pielou_e (Fig. 2A–D). The Good's coverage values for all five groups were above 0.9998, indicating that the sequencing results were representative and reliable. No significant difference in richness of bacterial community among these groups were found according to the Chao1 index. Whereas it seemed that the diversity and evenness of gut microbiota were affected by flaxseed diet according to the Simpson and Pielou_e indices, respectively. The diversities of bacterial community of gut gradually decreased and it was significantly down-regulated in 3% and 4% Fla groups ($P < 0.05$). Similarly, the evenness of gut microbiota gradually decreased as the proportion of flaxseed increased to 4%. However, the diversity and evenness seemed to be unaffected in 5% Fla group compared to Control group.

Beta diversity analysis based on unweighted UniFrac distance matrices demonstrated a significant changes in microbial communities in most Fla groups compared to Control group (Fig. 2E, $P < 0.01$). Moreover, the analysis of MRPP and PCoA showed that the composition and structure of bacterial community of Control group was distinct from that of Fla groups (Fig. 2F; Table S5).

Correlation analysis of microbial species with productive characteristics of duck

Correlations between the top 35 most abundant genera and EP, OW, HU and AH were analyzed (Fig. 3). Genera *Blautia*, *Butyricicoccus* and *Subdoligranulum* showed positive relationship with EP. Genera *Fourinierella*, *Fusobacterium* and *Intestinimonas* positively associated with OW, HU and AH. And *Mucispirillum* positively associated with HU and AH.

Discussion

Jinding ducks are a kind of indigenous laying duck in China known for their annual egg production of 260~300 eggs and a 2-year egg production period. Previous researches have showed that flaxseed consumption may be inversely associated with body weight of human [19, 20]. However, no significant difference in body weight between Fla and Control groups were found in the present study even though it slightly increased in the Fla groups. It has been widely proved that intestinal histomophometric characteristics and microbiota tightly connect with weight gain of human and animals [21]. In the present study, 4% and 5% Fla groups had higher VH, CD and VCR values in jejunum and ileum than Control group. Similarly, addition of flaxseed increased the

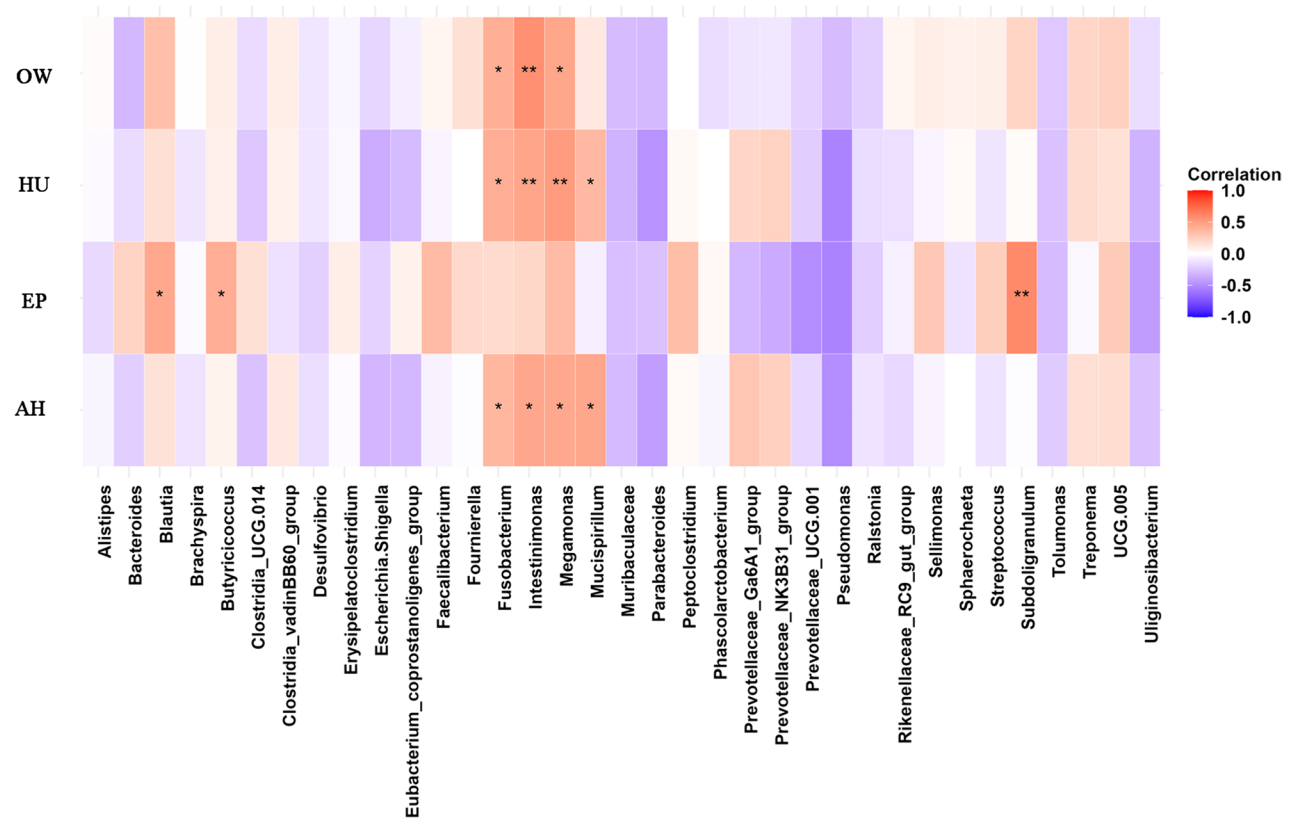

Fig. 3 Heatmap based on the correlation analysis between the top 35 abundant genera and OW, HU, EP and AH

ileal VH of pigs [22], and ω-3 PUFAs enriched diets upregulated the duodenal VH and VCR of hens [23]. Moreover, the dominant intestinal phyla *Firmicutes* and *Bacteroidota* have been proved to play a role in energy metabolism. And an increment of the relative abundance of *Firmicutes* with respect to *Bacteroidota* (F:B ratio) has been associated with obesity [24]. In this study, the F:B ratio were slightly upregulated in each Fla group, which seemed to be coincident with the elevation of body weight in Fla groups.

Flaxseed had also increased the ovary weight and the number of preovulatory follicles of laying ducks in this experiment. Consistently, laying ducks in 2% Fla, 3% Fla and 4% Fla groups had significantly higher EP, EM but lower FCR. Previous researches have proved the regulatory role. of flaxseed on the female reproduction including ovarian growth, follicle development, puberty, hormone release and the resulting reproductive cycle [25, 26]. And This indicates that moderate supplementation of flaxseed can improve the reproductive performance of laying ducks. Short-chain ω-3 PUFAs, such as ALA, are commonly found in plant oils like flaxseed and soybean oil. While long-chain ω-3 PUFAs, such as DHA and EPA are primarily obtained from marine products like fish oil. Previous researches have implied that DHA and EPA are modulators of inflammatory and immune response [27, 28]. Although endogenous DHA and EPA can be converted from ALA, the conversion efficiency is quite low in human [29, 30], especially in infants and elderly individuals [31, 32]. In the present study, addition of flaxseed significantly increased the content of easily absorbed DHA in duck eggs, but decreased the content of ALA. Previous research showed that ALA and DHA were both upregulated in the egg yolk of laying hens or ducks fed with full-fat flaxseed meal or flaxseed oil [14, 15]. Whether if the duck breed or procession method of flaxseed contributed to this discrepancy needs further research. On the other hand, flaxseed might enhanced the overall health of laying ducks owing to the elevated levels of IgA, IgG and IgM in Fla groups. This might reflect the positive role of ω-3 PUFAs in immunity [33, 34].

Dietary flaxseed have been proved to improve the health of gut through enhancing the intestinal barrier or regulating the composition of gut microbiota [35, 36]. Analysis of ASVs revealed that the Control and Fla groups shared a quite large number of ASVs, although the relative abundance of some ASVs exhibited significant differences. At the phylum level, 7/10 microbiota were common to both ducks and hens. And the proportion of *Firmicutes* was significantly increased by the flaxseed diet in the hen's ceca, which was consistent with the findings of this study [37]. Unlike *Firmicutes*, the relative abundance of *Proteobacteria* and *Desulfobacterota*

decreased in all Fla groups. *Proteobacteria* consists of many pathogens, such as *Vibrio cholerae*, *Shigella* and *Salmonella*. Abnormal expansion of *Proteobacteria* has been demonstrated to be positively correlated chronic colitis [38]. *Desulfobacterota* is a group of sulfate-reducing anaerobic bacterium that can bind to human colonic mucin, which has been proved to have pro-inflammatory and pathological effects in the gut [39, 40]. Nowadays, a growing number of researches have shown the relationship between intestinal microbiota with productive performance in poultry [41, 42]. In this study, seven genera were identifed positively correlated with the production and/or quality of egg in this experiment. Among them, *Intestinimonas* and *Butyricicoccus* are two butyrate-producing micrbiota which have been proved to benefits for intestinal barrier functions, resistance to pathogenic bacteria and anti-inflammation [43–46]. Another *Blautia* has been reported to be able to alleviate inflammatory diseases and metabolic diseases and prevent pathogen colonization by producing bacteriocins [47, 48]. Whereas, *Fusobacterium* has been identified as a kind of opportunist bacteria, some of them can induce the host to produce pro-inflammatory cytokines [49]. These findings suggested that flaxseed could indirectly improving productive performance through promoting the health of gut.

In summary, addition of flaxseed improved the productive performance including egg production, egg mass, feed conversion rate, egg quality and the content of DHA in eggs of laying ducks. Flaxseed diets may improve the body immunity through the elevating the plasma levels of IgA, IgG and IgM. Moreover, dietary flaxseed altered the composition of intestinal microbiota and increased the relative abundance of several anti-inflammatory bacteria, some of which showed positive correlation with several indices of production.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s11033-024-09858-y.

Author contributions LY, YS, TZ and ZY conceived and designed the study. LY, YS, WZ, LZ, DZ and KZ performed the experiments. YS and WZ analyzed the data. LY and YS wrote the manuscript. ZY and TZ supervised the experiment. All authors reviewed and approved the final version of the manuscript.

Funding This study was financially supported by the Natural Science Foundation Program (Joint Fund Project) in Hubei Province (Grant No.2023AFD051), the Key Science and Technology Plan Project in Xiangyang (Grant No. 2022-31663) and the Science Research Program Guidance Project of Education Department in Huber Province (Grant No. B2022537).

Data availability The datasets generated for this study has been deposited in the Sequence Read Archive (https://www.ncbi.nlm.nih.gov/sra, Accession Number PRJNA1085023) at NCBI.

Declarations

Conflict of interest The authors declare no competing interests.

Ethical approval All experimental procedure with animals were conducted with care and obey the "Guidelines for Experimental Animals"of the Ministry of Science and Technology (Beijing, China). This study was supervised and approved by the Experimental Ethics Committee of Xiangyang Polytechnic (permit No.2022000600).

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