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Effects of xylo-oligosaccharide supplementation on the production performance, intestinal morphology, cecal short-chain fatty acid levels, and gut microbiota of laying hens

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ABSTRACT This study investigated the effects of xylo-oligosaccharide supplementation on the production performance, intestinal morphology, cecal short-chain fatty acid levels, and gut microbiota of laying hens. A total of 800 Lohmann pink layers, each 48 wk old, were randomly divided into 5 dietary treatment groups, namely XOS at 0 (CON), 100 (XOS1), 200 (XOS2), 300 (XOS3) and 400 (X0S4) mg/kg. The experimental period was 24 wk. The results revealed that the egg production rate and the number of eggs laid by each layer between 1 to 12 wk increased as the XOS concentration increased ($P_{linear} < 0.05$). The sand-shell egg percentage decreased significantly from 1 to 12 wk in the XOS1, XOS2, and XOS3 groups ($P_{ANOVA} < 0.05$). Compared with the CON group, the 4 XOS dosage groups presented significant increases in the villus height and the ratio of villus height to crypt depth in the jejunum ($P_{ANOVA} < 0.05$), whereas a linear decrease in jejunal crypt depth ($P_{linear} < 0.05$) was noted. In addition, XOS supplementation significantly increased the concentrations of butyric acid and isovaleric acid in the caeca ($P_{ANOVA} < 0.05$). High-throughput sequencing analysis of bacterial 16S rRNA revealed that dietary XOS supplementation influenced the cecal microbiota. The alpha diversity analysis indicated that the richness of cecal bacteria was greater in the laying hens fed XOS. The modulation of the cecal microbiota composition upon the addition of XOS was characterized by an increased abundance of *Firmicutes* and *Bifidobacteriaceae*, and decreased abundance of *Bacteroidetes*. At the genus level, dietary XOS supplementation resulted in decreases in the abundances of *Bacteroides* and *Rikenellaceae*_RC9_gut_group and an increase in the abundance of *Lactobacillus*. In conclusion, dietary XOS supplementation improved the production performance of laying hens by increasing the production of short-chain fatty acids and improving their intestinal morphology, which was achieved mainly through changes in the composition of the intestinal microbiota. The recommended level of XOS in the diet of laying hens is 200 mg/kg.

Key words: xylo-oligosaccharide, laying hen, microbiota, intestinal health, production performance

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INTRODUCTION

A favorable gut microbiota is essential for the performance of chickens (Kogut, 2019). Therefore, in the poultry industry, the regulation of gut microbiota development is considered an effective approach to prevent disease and improve production performance (Broom and LJ, 2017; Kogut, 2019; Nurmi and Rantala, 1973). Antibiotics are widely used for altering the gut microbiota to improve productivity (Markowiak and Śliżewska, 2018). However, several nations have severely restricted or even outlawed the use of antibiotics in animals because of the negative effects observed after long-term abuse of such antibiotics (Er et al., 2013; van den Bogaard et al., 2002). Therefore, it is crucial to search for alternatives to antibiotics for application in the animal industry.

Therapies that increase the abundance of beneficial bacteria in the gut have promoted advancements in the development of probiotics and prebiotics (Ducatelle et al., 2015; Pourabedin and Zhao, 2015). Prebiotics are proposed as alternatives to antibiotics in livestock production (Hajati and Rezaei, 2010). Xylo-oligosaccharide (**XOS**), a typical prebiotic, that is chemically a

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functional oligosaccharide comprising mainly xylose units linked via β-1,4-glycosidic bonds (Chen et al., 2021). Chickens lack enzymes capable of degrading these β-1, 4-glycosidic bonds, facilitating the transport of xylo-oligosaccharides to the distal intestine, where they are metabolized by xylanolytic microorganisms, without requiring any structural alterations (Pourabedin and Zhao, 2015). As typical microbiota-accessible carbohydrates, XOS are broken down by bacteria in the cecum to produce SCFAs, which are the main metabolites (Koh et al., 2016). Other microorganisms can further utilize acetate and lactate generated by the fermentation of *Bifidobacteria* and *Lactobacillus* to form the end products propionate and butyrate (Hosseini et al., 2011; Rivière et al., 2016). Propionate and acetate are the final products of the fermentation process by the representatives of *Bacteroidetes*, whereas butyrate is the preferred substrate for intestinal epithelial cells to fulfill the energy requirements during the production process, with the main producers of butyrate belonging to *Firmicutes* (Duncan et al., 2007; Guilloteau et al., 2010; Roediger, 1982; Walker et al., 2005; Wrzosek et al., 2013; Yang et al., 2013)An *in vitro* experimental study indicated that XOS promoted the growth of beneficial bacteria by serving as a source of energy and carbon (Moura et al., 2007). Animal experiments have also revealed that dietary XOS supplementation can increase the abundance of beneficial bacteria in the cecum while decreasing the abundance of pathogenic bacteria, however, most of these studies focused on broilers, and relevant research on laying hens, particularly aged laying hens, is lacking (De Maesschalck et al., 2015; Eeckhaut et al., 2008; Pourabedin et al., 2017).The composition of intestinal microbial communities differs between laying hens and broilers (Videnska et al., 2014). According to previous studies, two-thirds of the microbiota in aged laying hens comprises representatives of *Bacteroidetes*, suggesting the age-dependent development of the gut microbiota (Arumugam et al., 2011; Callaway et al., 2009; Suzuki et al., 2004). To the best of our knowledge, few studies have investigated the effects of XOS on the gut microbiota of aged laying hens, with the exception of a recent report by Zhou et al. (2021a), which noted that dietary supplementation with 200 mg/kg XOS has the most significant effect. Therefore, the objective of the present study was to assess the effects of different XOS concentrations on the production performance, egg quality, intestinal morphology, cecal short-chain fatty acid levels, and gut microbiota of laying hens to determine the optimal dosage and potential as feed additives.

MATERIALS AND METHODS

Experimental Design, Birds, and Feed

The experimental protocols used in the present study were approved by the Animal Care and Use Committee of Sichuan Agricultural University, China. A total of eight hundred Lohmann laying hens aged 48 to 71 wk were adaptively prefed with a control diet for 2 wk and then randomly divided into 1 control group and 4 treatment groups. The hens were fed a corn−soybean meal-based diet supplemented with XOS at 0 (CON), 100 (XOS1), 200 (XOS2), 300 (XOS3), or 400 (XOS4) mg/kg. Each group had 8 replicates, with 20 hens per replicate. For each treatment, 5 replicates were employed, with 4 chickens assigned to a cage (45 × 42 × 46 cm). A group of 5 consecutive cages formed 1 replicate. The XOS used in the present study was purchased from Yibin Yatai Biotechnology Co., Ltd. The XOS test product contained 35% XOS, the remainder being made up of maltodextrin, and the XOS present ranged from DP2 −DP6, with DP2 = 25.35%, DP3 = 8.58%, DP4 = 2.71% DP5 = 1.92% and DP6 = 0.03% of the XOS; the botanical source of the XOS is poplar. The basic feed was based on NRC (1994) and the "Chicken Feeding Standard" (NY/T 33-2004). The test feed was ground into powder. Table 1 summarizes the composition and nutritional levels of the basic feed. Animal experiments were conducted at the Ya'an Institute of Animal Nutrition, Sichuan Agricultural University. All treatment replicates were randomly and evenly distributed within the coop. The birds were reared in an environment under controlled conditions, with a temperature of 24°C, humidity levels between 50% and 65%, and a 16 h light−dark cycle. All birds had unrestricted access to both water and the experimental diets throughout the 24-wk experimental period. At 9:00 am, the time of first feeding, chickens were observed for any abnormalities. The second feeding time was at 3:00 pm, and at this time, the chickens were

Table 1. Basic feed composition and nutritional level (air-dried basis, %).

Ingredients	Contents
Corn	58.06
Soybean meal (43% of CP)	24.50
Wheat bran	3.50
Soybean oil	2.30
Grainy limestone	4.75
Powdery limestone	4.75
$CaHPO_4$	1.10
DL ~ Met (99%)	0.11
NaCl	0.03
Choline chloride (60%)	0.10
Vitamin premix[1]	0.03
Mineral premix[1]	0.50
Total	100.00
Nutrient levels[2]	
ME, MJ/kg	10.85
CP, %	15.50
Calcium, %	4.00
Total phosphorus, %	0.51
Available phosphorus, %	0.32
Digestible lysine, %	0.78
Digestible methionine, %	0.35
Digestible threonine, %	0.58
Digestible tryptophan, %	0.17
Digestible methionine + cystine, %	0.59

[1]Vitamin and mineral premixes are provided per kilogram of feedstock: VA 12,000 IU, VD_3 3,000 IU, VE 30 IU, VB_1 3 mg, VB_2 9.6 mg, VB_6 6 mg, VB_{12} 0.3 mg, VK_3 4.8 mg, D ~ pantothenic acid 18mg, D ~ Biotin 1.665 mg, folic acid 1.5 mg, niacinamide 60 mg, Cu ($CuSO_4$)10 mg, Fe ($FeSO_4$) 60 mg, Mn ($MnSO_4$) 100 mg, Zn ($ZnSO_4$) 60 mg, I (KI) 0.36 mg, Se (Na2SeO3) 0.3 mg.

[2]The nutrient level is the calculated value.

observed again. Eggs were collected and weighed every day, and the enclosure was cleaned regularly.

Productive Performance Measurement and Sample Collection

The total number of eggs produced, total egg weight, qualified eggs (excluding sand-shell, soft, broken, malformed, dirty, large [>70 g], and small [<50 g] eggs), qualified egg weight, each category of unqualified eggs and the number of dead chickens were recorded every day. In addition, the weekly feed intake of laying hens was measured as the difference between the amount of feed consumed and the amount of feed that remained unused. The feed-egg ratio, egg production rate, and average egg weight were calculated as follows: egg production rate (%) = (number of eggs laid/number of chickens) × 100; feed-egg ratio (g/g) = total feed intake/total egg weight; and qualified egg rate (%) = (number of qualified eggs/total number of eggs) × 100.

At the end of the 24th wk of the trial, 1 laying hen from each replicate was selected and weighed. Afterward, all the chosen hens were sacrificed via cervical dislocation. About 2 cm-long jejunal and ileal segments were then removed and preserved in 4% neutral formaldehyde for histological analysis. To explore the intestinal microbial populations and short-chain fatty acids, the cecal contents were extracted fresh and immediately placed in sterile microtubes for preservation at −80°C.

Determination of Egg Quality

At 6, 12, 18, and 24 wks of the trial, 6 eggs were randomly selected from each replicate, and all 48 eggs were subjected to egg quality determination. The egg quality determination included eggshell strength, yolk color, Haugh unit, and albumen height measurements. Eggshell strength was measured using an eggshell strength tester (model: ETG-1601A, Japan Robotmation Company). Yolk color, Haugh unit, and albumen height were determined via an automatic egg-quality analyzer (model: EMT-5200 type).

Intestinal Morphology Analysis

The intestinal segments were soaked in 4% paraformaldehyde, dehydrated with ethanol, cleaned with xylene, and embedded in paraffin wax. The embedded tissues were then cut into thin sections 3 mm in size via a Leica CM1860 microtome. Each section was then placed on a glass slide and stained with hematoxylin−eosin, and ten straight and intact villi were selected from every sample to observe their morphology via Image-Pro Plus 6.0 (Media Cybernetics, Inc., Bethesda, MD).

SCFA Concentration Determinations

The concentrations of acetic acid, propionic acid and butyric acid in the cecal chyme were determined using a gas chromatography (VARIAN CP-3800, Agilent Technologies Inc., Santa Clara, CA). About 0.7 g of the sample (with its mass accurately recorded) was placed into a 2 mL centrifuge tube, followed by dilution in 1.5 mL of ultrapure water. The mixture was allowed to stand for 30 min and then centrifuged at 20,000× g for 15 min to obtain the extract, with a sample concentration that was denoted as M. Subsequently, 1 mL of the supernatant was transferred to a new tube to which 23.3 mL of 210 mmol/L crotonic acid and 0.2 mL of 25% metaphosphoric acid were added. The mixture was incubated for 30 min at 4°C, followed by centrifugation at 20,000× g for 10 min and then filtration. The filtrate was collected in a 1.5 mL tube to which methanol (0.9 mL) was added, followed by 5 min of centrifugation at 10,000× g and then filtration of the supernatant through a 0.22 mm membrane. This filtrate was collected in 1.5 mL tubes for further analysis (Xiong, et al., 2024).

The 16S rRNA Analysis

Microbial DNA was extracted from the cecal content samples via the Zymo Research DNA Kit (Zymo Research BIOMICS DNA Microprep Kit, Cat D4301) according to the manufacturer's instructions. Using the isolated DNA as a template, the v4 region of the bacterial 16S rRNA gene was amplified with the following universal primers: 515F (5′-GTGYCAGCMGCCGCGGTAA-3′) and 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The PCR conditions were as follows: 1 min of denaturation at 94°C, followed by 25 to 30 cycles of 20 s at 94°C (denaturation), annealing at 54°C for 30 s, and extension at 72°C for 30 s, with a final extension of 5 min at 72°C. Amplicons were extracted from 2% agarose gels and purified using the ZYMO REASERCH DNA Gel Extraction Kit (Zymoclean Gel Recovery Kit, Cat D4008) according to the manufacturer's instructions. Purified amplicons were qualified and sequenced using the NovaSeq 6000 platform with the NovaSeq 6000 SP Reagent Kit v1.5 at Roning Biotechnology Co., Ltd. (Chengdu, China).

Statistical Analysis

The data were subjected to 1-way analysis of variance (**ANOVA**) for 1-way ANOVA, linear and quadratic regression analysis, and Duncan's method for multiple comparisons via SPSS 25.0 software. All the data are expressed as the means and standard errors of the means. A P-value of < 0.05 was considered to indicate statistical significance.

RESULT

Production Performance

As presented in Table 2, the egg production rate increased linearly from 13 to 24 wk ($P_{linear} < 0.05$), and

Table 2. Effects of xylo-oligosaccharides on production performance of laying hens.

Item	XOS level(mg/kg)					SEM	*P*-value		
	0	100	200	300	400		ANOVA	Linear	Quadratic
Egg production rate/ (%)									
Wk 1−12	85.47	86.06	87.51	86.29	86.94	0.47	0.703	0.357	0.526
Wk 13−24	78.72	80.00	80.71	81.74	83.38	0.66	0.217	0.020	0.846
Wk 1−24	82.26	83.13	84.21	84.10	85.25	0.53	0.460	0.072	0.885
Feed to egg ratio/ (g/g)									
Wk 1−12	1.93	1.93	1.91	1.93	1.95	0.01	0.859	0.746	0.388
Wk 13−24	2.16	2.09	2.06	2.08	2.05	0.02	0.247	0.062	0.298
Wk 1−24	2.04	2.01	1.98	2.00	2.00	0.01	0.675	0.338	0.331
Qualified egg rate/ (%)									
Wk 1−12	96.22	97.38	97.03	96.88	97.02	0.29	0.796	0.599	0.461
Wk 13−24	93.88	95.03	94.88	95.70	95.58	0.40	0.639	0.165	0.641
Wks 1−24	95.17	96.29	96.03	96.34	96.34	0.30	0.724	0.285	0.527

Data were presented at mean (n = 8).

increased linearly from 1 to 24 wk ($P_{linear} = 0.072$). Furthermore, the feed-egg ratio tended to decrease with increasing XOS from 13 to 24 wk ($P_{linear} = 0.062$). No significant difference was noted in the percentage of qualified eggs between the CON and XOS groups ($P_{ANOVA} > 0.05$).

The sand-shell egg percentage decreased significantly ($P_{ANOVA} < 0.05$) in the XOS1, XOS2, and XOS3 groups compared with the CON group from 1 to 12 wk, and exhibited significant quadratic function changes ($P_{Quadratic} < 0.05$) (Table 3). However, no difference was noted in the rates of dirty, broken, and soft eggs among the groups ($P_{ANOVA} > 0.05$).

As presented in Table 4, the intensity of the egg yolk color increased linearly with increasing XOS at the 12th week ($P_{linear} < 0.05$), although dietary XOS supplementation exerted no significant effect on eggshell strength, Haugh unit, or albumen height ($P_{ANOVA} > 0.05$).

Intestinal Morphology

As depicted in Table 5, compared with the CON group, the groups that received the 4 doses of XOS presented a significant increase in the villus height and villus height-to-crypt depth ratio (V/C) in the jejunum ($P_{ANOVA} < 0.05$), whereas the jejunal crypt depth decreased linearly with increasing XOS levels ($P_{linear} < 0.05$). A linear decrease was also noted in the ileal villus height-crypt depth ratio ($P_{linear} = 0.078$).

SCFA Concentrations

Table 6 presents the effects of XOS on cecal short-chain fatty acid levels. XOS supplementation significantly increased the concentrations of butyric acid and isovaleric acid ($P_{ANOVA} < 0.05$). The concentration of propionic acid tended to increase with the addition of XOS ($P_{linear} = 0.096$).

Cecum Microorganisms

The results for the rate of emergence of new OTUs under continuous sampling are presented in Figure 1A. As illustrated in the figure, the rarefaction curve eventually flattened out, demonstrating that the sequenced sequence basically covered all the species in the sample and that the sampling was sufficient for data analysis. As depicted in Figure 1B, the number of OTUs belonging to each group was 571. The number of unique OTUs

Table 3. Effects of xylo-oligosaccharides on unqualified eggs of laying hens.

Item	XOS level(mg/kg)					SEM	*P*-value		
	0	100	200	300	400		ANOVA	Linear	Quadratic
Dirty egg rate/ (%)									
Wk 1−12	0.31	0.52	0.43	0.32	0.52	0.07	0.770	0.678	0.954
Wk 13−24	0.69	0.99	0.70	0.66	0.84	0.09	0.740	0.959	0.982
Wk 1−24	0.49	0.74	0.55	0.48	0.67	0.07	0.764	0.855	0.987
Broken egg rate/ (%)									
Wk 1−12	1.88	1.46	1.27	1.78	1.44	0.20	0.857	0.711	0.628
Wk 13−24	2.59	1.57	1.14	1.97	1.86	0.22	0.304	0.483	0.094
Wk 1−24	2.19	1.51	1.21	1.87	1.64	0.19	0.591	0.597	0.268
Soft egg rate/ (%)									
Wk 1−12	0.54	0.26	0.49	0.44	0.33	0.08	0.790	0.651	0.915
Wk 13−24	0.85	0.22	0.44	0.54	0.42	0.09	0.227	0.375	0.217
Wk 1−24	0.68	0.24	0.47	0.49	0.37	0.07	0.420	0.478	0.471
Sand-shell egg rate/ (%)									
Wk 1−12	0.47[a]	0.11[b]	0.10[b]	0.11[b]	0.26[ab]	0.04	0.015	0.120	0.002
Wk 13−24	0.86	0.32	0.66	0.60	0.32	0.07	0.338	0.239	0.878
Wk 1−24	0.64	0.21	0.36	0.33	0.29	0.05	0.111	0.113	0.175

Data were presented at mean (n=8).
[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

Table 4. Effects of xylo-oligosaccharides on egg quality of laying hens.

	XOS level(mg/kg)						P-value		
Item	0	100	200	300	400	SEM	ANOVA	Linear	Quadratic
Albumen height (mm)									
Wk 6	8.83	8.77	8.85	8.61	8.26	0.10	0.393	0.086	0.305
Wk 12	8.42	8.42	8.79	8.19	8.14	0.11	0.237	0.234	0.180
Wk 18	8.38	8.09	8.15	8.21	7.93	0.10	0.722	0.289	0.972
Wk 24	7.56	7.43	7.06	7.13	7.35	0.08	0.294	0.212	0.096
Haugh unit									
Wk 6	91.03	94.09	95.10	92.65	90.98	0.78	0.360	0.779	0.049
Wk 12	91.67	91.96	92.57	90.53	90.12	0.48	0.499	0.192	0.322
Wk 18	91.47	89.39	90.08	90.31	88.47	0.55	0.525	0.199	0.996
Wk 24	87.93	85.90	86.24	86.65	86.31	0.55	0.811	0.529	0.458
Egg yolk color									
Wk 6	12.98	13.10	13.05	13.28	13.14	0.06	0.617	0.248	0.229
Wk 12	12.77	13.25	13.12	13.14	13.33	0.07	0.140	0.039	0.451
Wk 18	13.19	13.08	13.31	13.26	13.23	0.05	0.603	0.412	0.078
Wk 24	13.52	13.24	13.40	13.33	13.43	0.05	0.631	0.791	0.265
Eggshell strength (kg/cm2)									
Wk 6	4.25	4.23	4.02	4.37	4.35	0.07	0.495	0.463	0.326
Wk 12	4.26	4.09	4.09	4.26	4.00	0.07	0.728	0.662	0.454
Wk 18	4.28	4.24	3.85	4.14	3.66	0.13	0.518	0.160	0.843
Wk 24	4.18	4.10	4.44	3.90	4.08	0.13	0.798	0.674	0.753

Data were presented at mean (n = 8).

Table 5. Effects of xylo-oligosaccharides on intestinal morphology of laying hens at 71 wk of age.

	XOS level(mg/kg)						*P*-value		
Item	0	100	200	300	400	SEM	ANOVA	Linear	Quadratic
Jejunum									
Villus height (mm)	904.88[a]	1,160.85[b]	1,200.99[b]	1,184.37[b]	1,189.82[b]	35.53	0.015	0.008	0.032
Crypt depth (mm)	209.41	196.70	196.14	177.74	184.79	4.23	0.119	0.021	0.524
Villus height/crypt depth (VH/CD)	4.24[a]	5.82[b]	6.11[b]	6.28[b]	6.40[b]	0.25	0.033	0.008	0.117
Ileum									
Villus height (mm)	599.93	755.99	763.82	754.44	793.58	31.92	0.381	0.111	0.367
Crypt depth (mm)	145.60	140.57	136.12	136.81	141.98	3.11	0.919	0.667	0.401
Villus height/crypt depth (VH/CD)	4.20	5.53	5.56	5.46	5.51	0.18	0.160	0.078	0.095

Data were presented at mean (n = 8).
[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

in each XOS group was greater than that in the CON group, suggesting that dietary XOS supplementation influenced the composition of the intestinal microbiota.

The diversity and richness indices, namely, the Shannon, Simpson, Ace and Chao indices were employed to estimate the bacterial α diversity of the cecal microbiota (Table 7). No significant differences were noted in the Shannon or Simpson indices between the CON and XOS groups. The Ace and Chao index values noted for the XOS2 group were significantly greater than those noted for the CON group. These findings indicated that the addition of 200 mg/kg XOS increased the overall bacterial richness of the cecal microbiota ($P_{ANOVA} < 0.05$).

The results of the NMDS analysis at the OTU level are presented in Figure 2. In the figure, the distance between each point denotes the degree of difference, each point represents a sample, and the same hue denotes samples from the same group. An accurate reflection of the degree of variation between samples is a stress value of less than 0.2. As shown in the figure, the XOS2 group had the least overlap with the CON group and presented the greatest difference in the microbial community compared with the control group.

Table 6. Effects of xylo-oligosaccharides on short-chain fatty acids of cecal digesta of laying hens at 71 wk of age.

	XOS level(mg/kg)						*P*-Value		
Item	0	100	200	300	400	SEM	ANOVA	Linear	Quadratic
Acetic acid (mmol/L)	43.63	54.81	55.74	55.38	54.44	1.99	0.278	0.126	0.136
Propionic acid (mmol/L)	14.88	20.82	20.93	20.29	20.72	2.01	0.176	0.096	0.132
Butyric acid (mmol/L)	6.75[b]	8.61[a]	9.04[a]	8.92[a]	8.46[a]	0.29	0.004	0.014	0.01
Valeric acid (mmol/L)	1.96	2.17	2.13	2.25	2.15	0.12	0.965	0.615	0.657
Isobutyric acid (mmol/L)	0.99	1.30	1.28	1.35	1.27	0.06	0.359	0.179	0.205
Isovaleric acid (mmol/L)	1.34[c]	2.03[ab]	1.96[b]	2.14[ab]	2.31[a]	0.08	< 0.01	< 0.01	0.046

Data were presented at mean (n = 8).
[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

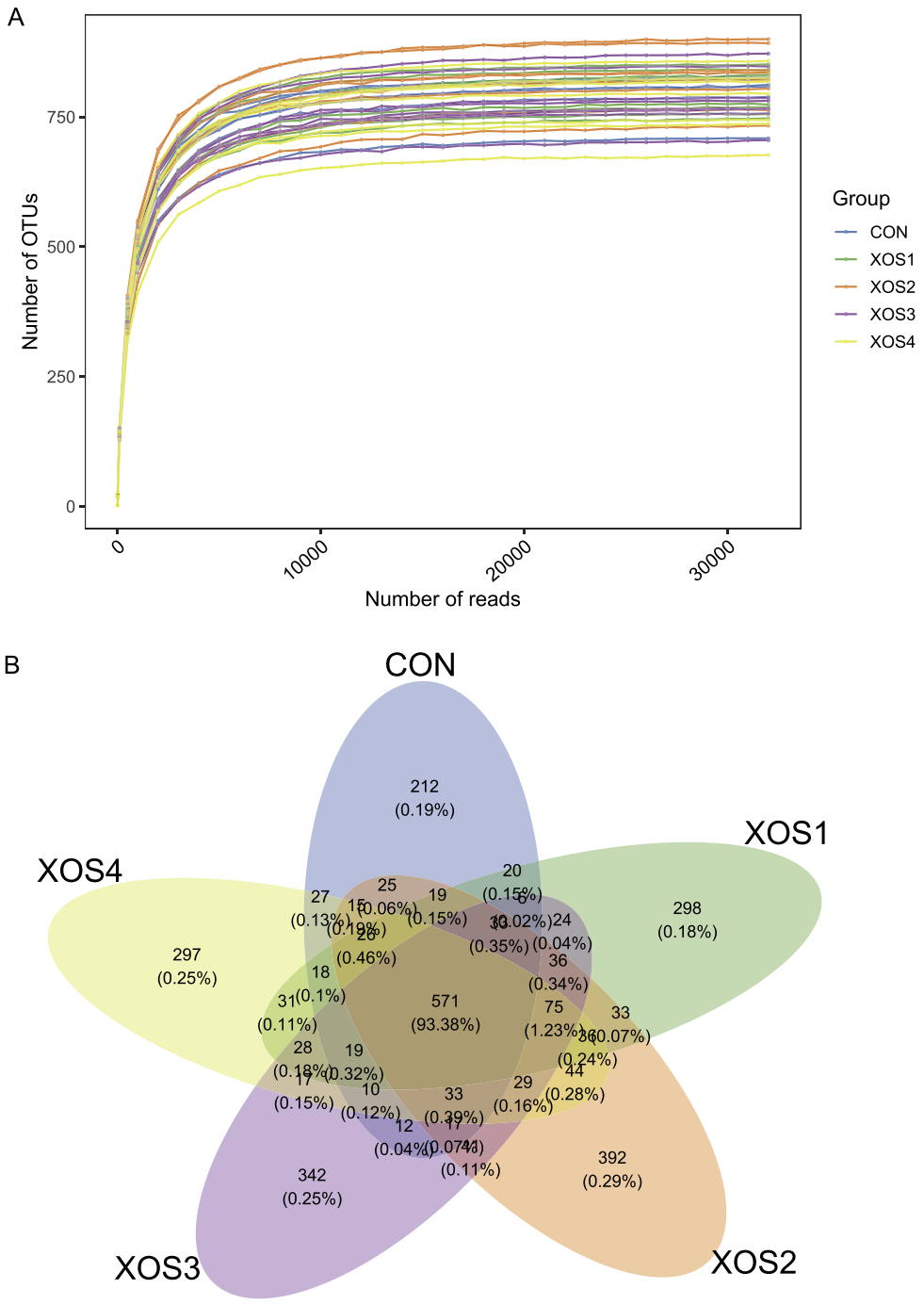

Figure 1. (A) Rarefaction curve of species. The abscissa is the sample size; the ordinate is the number of sequences after sampling. (B) Venn diagram showing cecal microorganisms in the samples.

Table 7. Effects of xylo-oligosaccharides on microbial alpha diversity in the cecum of laying hens at 71 wk of age.

	XOS level(mg/kg)						P-value		
Item	0	100	200	300	400	SEM	ANOVA	Linear	Quadratic
Shannon	5.26	5.24	5.28	5.19	5.22	0.02	0.648	0.369	0.929
Simpson	0.99	0.99	0.99	0.99	0.99	0.00	0.675	0.380	0.690
Chao1	467.16[b]	492.09[b]	543.60[a]	505.38[ab]	491.62[b]	7.55	0.021	0.183	0.005
ACE	462.71[b]	482.50[b]	532.50[a]	504.08[ab]	490.93[ab]	7.32	0.020	0.090	0.010

Data were presented at mean (n = 8).
[a,b]Average with diverse superscripts in the column shows a significant difference ($P < 0.05$).

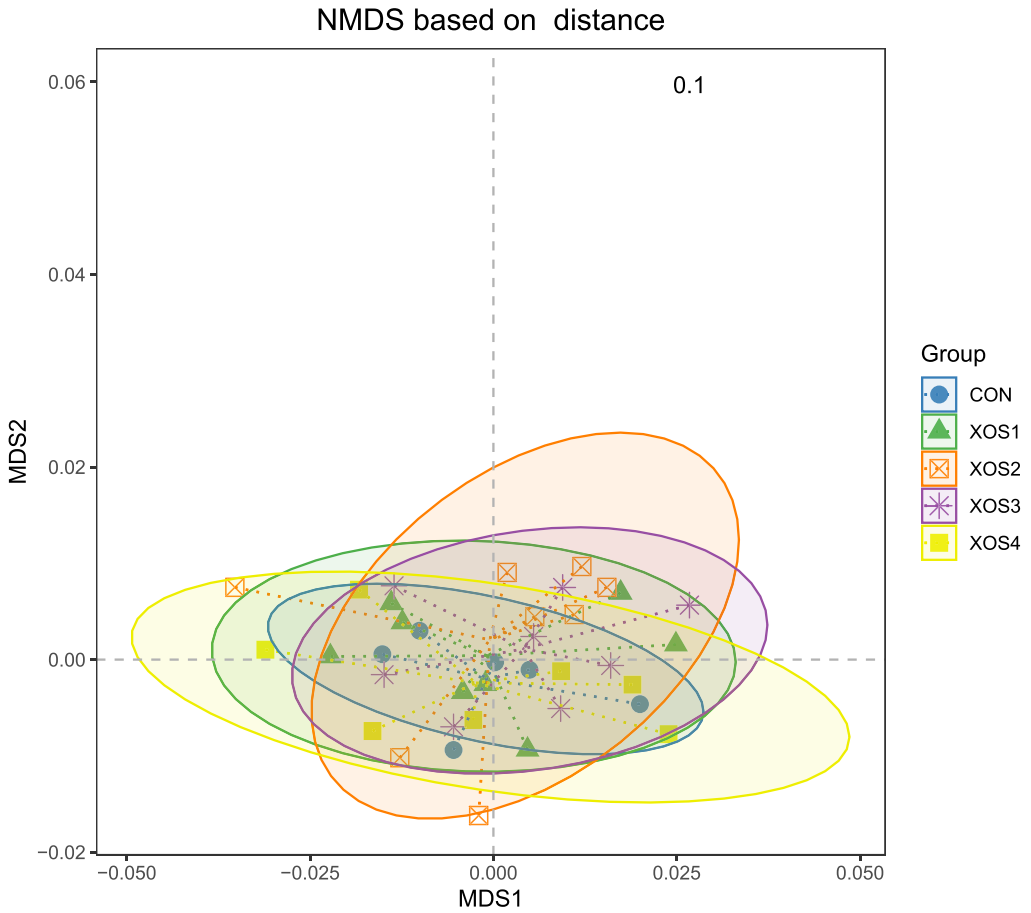

Figure 2. NMDS analysis results based on OTU levels.

The taxonomic composition of the microbiota was analyzed at the phylum and genus levels. In Figure 3A, the top 10 phyla in terms of the relative abundance of species are shown, whereas the other phyla are collectively represented as "Others", The phyla with the highest relative abundances of species are *Bacteroidetes* and *Firmicutes*, followed by *Proteobacteria* and *Euryarchaeota*. Furthermore, compared with the CON group, the XOS groups, particularly the XOS2 group, were characterized by a reduced abundance of *Bacteroidetes* and a greater abundance of *Firmicutes*. Figure 3B presents the top 10 [most abundant] genera. As shown in Figure 3B, the XOS2 group presented a relatively low abundance of *Bacteroides* and *Rikenellaceae*_RC9_gut_group, whereas the XOS3 group presented a relatively high abundance of *Lactobacillus*. At the family level, the XOS2 group presented a greater abundance of *Bifidobacteriaceae* than did the CON group.

To determine the associations among the phylotypes in the gut microbiota and the different phenotypes of hens, a Pearson correlation analysis was performed. The results presented in the form of a heatmap revealed that the abundance of *Prevotellacae* UCG-001 ($P < 0.05$; $R = 0.9$) and the abundance of uncultured_*Muribaculaceae* ($P < 0.05$; $R = 0.9$) were positively related to jejunal crypt depth. The abundance of the [*Eubacterium*] coprostanoligenes group ($P < 0.01$; $R = 1$) was positively correlated with jejunal villus height. In contrast, the abundances of uncultured_*Rikenellace* ($P < 0.05$; $R = -0.9$), *Bacteroides* ($P < 0.05$; $R = -0.9$), and *Faecalibacterium* ($P < 0.05$; $R = -0.9$) were negatively correlated with jejunal villus height (Figure 4A). Moreover, the abundance of *Shuttleworthia* ($P < 0.05$; $R = 0.9$) was positively related to cecal propionic acid. *Lactobacillus* was positively related to cecal acetic acid ($P < 0.05$; $R = 0.9$), butyric acid ($P < 0.05$; $R = 0.9$), and isobutyric acid ($P < 0.05$; $R = 0.9$) contents. The abundance of the (*Ruminococcus*) torques group was positively related to cecal acetic acid ($P < 0.05$; $R = 0.9$), butyric acid ($P < 0.05$; $R = 0.9$), and isobutyric acid ($P < 0.05$; $R = 0.9$). Conversely, the abundance of *Rikenellaceae*_RC9_gut_group was negatively correlated with cecal propionic acid ($P < 0.05$; $R = -0.9$), acetic acid ($P < 0.05$; $R = -0.9$), and butyric acid ($P < 0.05$; $R = -0.9$) contents. The abundance of *Faecalibacterium* was negatively correlated with cecal acetic acid ($P < 0.05$; $R = -0.9$) and butyric acid ($P < 0.05$; $R = -0.9$) contents. The abundance of *Bacteroides* was negatively correlated with cecal acetic acid ($P < 0.05$; $R = -0.9$) and butyric acid ($P < 0.05$; $R = -0.9$). A negative relationship was also observed between the abundance of *Parabacteroides* and cecal isovaleric acid ($P < 0.05$; $R = -0.9$) (Figure 4B). These findings suggest that the gut microbiota in laying hens is related to intestinal morphology and the content of short-chain fatty acids.

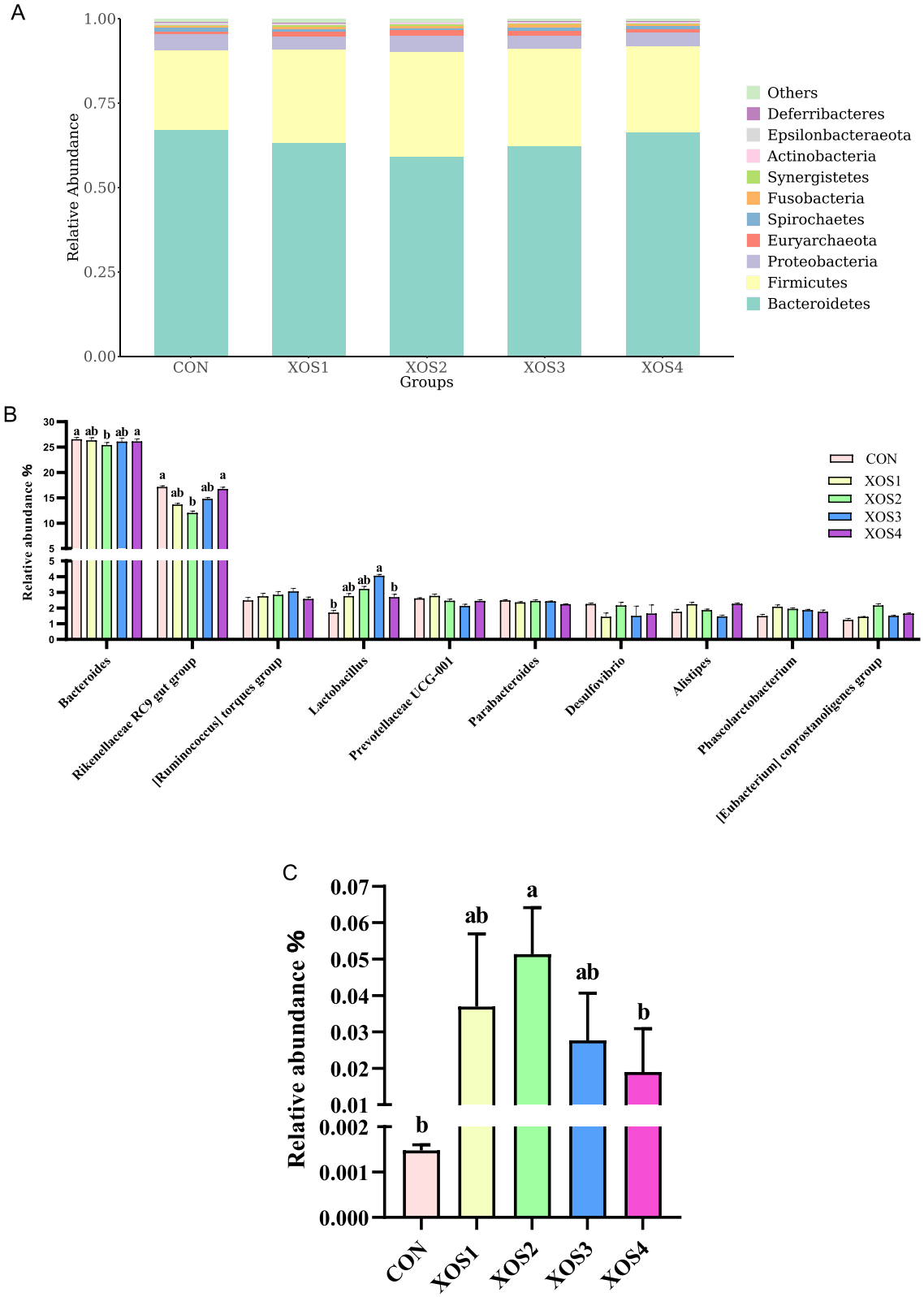

Figure 3. (A) Histogram of relative abundance of species at the phylum level. (B) Relative abundance of the most abundant 10 bacterial genera in 5 treatment groups. Bars with asterisks mean that the genera in the XOS-supplemented group were significant different compared with the control group ($P < 0.05$), and bars with no asterisks mean no significant difference in the genera between 2 groups ($P > 0.05$). (C) Relative abundance of *Bifidobacteriaceae* detected in the samples. Bars with asterisks mean that in the XOS-supplemented group were significant different compared with the control group ($P < 0.05$).

DISCUSSION

Xylo-oligosaccharides have been confirmed to improve production performance in previous reports (Craig et al., 2020; Rao et al., 2024; Zhou et al., 2021b). In the present study, from 13 to 24 wk, he egg production rate increased linearly in the XOS supplementation group, which indicated that XOS takes time to exert positive

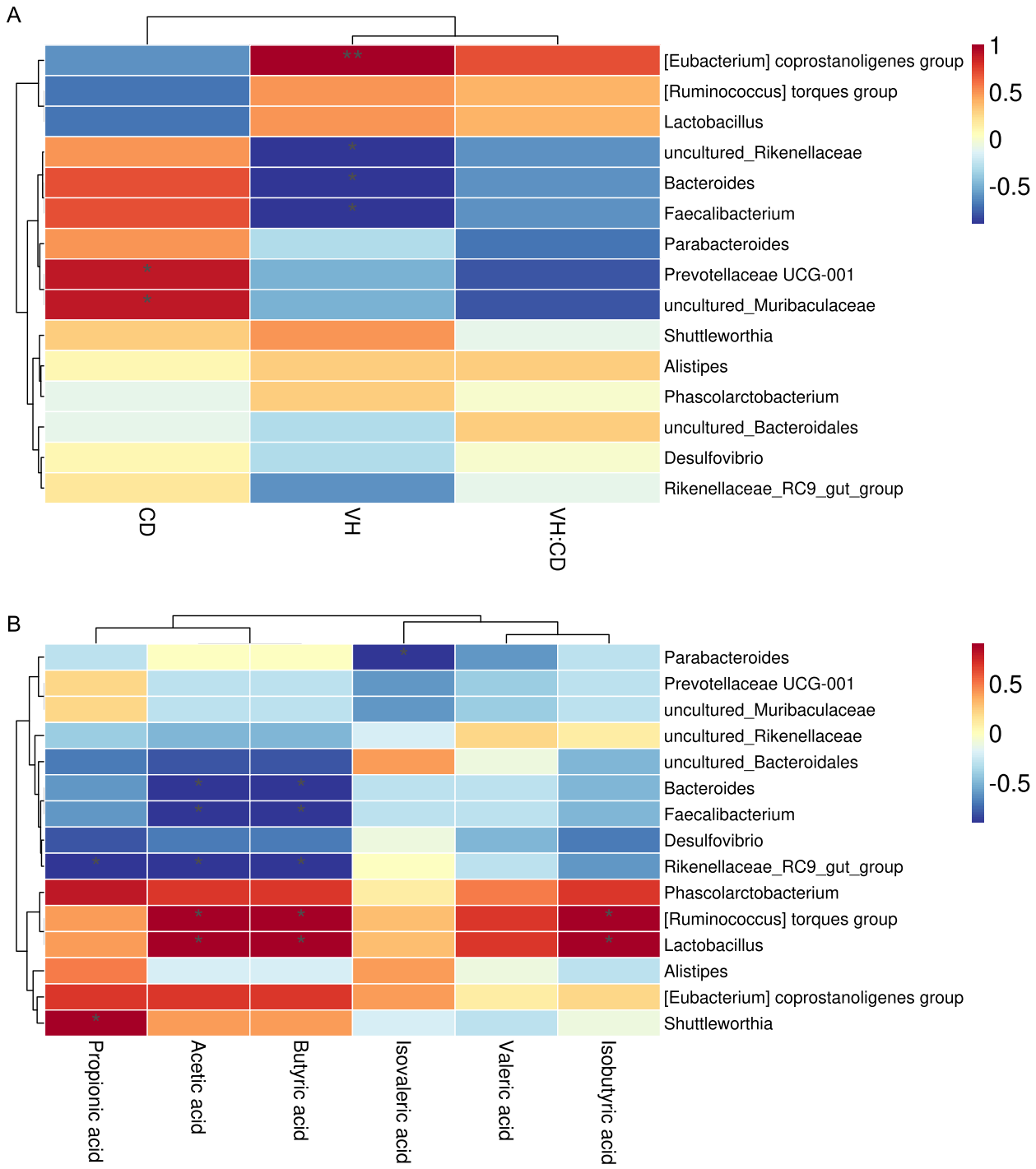

Figure 4. Associations of 15 key phylotypes (at genus level) with phenotypes. The depth of colors ranging from blue to red represents the magnitude of correlation. The OTU were organized according to their Pearson correlation coefficient. Significant correlations are noted by: *, $0.01 < P < 0.05$; **, $0.001 < P < 0.01$. (A) Associations of 15 key phylotypes (at genus level) with the morphology of jejunum; VH- villus height; CD- crypt depth; VH:CD- villus height-to-crypt depth ratio. (B) Associations of 15 key phylotypes (at genus level) with the cecal short-chain fatty acid.

effects. Similar "time dependence" effects have been reported by Zhou et al. (2021a). The possible mechanism underlying this effect of XOS on FCR could be that it sends signals to induce specific bacteria to ferment non-digestible carbohydrates and interact with the digestive tract, resulting in increased digestive efficiency (Ribeiro et al., 2018). Moreover, XOS supplementation resulted in lower sand-shell egg rates in the present study, and the XOS2 group (200 mg/kg) presented the lowest sand-shell egg rates. The possible reason for this could be that XOS promoted calcium absorption (Li et al., 2017; Xu et al., 2011). A previous study indicated that XOS selectively promotes the colonization of beneficial bacteria, following which the metabolites such as organic acids and fatty acids produced by the microbial fermentation of XOS result in a lowered pH of the intestine, which leads to increased mineral solubility and absorption (Tuohy et al., 2005). In addition, xylo-oligosaccharide as

prebiotics, reportedly increase the expression of Ca-binding proteins (Stefanello et al., 2014). Zhou et al. (2021a) demonstrated that XOS supplementation might be associated with greater egg yolk color intensity, which is consistent with the findings of the present study. One explanation for this could be that XOS regulates lipid metabolism and thus affects the absorption and deposition of carotenoids in egg yolks (Li et al., 2017; Ooi and Liong, 2010).

Intestinal morphology is an important indicator of intestinal health and affects animal performance. Ding et al. (2018) reported that supplementing the diet of laying hens with XOS increases the villus height and the V/C in the jejunum. Another study reported that XOS significantly improved the intestinal morphology of both broilers and laying hens (Min et al., 2016; Zhou et al., 2021b). These findings were consistent with the results of the present study, in which dietary supplementation with XOS led to an increase in jejunal villus height and V/C. Moreover, improvement in the ileal V/C was observed. These results were attributed to the fermentation of XOS. XOS supplementation reportedly promotes butyrate production by butyrate-producing bacteria (Scott et al., 2014; Zhang et al., 2022). In our study, the butyric acid content dramatically increased with XOS supplementation, and the butyric acid content was highest in the XOS2 group (200 mg/kg). Butyrate may be used as an energy source in epithelial cells and could stimulate the growth of villi and improve intestinal morphology (Guilloteau, et al., 2010). In addition, butyrate promoted the proliferation of intestinal epithelial cells by increasing p-mTOR expression. It can also improve intestinal morphology by inducing the expression of glucagon-like peptide-2 (GLP-2) (Hu et al., 2010; Zeng et al., 2022).

Since animals are unable to directly digest XOS, they mainly ferment XOS through intestinal bacteria. The acetate and lactate produced by *Bifidobacteria* and *Lactobacillus* fermentation are further utilized by other microorganisms to form the end products propionate and butyrate. Therefore, short-chain fatty acids are the major products of intestinal bacterial metabolism (Hosseini et al., 2011; Koh et al., 2016; Rivière et al., 2016). In the present study, dietary XOS supplementation increased the levels of isovaleric acid, acetic acid, propionic acid, and butyric acid. Similarly, previous studies have demonstrated that consuming XOS increased the levels of short-chain fatty acids in the cecum (De Maesschalck et al., 2015; Pourabedin et al., 2015). Short-chain fatty acids are reportedly involved in energy and nutrition absorption, which could also explain the improved production performance observed in the present study (Schönfeld and Wojtczak, 2016; Zhou, et al., 2021b).

Studies have reported that XOS modulates the gut microbiome of animals (De Maesschalck et al., 2015; Ebersbach et al., 2012; Pan et al., 2009; Wang et al., 2023). The cecum provides the most comprehensive information on the microbiota of chicken stomachs. In addition, the cecum is a crucial region for the fermentation of indigestible carbohydrates by bacteria and a major location of pathogen colonization (Pourabedin and Zhao, 2015). In the present study, the cecal microbiota was analyzed to explore the effect of XOS on the intestinal microbiota of aged laying hens. The results of the α-diversity and β-diversity analyses indicated that XOS altered the organization of the microbiota and increased microbial richness. Greater diversity in the digestive tract microbial community is thought to benefit the well-being and productivity of the host bird (Janczyk et al., 2009). In addition, the gut microbial ecosystem changed at the phylum level upon XOS supplementation. Similar to the findings of a previous study, the XOS groups presented a reduced abundance of *Bacteroidetes* and a greater relative abundance of *Firmicutes*, especially the XOS2 group (200 mg/kg) (Zhou et al., 2021b). Videnska et al. (2014) reported that during egg production, a gradual increase in *Bacteroidetes* occurs at the expense of *Firmicutes* in aged laying hens, causing the *Bacteroidetes* to account for two-thirds of the total. However, the preferred substrate used in intestinal cells, butyric acid, is produced mainly by members of *Firmicutes* (Ahmad et al., 2000; Roediger, 1982). The ratio of *Firmicutes* to *Bacteroidetes* reportedly affects the energy harvest and weight gain, and the abundance of *Firmicutes* has been demonstrated to be positively correlated with energy and nutrient absorption, whereas an increase in the abundance of fecal *Bacteroidetes* is linked to insufficient nutrient digestibility. (Bäckhed et al., 2004; Jumpertz et al., 2011; Singh et al., 2013; Turnbaugh et al., 2006). Therefore, an increased abundance of *Firmicutes* and a decreased abundance of *Bacteroidetes* could contribute to the absorption of nutrients in aged laying hens, which ultimately results in improved production performance.

Differences between the CON and XOS groups were also noted at other taxonomic levels. At the genus level, the XOS groups presented a lower abundance of *Bacteroides* and *Rikenellaceae*_RC9_gut_group and a greater abundance of *Lactobacillus*. A further analysis revealed that the abundance of *Bifidobacteriaceae* in the XOS group, particularly the XOS2 group was greater than that in the CON group, which is consistent with the findings of Zhou et al. (2021b). Pourabedin et al. (2015) reported that the relative abundance of the *Lactobacillus* genus in the broiler chick cecal microbiome increased with a diet containing 2 g XOS/kg. Ding et al. (2018) reported that XOS-supplemented diets could increase the number of *Bifidobacteria* in the cecum and decrease *Escherichia coli* enumeration. As predominant members of the gut microbiota, *Lactobacillus* and *Bifidobacteria* competitively inhibit pathogenic bacteria through antagonistic activities (Servin, 2010; Yang et al., 2019). The accumulation of acetic acid and lactic acid produced by *Bifidobacterium* and *Lactobacillus* reduces the intestinal pH, which is not conducive to pathogen colonization, whereas the populations of butyrate-producing bacteria and butyrate production might increase at a lower pH (Walker

et al., 2005). For example, in our trial, the XOS2 (200 mg/kg) group presented the highest abundance of *Bifidobacteriaceae* and the highest concentration of butyric acid in the cecum.

In terms of alterations in the cecal microbiota, the Pearson correlation analysis revealed that the genera *Prevotellacae* UCG-001, uncultured_*Muribaculaceae*, [*Eubacterium*] coprostanoligenes group, *Shuttleworthia, Lactobacillus*, and (*Ruminococcus*) torques group exhibited markedly positive correlations with jejunal morphology and levels of cecal short-chain fatty acid, whereas the genera uncultured_*Rikenellace, Bacteroides, Faecalibacterium, Rikenellaceae*_RC9_gut_group, and *Parabacteroides* presented markedly negative correlations with jejunal morphology and levels of cecal short-chain fatty acid, further corroborating that dietary XOS supplementation mediates intestinal functions by targeting the gut microbiota. As observed in our experiment, the XOS group had a greater abundance of *Bacteroides* and *Lactobacillus* and a lower abundance of *Firmicutes* and *Rikenellaceae*_RC9_gut_group. Compared with the CON group, the intestinal morphology was improved in the XOS group, and the levels of cecum short-chain fatty acid were greater than those in the CON group. Zhou et al. (2021b) reported that the abundance of *Lactobacillus* was positively correlated with the ileal villus height and villus height-to-crypt depth ratio. Pourabedin et al. (2015) reported that the relative abundance of the *Lactobacillus* genus in the cecum was positively related to cecal acetate production, although a positive correlation was noted between the relative abundance of ileal *Propionibacterium* and cecal propionate concentrations. The similarities and differences noted between the above findings and the results of the present study imply that specific conditions might affect the microbial alterations caused by dietary interventions (Dethlefsen and Relman, 2011)

CONCLUSIONS

In summary, this study provides evidence that incorporating XOS into the diet can increase the production performance and egg quality of laying hens. It improved the intestinal morphology and increased the content of short-chain fatty acids in the cecum of laying hens, regulated the structure of the intestinal microflora, and promotes increased diversity of the intestinal microbial population. The recommended level of XOS in the diet of laying hens is 200 mg/kg.

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DISCLOSURES

All authors declare that they have no conflicts of interest.

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