BMC Microbiology

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The single degree of polymerization influences the efficacy of xylooligosaccharides in shaping microbial and metabolite profiles in chicken gut to combat avian pathogenic *Escherichia coli*

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Abstract

Background Avian pathogenic *Escherichia coli* (APEC) threatens both poultry production and human health. Xylooligosaccharides (XOS) may suppress pathogenic bacteria through prebiotic actions. However, the influences of single degree of polymerization (DP) on the inhibition of APEC by XOS remain unknown. This study aimed to probe if XOS and their major monomers (xylobiose, xylotriose and xylotetraose) could differentially combat APEC via prebiotic actions using an in vitro fermentation model with chicken cecal microbiota.

Methods Microbiota were randomly divided into 7 groups (5 replicate tubes/group). Control group (CON) received no treatment; XOS group received commercial XOS mixtures; APEC group received APEC; XA, X2, X3 and X4 groups received APEC combined with commercial XOS mixtures, xylobiose, xylotriose and xylotetraose, respectively.

Results XOS and their major monomers mitigated APEC-induced decline ($p < 0.05$) in gut microbial α-diversity, with xylotetrose showing the least effect. Gut microbiota in XA, X2, X3 and X4 groups clustered together, with a relative separation observed in X4 group. XOS and their monomers elevated ($p < 0.05$) the abundances of *Firmicutes*, *Bacteroidota* and several probiotics (*Lactobacillus*, *Bacteroides* and *Megamonas*), but reduced ($p < 0.05$) the abundances of *Proteobacteria* and *Escherichia-Shigella*, with xylotetraose exhibiting the least efficacy. Besides, xylotriose and xylotetrose had an advantage over xylotetraose in promoting microbial production of short-chain fatty acids. Metabolomics analysis revealed that APEC challenge mainly downregulated ($p < 0.05$) several amino acids metabolism pathways of gut microbiota, while xylotriose had an inferiority to XOS in upregulating ($p < 0.05$) histidine metabolism

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pathway. Furthermore, microbial fermentation metabolites of all XOS monomers lowered ($p < 0.05$) certain virulence genes expression in APEC, with xylotriose being the most advantageous.

Conclusions XOS and their major monomers differentially improved gut microbiota and metabolite profiles in chicken gut against APEC challenge. Overall, xylotriose exhibited the greatest inhibition against APEC abundance and virulence. Our findings underscore the role of single DP in influencing the prebiotic actions of XOS against APEC, providing a basis for the reasonable application of XOS in diets to combat bacterial challenge.

Keywords Xylooligosaccharide, Xylobiose, Xylotriose, Xylotetrose, Polymerization degree, Gut microbiota, Avian pathogenic *Escherichia coli*, Virulence

Background

As the most prevalent pathogen in poultry, avian pathogenic *Escherichia coli* (APEC) mainly inhabits the intestine and opportunistically causes colibacillosis [1], leading to huge economic losses in poultry production. Furthermore, APEC-colonized poultry can serve as the reservoirs for the spread of drug-resistant plasmids and virulence factors to other pathogenic microbes that may endanger human health [2]. Particularly, APEC represents a potential zoonotic pathogen based on its genetic similarities with specific pathogenic *Escherichia coli* infecting humans [1, 2]. The side effects such as the increasing antibiotic resistance and antibiotic residuals underscore a pressing necessity for the development of sustainable countermeasures to combat APEC.

Our previous study has revealed that the usage of prebiotics such as xylooligosaccharides (XOS) represents a promising strategy to limit intestinal APEC infection in chickens [3, 4]. XOS can be mass-produced from xylans that spread widely in the cell walls of various plants including agricultural byproducts like corncobs, which make it easily accessible and cost-effective for the application in animal production. Commercial XOS are usually composed of β-1,4-linked xylopyranosyl units with degree of polymerization (DP) ranging from 2 to 7, particularly xylobiose (DP = 2), xylotriose (DP = 3), and xylotetraose (DP = 4), which may display different efficacy in their beneficial actions. For example, it was reported that *in ovo* feeding of xylobiose had some advantages over xylotriose in improving gut microbiota of broiler chicks [5]. However, it was also found that *in ovo* feeding of xylobiose was inferior to xylotriose in improving intestinal structure and growth performance of broilers [6]. Within the intestine, *Bifidobacterium* and *Lactobacillus* are the conventional XOS-enriched bacteria with abilities to resist harmful bacteria in gut [7, 8]. The capacity of these probiotics to metabolize XOS is generally associated with the presence of enzymes capable of hydrolyzing specific XOS monomers [9]. Besides, other microbes such as *Bacteroides* possessing polysaccharide utilization loci also benefit XOS degradation [10]. Although DP has been indicated to play an important role in affecting the actions of prebiotics [11], an understanding of how the single DP affects the prebiotic actions of XOS against bacterial challenge remains unknown.

It is well-known that intestinal bacteria can opportunistically translocate from the gut lumen to other internal organs, resulting in local and systemic tissue damages of poultry [1]. In these processes, the virulence factors exert crucial roles due to their maintenance of APEC activity inside the body with a subsequent establishment of its infection and induction of inflammation of hosts [3, 4]. Improving gut microbiota is an approach to inhibit the colonization and virulence of pathogenic bacteria without causing development of bacterial resistance [12]. It has been indicated that certain probiotics *Limosilactobacillus*, *Megamonas* and *Bacteroides* that can be enriched by XOS protected animals against enteric pathogens [13, 14]. XOS-induced alterations in gut microbiota may change the profile of gut metabolites such as short-chain fatty acids (SCFA), which exert prominent roles in mediating the inhibitory effects of prebiotics against gut pathogens including pathogenic *E. coli* [15, 16]. Nevertheless, it is unclear whether the microbial metabolites produced from XOS can repress APEC virulence, and the extent to which the single DP contributes to this effect is not well understood.

Comprehensively, this study aimed to compare the protective effects of XOS and their major monomers (xylobiose, xylotriose and xylotetraose) on microbial composition and metabolite profiles in chicken gut under APEC challenge using an in vitro fermentation model, which has been employed to conveniently evaluate the microecology-regulating benefits of functional carbohydrates for hosts [17], followed by investigation of the effects of the above fermentation metabolites on the virulence of APEC. The findings of this study would provide a basis for the reasonable application of XOS in chicken diets to combat bacterial challenge.

Methods

Chemicals

Commercial XOS mixtures (> 95% purity; Longlive Biotech., Dezhou, China) contained 40% xylobiose, 33% xylotriose, 12% xylotetraose, 5% xylotentaose together with 5.5% xylhexaose and xylheptaose. The monomers

xylobiose (Macklin Biotech., Shanghai, China) along with xylotriose and xylotetraose (Zzstandard Biotech., Shanghai, China) had a purity higher than 98%. Other reagents were analytically pure or met the experimental requirements.

Cecal digesta collection and Preparation of fermented seed solution

The in vitro fermentation model of cecal digesta was developed according to a previous study [11]. Ten seven-day-old yellow-feathered (Mahuang) male broilers receiving a diet free of antibiotics and probiotics were housed on a flat floor with rice husk bedding. These broilers were euthanized by anesthesia via wing vein injection of pentobarbital sodium at 50 mg/kg of body weight, in order to avoid the violent struggle of broilers during bleeding that might affect the amount or composition of intestinal contents. Thereafter, the cecum of the above broilers was immediately removed and the contents were extracted in an aseptical environment. A pre-deoxidized sterile phosphate buffer saline-based diluent (including NaCl 8.0 g/L, KCl 0.2 g/L, Na_2HPO_4 1.15 g/L, KH_2PO_4 0.2 g/L and L-cysteine 0.5 g/L, pH 6.5) was used to prepare a 20% (w/v) suspension. The mixtures were vortexed and filtered through two layers of gauze to act as the seed solution for anaerobic fermentation.

In vitro anaerobic fermentation model of cecal digesta and experimental design

The basal nutrient medium was prepared according to a previous study [18] and sterilized using 0.22-μm filters, followed by an exposure to anaerobic gas to eliminate oxygen. The seed solution was mixed with the basal medium at a ratio of 1:9 (v/v) and distributed into Hengate tubes sealed with butyl rubber stoppers (5 replicate tubes/group). The experimental design is shown in Fig. 1. Control group (CON) received no treatments, XOS group received 4 mg/mL XOS, APEC group received 4×10^9 CFU of APEC (O78 strain, CVCC1570, China Center for Type Culture Collection), while XA, X2, X3 and X4 groups received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively. Anaerobic gas was passed through for 10 min to ensure deoxygenation. All Hengate tubes

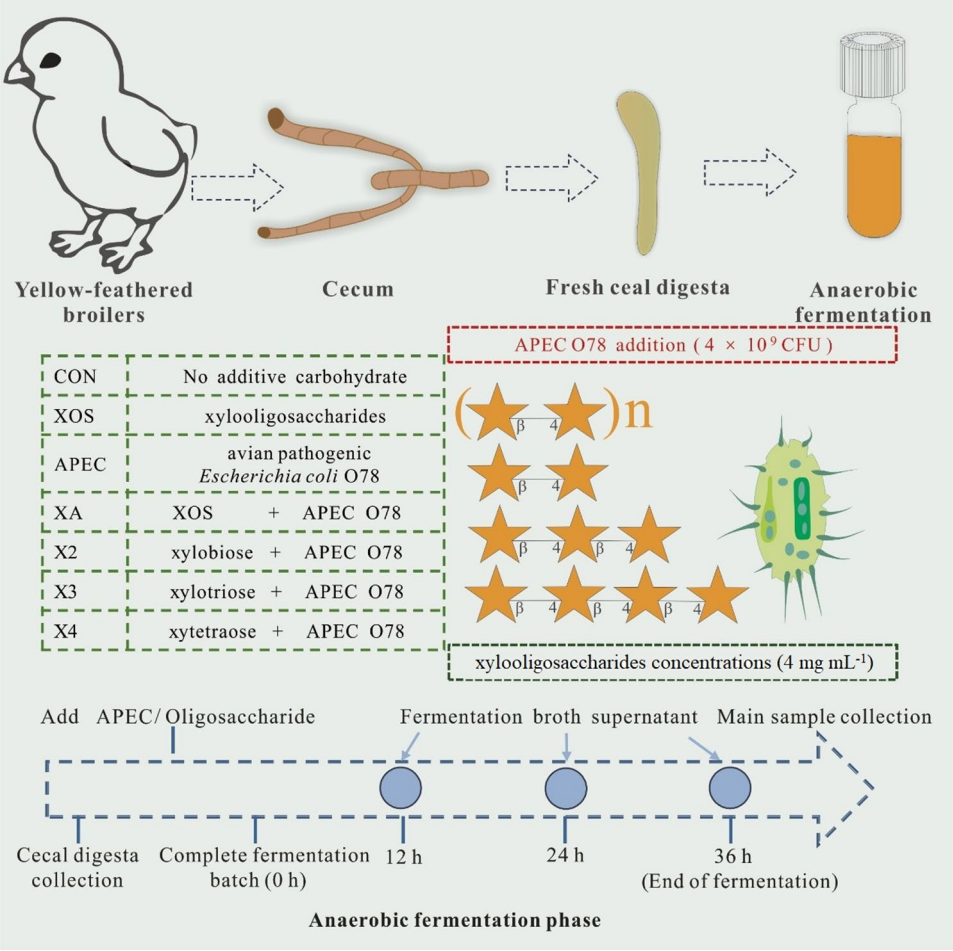

Fig. 1 Experimental design of in vitro anaerobic fermentation model. XOS, xylooligosaccharides; APEC, avian pathogenic *Escherichia coli*

were placed with anaerobic fermentation bags and incubated at 39 °C for 36 h, during which the fermentation supernatant was taken at 12 h intervals to quantify SCFA. At the end of fermentation, liquid nitrogen was used to halt fermentation.

Gut microbiota analysis

Gut microbiota analysis was conducted according to a previous study [19]. Firstly, bacterial DNA in the cecal fermentation system was extracted using the E.Z.N.A.®Stool DNA Kit (Omega, Norcross, USA). The quality and concentration of DNA were assessed via agarose gel electrophoresis and using an UV-spectrophotometer, respectively. Bacterial 16 S rRNA sequences spanning the variable regions were amplified using the primers 341 F (5′- CCTACGGGNGGCWGCAG-3′) and 805R (5′- GACTACHVGGGTATCTAATCC-3′). The polymerase chain reaction (PCR) products were verified by agarose gel electrophoresis and purified with AMPure XT beads (Beckman Coulter Genomics, Danvers, USA). The amplicon pools were prepared for sequencing, and the size and quantity of the amplicon libraries were analyzed using the Agilent 2100 Bioanalyzer (Agilent, Santa Clara, USA). Finally, bipartite sequencing was conducted on a NovaSeq PE250 sequencer (Illumina, Woburn, USA).

Table 1 Primer informations used for quantitative real-time PCR

Genes[1]	Primer sequences (5′-3′)	Product size (bp)
rpoA	F: GCACCAAAGAAGGCGTTCAG	139
	R: ATATCGGCTGCAGTCACAGG	
tnaA	F: TGTACACCGAGTGCAGAACC	105
	R: CGTCATACAGACCTACCGCC	
glnA	F: TCCGTTGCGACATCCTTGAA	118
	R: AACAGTACGGTGTCGGCAAT	
relA	F: GTTCGCCGGATGTTATTGGC	100
	R: CCGGCGCATCTTTTACTTCG	
entF	F: CGGACTAGCGCAAGCAGATA	123
	R: ATGTTGGTTCGCAGGTCGAT	
yddA	F: ACCGTCGTGCTCATTGTGAT	104
	R: CGAAATGTCGCTTCGCTACG	
fimH	F: GATGTTTCTGCTCGTGATG	261
	R: TACCGCCGAAGTCCCT	
csgD	F: ACTGGCCTCATATCAACGGC	98
	R: CGTAAAGTAGCATTCGCCGC	
luxS	F: TTGGTACGCCAGATGAGCAG	113
	R: GCCACACTGGTAGACGTTCA	
ompR	F: GCGTCGCTAATGCAGAACAG	142
	R: ATGATCGGCATCGGATTGCT	

[1]*rpoA*, DNA-directed RNA polymerase subunit alpha-encoding gene; *tnaA*, tryptophanase-encoding gene; *glnA*, glutamine synthetase-encoding gene; *relA*, (p)ppGpp synthetase gene; *entF*, enterobactin synthase component F-encoding gene; *yddA*, ABC transporter ATP-binding protein YddA-encoding gene; *fimH*, fimbria H-encoding gene; *csgD*, curli subunit gene D; *luxS*, S-ribosylhomocysteine lyase-encoding gene; *ompR*, DNA-binding dual transcriptional regulator OmpR-encoding gene

Quantification of SCFA in the fermentation supernatant

The fermentation broth from each group was centrifuged (12,000 rpm, 4 °C) for 10 min, followed by transferring of 0.5 mL supernatant to a tube added with 1.25 mL ultrapure water. The mixture was blended and centrifuged (10,000 rpm, 4 °C) for 10 min. The resulting supernatant (1 mL) was mixed with 0.2 mL of 25% (v/v) metaphosphoric acid solution containing 2 g/L of 2-ethylbutyric acid. After centrifugation (10,000 rpm, 4 °C) for 10 min, the supernatants were collected at different times during fermentation and filtered with filters. SCFA in the filtrate were quantified using a Shimadzu GC-17 A gas chromatograph (Kyoto, Japan) following the internal standard method [20].

Metabolomic analysis of the fermentation supernatant

A total of 100 µL of the fermentation broth was mixed with 400 µL of the extraction solution composed of methanol and acetonitrile at a ratio of 1:1 (v/v). The mixtures were blended and sonicated for 10 min and centrifuged (4 °C, 10,000 rpm) for 15 min. The supernatants were collected and transferred to an injection bottle for analysis using an Vanquish Ultra-high Performance Liquid Chromatograph (Thermo Fisher Scientific, Waltham, USA) according to a previous study [21]. Afterwards, the target compounds were separated using a Waters ACQUITY UPLC BEH Amide liquid chromatographic column (2.1 mm × 50 mm, 1.7 µm) and characterized using secondary spectra. The differential metabolites were identified based on the orthogonal partial least squares discriminant analysis (OPLS-DA) model with the following criteria:|fold change| > 2, variable importance of projection > 1, and $P < 0.05$.

Detection of the effect of fermentation supernatants on genes expression of APEC

At the end of fermentation, the fermentation supernatant was harvested and filtered with filters. The filtrate was separately mixed with Luria-Bertani (LB) medium at a ratio of 1:14 (v/v). APEC grown on LB agars was transferred to LB liquid medium and incubated overnight (37 °C, 200 rpm), followed by incubation (37 °C, 200 rpm) with fermentation supernatant-containing LB medium at a ratio of 1:50 (v/v) for 6 h. After centrifugation (12,000 rpm,10 min), bacterial precipitates were harvested and the RNA samples were extracted using the Bacterial RNA Extraction Kit (Vazyme, Nanjing, China). After evaluation of the quality and concentration, the extracted DNA was reverse-transcribed into complementary DNA samples. For determining genes expression, we used the quantitative real time-PCR (QuantStudio 3, Applied Biosystems, USA) with the designed primers (Table 1). The heating protocol as follow: 94 °C for 5 min, then proceeded to the amplification cycle, 94 °C for

30 s, 60 °C for 30 s, 72 °C for 1 min to complete a cycle, repeated 40 times, finally, 72 °C for 5 min. The relative mRNA expression was calculated by the $2^{-\Delta\Delta Ct}$ method [3].

Statistical analysis

The data are presented as mean ± standard error and analyzed using the one-way ANOVA of SPSS 26.0. Differences among groups were identified by the Tukey's tests, while $p < 0.05$ was considered statistically significant.

Results

XOS with different DP differentially affect the diversity of gut microbiota with APEC challenge

As shown in Fig. 2A, the Pielou evenness index in XOS group was lower ($p < 0.05$) than that of CON group but higher ($p < 0.05$) than APEC group. Both XA, X2, X3 and X4 groups had higher ($p < 0.05$) Pielou evenness index than APEC group. Compared with CON group, both XOS and APEC groups showed a reduction ($p < 0.05$) in the community richness index (Chao 1) (Fig. 2B), which

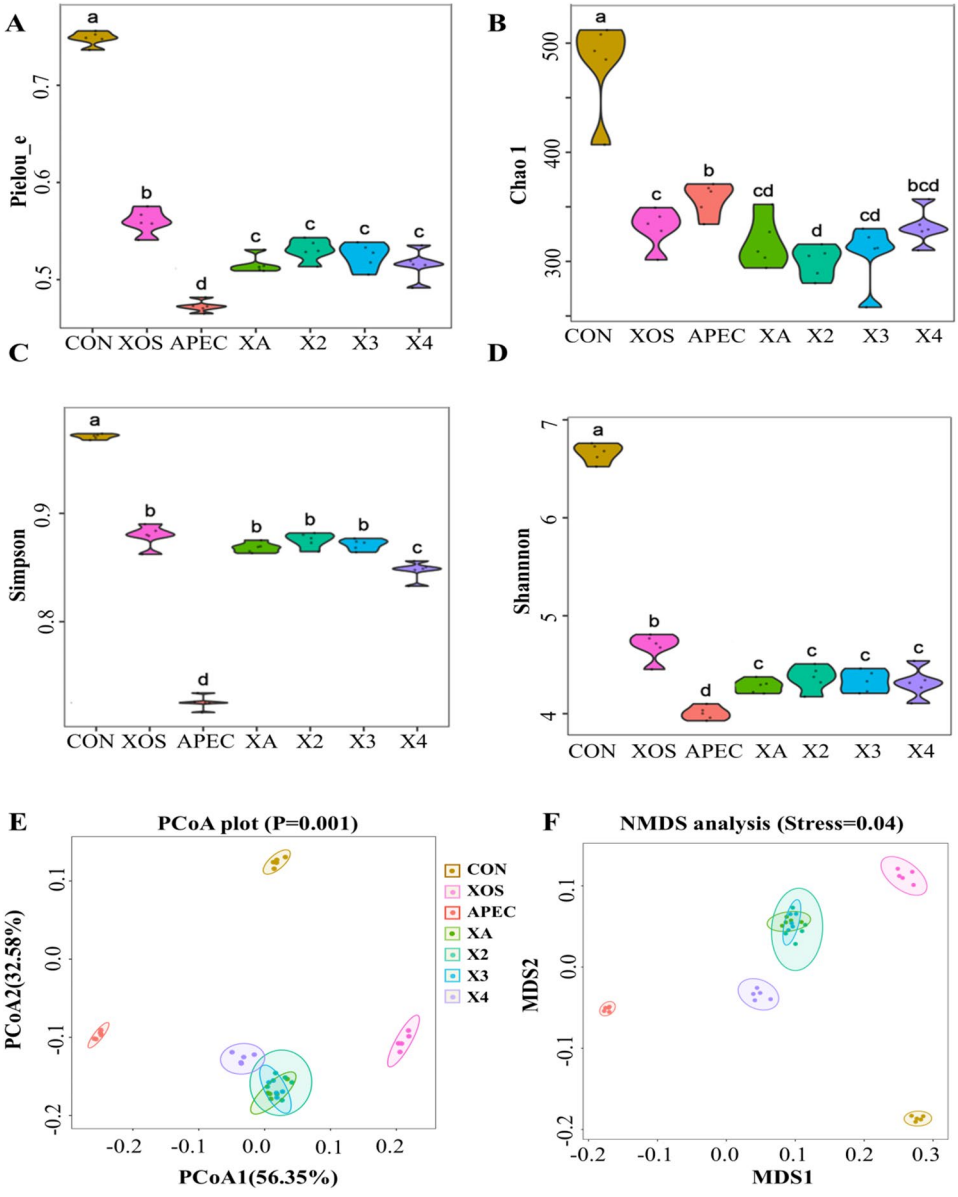

Fig. 2 The α- and β-diversity analysis of gut microbiota-treated by xylooligosaccharides (XOS) and their monomers with different degrees of polymerization. (A ~ D) The Pielou eveness index, Chao 1 index, Simpson index, and Shannon index, respectively; (E ~ F) Clustering analysis of gut microbiota using the Principal coordinates analysis (PCoA) and weighted UniFrac non-metric multidimensional scaling (NMDS), respectively. Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

was also found to be lower ($p < 0.05$) in XA, X2 and X3 groups relative to APEC group. The diversity indexes (Simpson and Shannon) in all XOS or monomers-treated groups were higher ($p < 0.05$) than that of APEC group but lower ($p < 0.05$) than CON group (Fig. 2C and D), with X4 group showing the least effect. There were differences ($p < 0.05$) in gut microbial β-diversity among groups (Fig. 2E and F). Microbial communities in XA, X2, X3, and X4 groups clustered together, with a relative separation of X4 group from other three groups. However, microbial communities of the above four groups showed a distinct separation from both CON, XOS and APEC groups.

XOS with different DP differentially shape the distribution of gut microbiota with APEC challenge

Firmicutes was the most abundant phylum in all groups except in APEC group (Fig. 3A), where *Proteobacteria* was the most prevalent phylum. *Proteobacteria* abundance was obviously elevated in APEC-containing groups (APEC, XA, X2, X3, and X4 groups) versus APEC-free groups (CON and XOS groups). However, addition of either XOS or their monomers reduced *Proteobacteria* abundance compared to APEC group. The bacterial dominance pattern at the genus level was extremely different between APEC-free groups and APEC-containing groups (Fig. 3B). Within APEC-free groups, the predominant genera were several probiotics such as *Limosilactobacillus*, *Bacteroides*, and *Megamonas*. Conversely, within APEC-containing groups, the most abundant genera was *Escherichia-Shigella*, followed by *Limosilactobacillus*, *Bacteroides*, and *Megamonas*. Remarkably, the distribution of genera in either XOS or APEC group was not as even as that in CON group, while both XA, X2, X3 and X4 groups showed a more even distribution of genera relative to APEC group.

The differences in gut microbial composition among groups

As depicted in Fig. 4A and B, APEC challenge decreased ($p < 0.05$) the abundances of the phyla *Firmicutes*, *Bacteroidota* and *Actinobacteriota* along with the genera *Lactobacillus*, and *Bacteroides*, but increased ($p < 0.05$) the abundances of the phylum Proteobacteria and genus *Escherichia-Shigella*, when compared to the CON group. Both XOS and their monomers treated-groups had a higher ($p < 0.05$) abundance of *Lactobacillus* while only X4 group had a higher ($p < 0.05$) abundance of *Bifidobacterium* than APEC group. Compared to APEC group, *Limosilactobacillus* abundance was higher ($p < 0.05$) in XA, X2 and X3 groups rather than in X4 group, while the abundances of *Bacteroides* and *Megamonas* were higher ($p < 0.05$) in both XOS and their monomers-treated groups, with X4 group being the most effective ($P < 0.05$). Treatment with either XOS or their monomers sharply elevated ($p < 0.05$) *Megamonas* abundance from a low

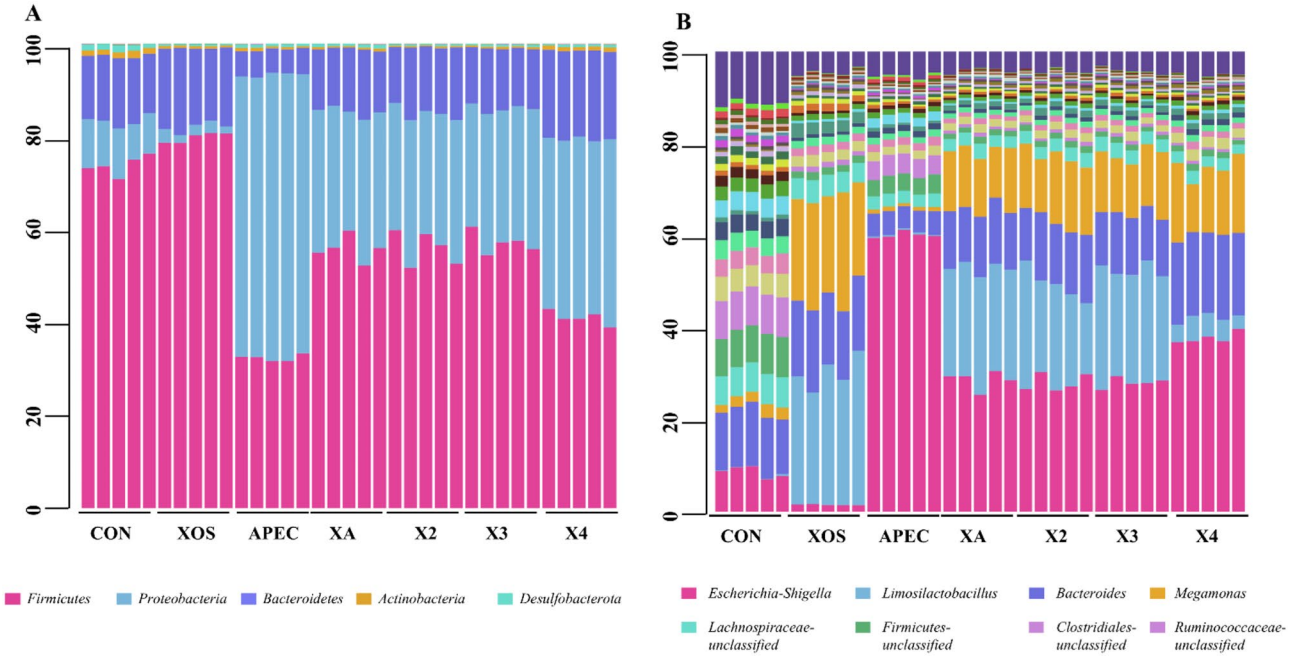

Fig. 3 Distributions at the phylum (**A**) and genus (**B**) levels of gut microbiota treated by xylooligosaccharides (XOS) and their monomers with different degrees of polymerization. Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

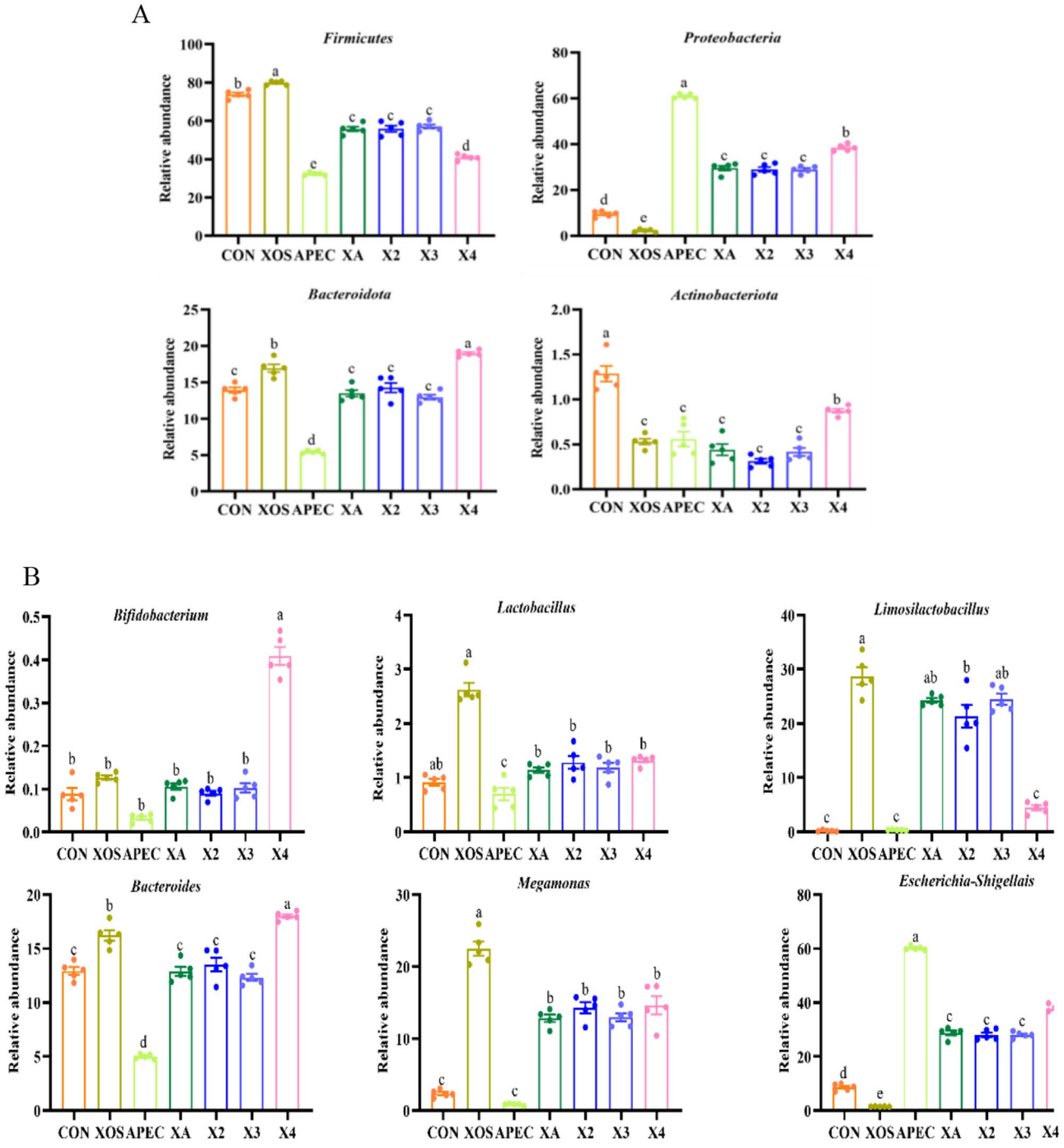

Fig. 4 The differences in bacterial members at the phylum (**A**) and genus (**B**) levels in gut microbiota treated by xylooligosaccharides (XOS) and their monomers with different degrees of polymerization. Different letters represent significant differences ($p < 0.05$) among groups. Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

baseline (Fig. 4B). Conversely, either XOS or their monomers-treated groups showed a lower ($p < 0.05$) *Escherichia-Shigellas* abundance than that in APEC group, with xylotetraose (X4 group) exhibiting the least effect ($p < 0.05$).

The core functional bacteria in gut microbiota enriched in different groups

The linear discriminant analysis (LDA) combined effect size measurements (LEfSe) analysis was employed to identify the biomarkers ($p < 0.05$, LDA score > 4.0)

among groups. As illustrated in Fig. 5, the core bacteria *Firmicutes*, *Clostridium* and *Oscillospirales*, the typical commensal microbes in broiler gut [20, 22], were enriched in CON group. Conversely, APEC group was differentially enriched with *Proteobacteria*, *Enterobacteriaceae* and *Escherichia-Shigella* that ought to derive from APEC inoculation. Several core beneficial bacteria such as *Firmicutes*, *Bacilli*, *Lactobacillales*, *Lactobacillaceae*, *Limosilactobacillus* and *Megamonas* were enriched in XOS group. The core bacteria enriched in XA, X2 and X3 groups were similar to those in XOS group, but their LDA scores in enrichment analysis were less than 4.0 (data not shown). Differently, X4 group was enriched with *Bacteroidota* and its affiliated members (e.g. *Bacteroides* and *Bacteroides uniformis*).

The correlations among gut microbes

As illustrated in Fig. 6, there were complicated relationships among different gut microbes. Notably, *Escherichia-Shigella* showed negative correlations ($p < 0.05$) with plentiful beneficial bacteria including XOS-enriched bacteria (*Limosilactobacillus*, *Megamonas*, *Lactobacillus*) along with several butyric acid-producing bacteria such as *Oscillibacter*, *Subdoligranulum*, *Blautia*, *Ruminococcus torques*, *Butyricococcus* and *Eubacterium hallii* [23, 24], while there were positive correlations ($p < 0.05$) among the majority of the above beneficial bacteria.

Dynamic changes of SCFA in fermentation supernatant of gut microbiota in different groups

Metabolites are the mediators in the interactions among intestinal probiotics, pathogens and hosts [24, 25]. SCFA serve as crucial metabolites produced from microbial fermentation of prebiotics. As indicated in Table 2, at 12 h during fermentation, no differences ($p > 0.05$) were noted in SCFA concentrations between CON and APEC groups. However, the groups receiving XOS or their

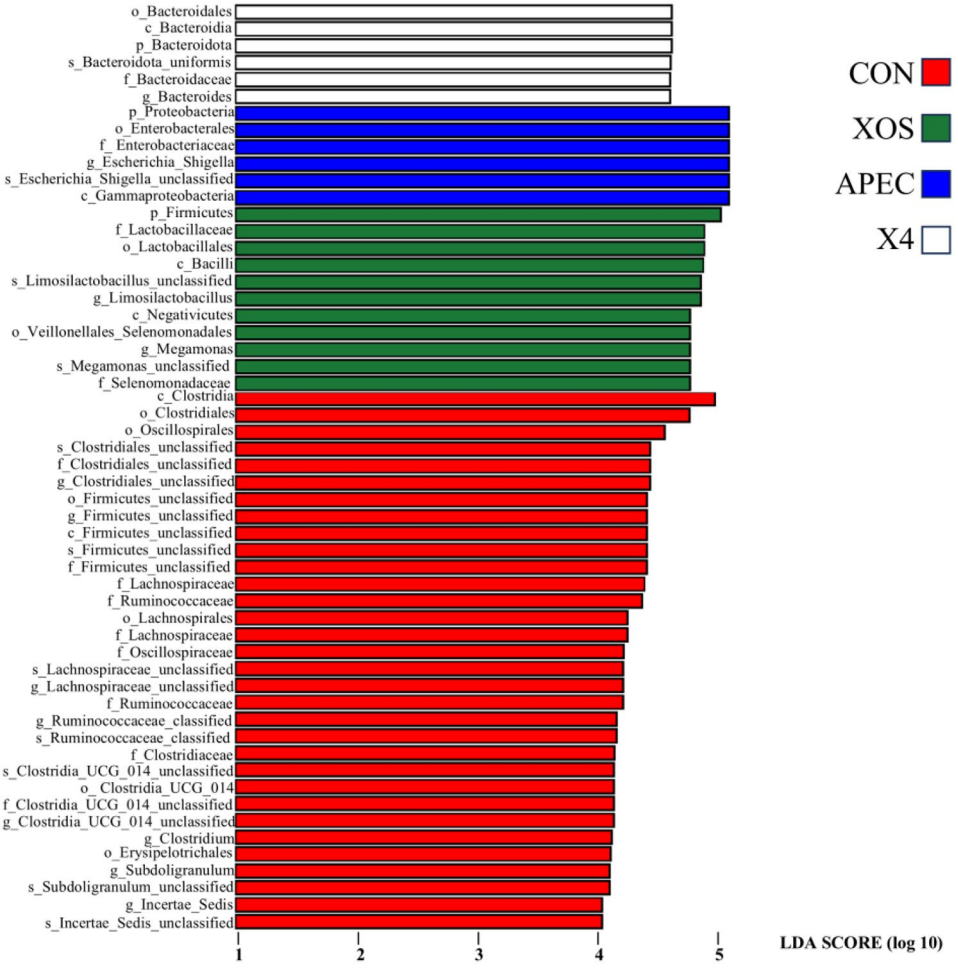

Fig. 5 Linear discriminant analysis (LDA) combined effect size measurements (LEfSe) analysis of the core functional microbes enriched ($p < 0.05$, LDA > 4.0) by xylooligosaccharides (XOS) and and their monomers with different degrees of polymerization. Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

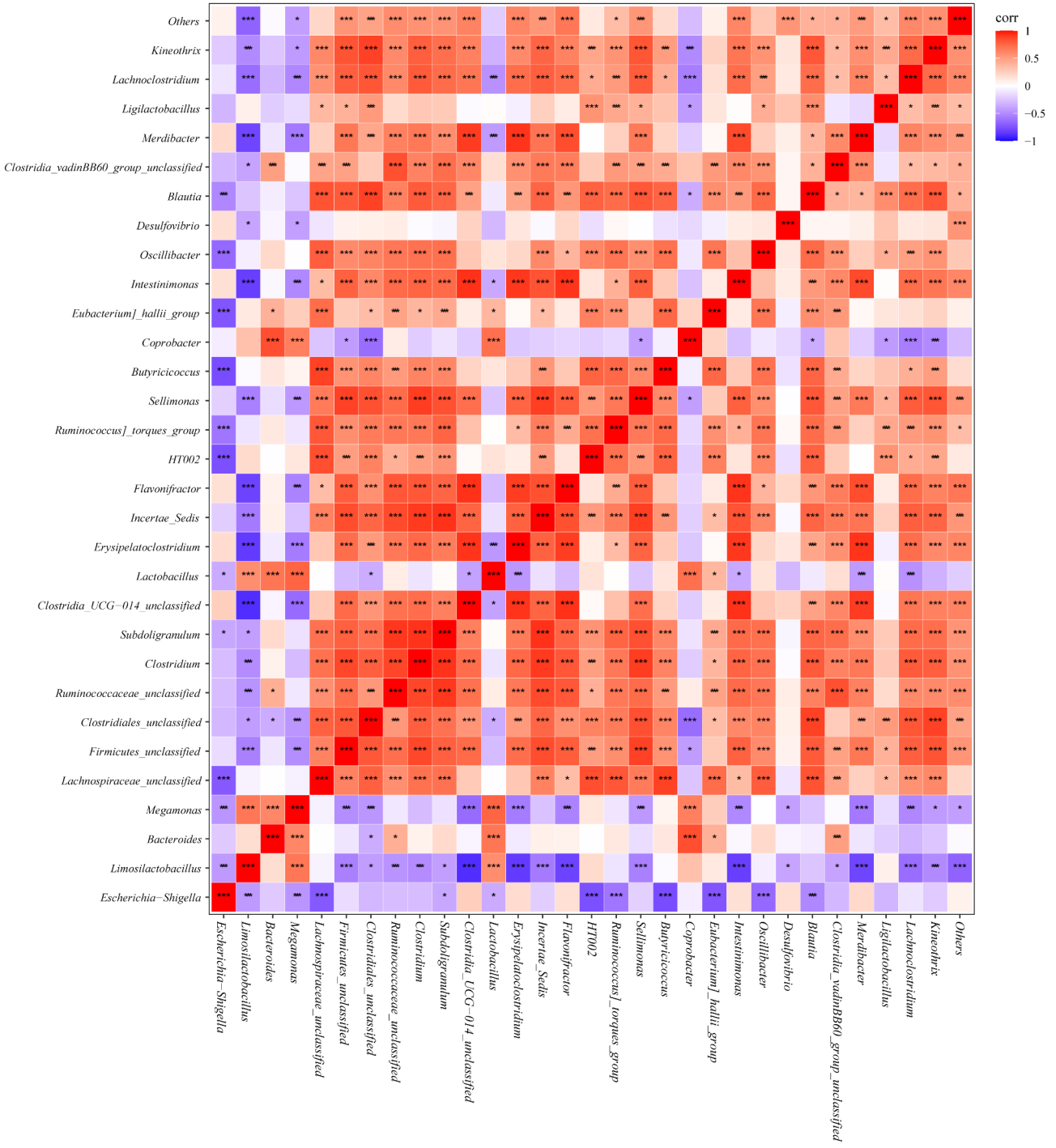

Fig. 6 Correlation analysis among gut microbes. The red and orange blocks indicate the positive correlations between microbes, while the blue blocks indicate negative correlations between microbes, with darker colors representing stronger correlations. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$

monomers had higher ($p < 0.05$) concentrations of acetic, propionic, butyric and valeric acids, along with lower ($p < 0.05$) concentrations of isobutyric and isovaleric acids than APEC group, with X2, X3 and X4 groups being more effective ($p < 0.05$) than XA or XOS group in increasing the concentrations of propionic, isovaleric and valeric acids. Notably, valeric acid concentration was the highest ($p < 0.05$) in X4 group. At 24 and 36 h during fermentation, APEC group only showed a reduction ($p < 0.05$) in butyric acid concentration versus CON group. However, the groups receiving XOS or their monomers had higher ($p < 0.05$) concentrations of acetic and propionic acids than those in APEC group, with X3 and X4 groups being

Table 2 Effects of xylooligosaccharides (XOS) and their monomers with different degrees of polymerization[1] on short-chain fatty acids production in the fermentation broth of gut microbiota at different time-points during fermentation

	Acetic acid (mmol/L)	Propionic acid (mmol/L)	Butyric acid (mmol/L)	Isobutyric acid (mmol/L)	Valeric acid (mmol/L)	Isovaleric acid (mmol/L)
12 h						
CON	27.55 ± 1.75^{b}	5.20 ± 0.20^{c}	9.30 ± 0.55	0.17 ± 0.02^{a}	0.11 ± 0.01^{c}	0.48 ± 0.04^{a}
XOS	60.44 ± 4.81^{a}	17.44 ± 0.8^{b}	5.45 ± 1.53	0.04 ± 0.01^{b}	0.11 ± 0.01^{c}	0.11 ± 0.01^{c}
APEC	23.39 ± 0.50^{b}	4.24 ± 0.07^{c}	8.22 ± 0.17	0.21 ± 0.00^{a}	0.09 ± 0.00^{c}	0.63 ± 0.02^{a}
XA	58.23 ± 4.30^{a}	19.31 ± 0.5^{b}	6.38 ± 0.32	0.06 ± 0.01^{b}	0.12 ± 0.00^{c}	0.09 ± 0.02^{c}
X2	55.76 ± 3.07^{a}	27.91 ± 0.96^{a}	6.88 ± 0.12	0.05 ± 0.00^{b}	0.17 ± 0.01^{b}	0.24 ± 0.03^{b}
X3	60.99 ± 2.74^{a}	25.53 ± 1.07^{a}	10.01 ± 3.17	0.07 ± 0.02^{b}	0.15 ± 0.00^{b}	0.16 ± 0.01^{b}
X4	57.73 ± 16.95^{a}	27.11 ± 1.32^{a}	8.29 ± 1.05	0.06 ± 0.01^{b}	0.29 ± 0.02^{a}	0.17 ± 0.03^{b}
p-value	< 0.001	< 0.001	0.280	< 0.001	< 0.001	< 0.001
24 h						
CON	34.76 ± 1.75^{d}	4.91 ± 0.31^{e}	12.06 ± 0.53^{a}	U.D[2]	0.15 ± 0.10^{b}	U.D
XOS	88.93 ± 1.67^{a}	18.41 ± 0.41^{d}	5.68 ± 0.16^{c}	U.D	2.85 ± 0.08^{a}	U.D
APEC	32.92 ± 1.75^{d}	3.64 ± 1.15^{e}	5.93 ± 1.99^{c}	U.D	0.29 ± 0.09^{b}	U.D
XA	78.62 ± 2.16^{bc}	21.63 ± 0.57^{cd}	7.59 ± 0.30^{bc}	0.06 ± 0.00	0.15 ± 0.01^{b}	U.D
X2	78.23 ± 1.31^{bc}	28.58 ± 0.43^{b}	7.33 ± 0.07^{bc}	U.D	0.18 ± 0.02^{b}	U.D
X3	109.72 ± 8.21^{a}	37.64 ± 2.91^{a}	10.24 ± 0.96^{ab}	0.06 ± 0.00	0.21 ± 0.06^{b}	U.D
X4	72.36 ± 1.98^{c}	24.99 ± 0.85^{bc}	8.21 ± 0.52^{abc}	0.05 ± 0.00	0.26 ± 0.02^{b}	U.D
p-value	< 0.001	< 0.001	0.002	-	< 0.001	U.D
36 h						
CON	32.88 ± 1.55^{c}	4.52 ± 0.25^{d}	11.76 ± 0.40^{b}	U.D	U.D	U.D
XOS	79.08 ± 3.45^{b}	21.49 ± 0.10^{c}	20.18 ± 0.93^{a}	U.D	U.D	U.D
APEC	22.56 ± 9.31^{c}	3.28 ± 1.37^{d}	5.35 ± 0.12^{d}	U.D	U.D	U.D
XA	93.32 ± 3.92^{b}	23.81 ± 0.81^{c}	6.61 ± 0.21^{cd}	U.D	U.D	U.D
X2	65.51 ± 16.88^{b}	29.68 ± 1.52^{b}	6.55 ± 0.33^{cd}	U.D	U.D	U.D
X3	79.29 ± 3.16^{b}	26.49 ± 1.02^{bc}	7.36 ± 0.24^{cd}	U.D	U.D	U.D
X4	153.98 ± 4.78^{a}	49.92 ± 0.66^{a}	8.40 ± 0.60^{c}	U.D	U.D	U.D
p-value	< 0.001	< 0.001	< 0.001	-	-	-

[a–d] Different letters represent significant differences ($p < 0.05$) among groups

[1] Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

[2] U.D, undetectable

the most effective ($p < 0.05$) at 24 and 36 h, respectively, during fermentation.

Metabolomic profiling of fermentation supernatant of gut microbiota in different groups

The results of 16 S rRNA sequencing revealed that XOS, xylobiose and xylotriose had a similar role in shaping gut microbiota challenged by APEC, whilst xylotetrose showed a relatively different effect. Therefore, the fermentation supernatants from CON, APEC, XA and X4 groups were selected for metabolomic analysis, which revealed multiple differential metabolites between CON group and APEC group (Fig. S1), whereas the differential metabolites between APEC group and XA group as well as between XA group and X4 group were more abundant. These differential metabolites were primarily linked with amino acid and carbohydrate metabolism.

Pathway enrichment analysis was conducted based on the criterium of $p < 0.05$ (- ln *p*-value > 3.0). As illustrated in Fig. 7A and Fig. S2A, the pathways of pantothenate and CoA biosynthesis, phenylalanine, tyrosine and tryptophan biosynthesis, β-alanine metabolism, and histidine metabolism were downregulated in APEC group versus CON group. Only the pathway of histidine metabolism was extremely upregulated in XA group relative to APEC group (Fig. 7B and Fig. S2B). In comparison, X4 group showed a similar ($p > 0.05$) enrichment pattern of pathways to XA group (Fig. 7C and Fig. S2C), with some pathways including histidine metabolism pathway tending to be downregulated in X4 group relative to XA group.

Fermentation supernatant from different groups differentially modulates genes expression of APEC

Bacteria can perceive specific metabolites to adjust their activities in adverse environments [26]. Accordingly, the expression of survival- and virulence-related genes in APEC was analyzed. As presented in Fig. 8A, there was no difference ($p > 0.05$) in *tnaA* and *glnA* expression

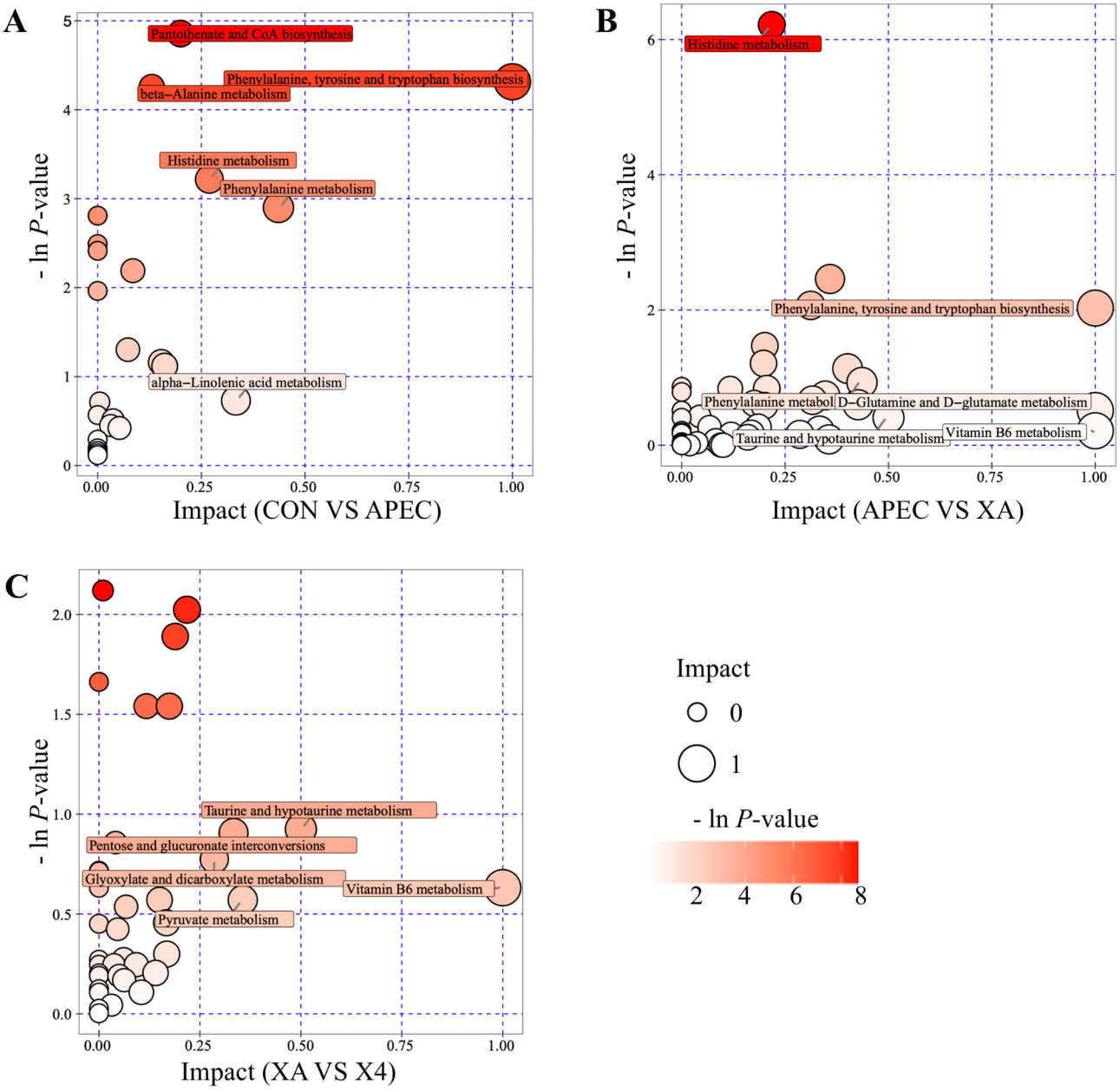

Fig. 7 Enrichment analysis of metabolic pathways in the fermentation supernatant of gut microbiota. Control group (CON), gut microbiota without treatment; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS and xylotetraose, respectively

between CON group and APEC group. Compared with APEC group, XOS or their monomers-treated groups displayed no change ($p>0.05$) in *tnaA* and *glnA* expression, except for a decrease ($p<0.05$) in *tnaA* expression in X2 group. Fermentation supernatant from XOS group increased ($p<0.05$) *entF* expression but reduced ($p<0.05$) *yddA* expression of APEC compared with that from CON group. Fermentation supernatant from X2 and X3 groups reduced ($p<0.05$) *yddA* expression compared with that from APEC group. The expression of *fimH* in APEC was increased ($p<0.05$) by fermentation supernatant from APEC group versus CON group (Fig. 8B), whilst APEC treated with fermentation supernatant from X2 and X3 groups exhibited a reduction ($p<0.05$) in *fimH* and *csgD* expression. Fermentation supernatant from X4 group lowered ($p<0.05$) *ompR* expression compared with that from APEC group (Fig. 8C), however, fermentation supernatant from X2 group caused higher ($p<0.05$) expression of *luxS* and *ompR* of APEC relative to that from either X3 or X4 group.

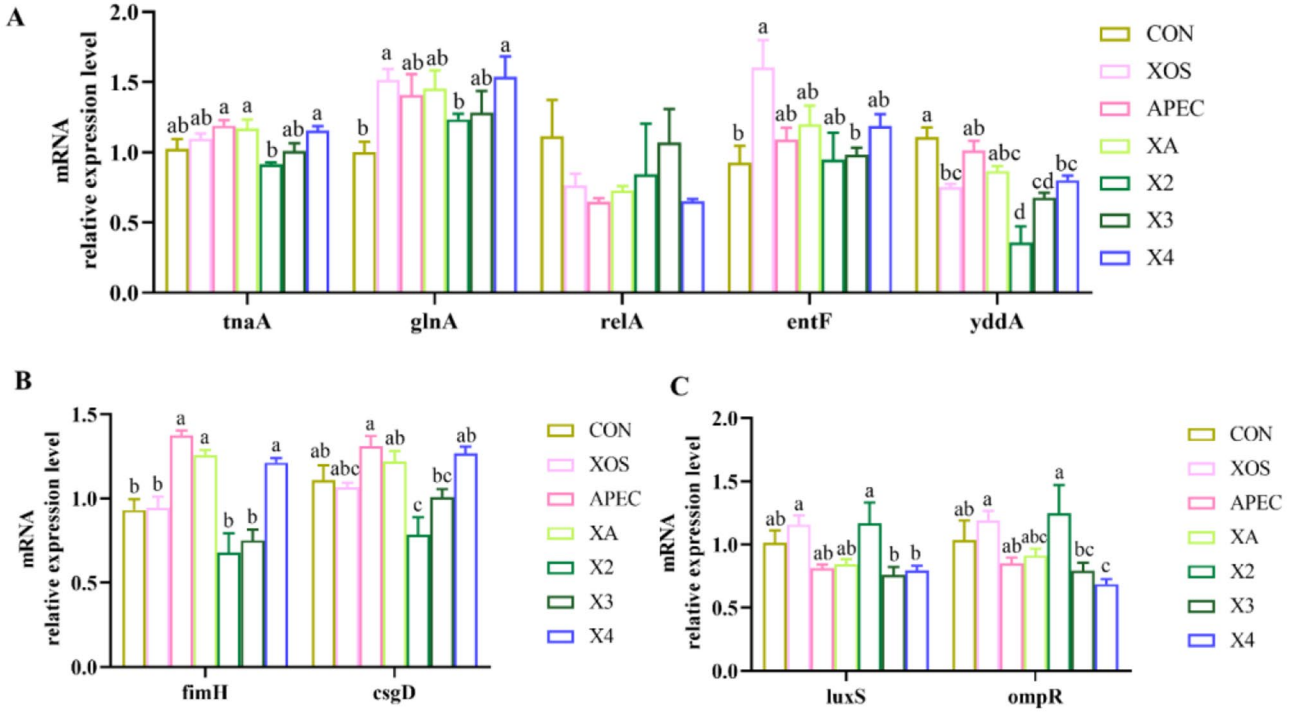

Fig. 8 Effects of fermentation supernatant from gut microbial fermentation of xylooligosaccharides (XOS) and and their monomers with different degrees of polymerization on virulence factors expression in APEC. (**A**) The expression of survival- and tolerance-related genes; (**B**) The expression of adhesion-related genes; (**C**) The expression of invasion-related genes. Different letters represent significant differences ($p < 0.05$) among groups. Control group (CON), gut microbiota without treatment; XOS group, gut microbiota was added with 4 mg/mL XOS; APEC group, gut microbiota received 4×10^9 CFU of avian pathogenic *Escherichia coli* (APEC); XA, X2, X3 and X4 groups, gut microbiota received 4×10^9 CFU of APEC combined with 4 mg/mL XOS, xylobiose, xylotriose and xylotetraose, respectively

Discussion

Microbial α-diversity that composes of evenness and richness was first analyzed in this study. Intriguingly, both XOS addition alone and APEC challenge alone especially the latter reduced the evenness (Pielou index) and richness (Chao 1 index) in gut microbiota, subsequently reducing microbial α-diversity (Shannon and Simpson indexes). This could be responsible by that the consumption of available nutrients arising from the prevalence of pathogenic *E. coli* restrained the growth of commensal microbes [27]. Comparatively, XOS conferred growth advantages to certain probiotics over other microbes, thus leading to a decrease in overall microbial α-diversity [11]. Nevertheless, under APEC challenge, both XOS and their monomers (xylobiose, xylotriose, and xylotetraose) treatments increased microbial evenness and α-diversity, which might benefit the protection of gut microbiota against APEC challenge, with xylotetraose being the least effective. It was probable that the nutrient blocking from the prebiotic actions of XOS antagonized the detriment of microbial α-diversity by APEC [28], thus accounting for the observed increased evenness and α-diversity of gut microbiota treated with XOS or their monomers. In support of α-diversity analysis results, β-diversity analysis revealed that XOS addition alone and APEC challenge alone differentially changed gut microbial structure. However, under APEC challenge, gut microbiota treated with XOS or their monomers shared a similarity in structure, with a relative seperation noted in xylotetraose-treated gut microbiota from those treated by XOS, xylobiose and xylotriose. These results implied a different efficacy of xylotetraose (versus XOS, xylobiose and xylotriose) in shaping gut microbial composition against APEC.

In order to expound the results of diversity analysis, gut microbial distributions were then analyzed. As expected, there was an obvious change in bacterial dominance following APEC challenge. Concretely, it dramatically increased the abundance of *Proteobacteria* at the expense of *Firmicutes* and *Bacteroidota*, as well as dramatically increased the abundance of *Escherichia-Shigella* at the expense of multiple probiotic bacteria such as *Lactobacillus* and *Bacteroides*. These results substantiated a disturbance of gut microbiota following APEC challenge, because *Proteobacteria* includes a mass of harmful bacteria (e.g. *Escherichia-Shigella*) and their expansions indicate gut microbiota dysbiosis that can cause intestinal dysfunction [29, 30], while *Firmicutes* and *Bacteroidota* encompass abundant probiotics including the renowned *Lactobacillus* and *Bacteroides* that favor intestinal

homeostasis [31, 32]. LEfSe analysis corroborated that APEC challenge caused gut microbiota dysbiosis, mainly characterized by the enrichment of *Escherichia-Shigella*. However, similar to previous studies [7–9], we observed that both XOS and their monomers (xylobiose, xylotriose, and xylotetraose) treatments expanded *Firmicutes* and *Bacteroidota* at the expense of *Proteobacteria*, as well as expanded several probiotics (e.g. *Lactobacillus*, *Megamonas*, and *Bacteroides*) at the expense of *Escherichia-Shigella.* These actions could conduce to the observed increased α-diversity of APEC-challenged gut microbiota following XOS or their monomers treatment [33, 34]. It was presumable that the growth-promoting effects of XOS on the above-mentioned probiotics competitively inhibited the growth of APEC or along with some other harmful bacteria, therefore depleting *Escherichia-Shigella* and *Proteobacteria* that favor to maintain intestinal homeostasis [9, 10]. Strikingly, different monomers of XOS exhibited a disparity in prebiotic properties. In specific, xylobiose and xylotriose were almost as efficacious as XOS in expanding the probiotics *Lactobacillus*, *Limosilactobacillus*, *Megamonas*, and *Bacteroides*. Comparatively, xylotetraose displayed a higher efficacy than xylobiose and xylotriose in expanding the probiotics *Bacteroides* and *Bifidobacterium*, but showed little efficacy in expanding the probiotic *Limosilactobacillus* together with a lower efficacy in depleting *Escherichia-Shigella.* The absense of expansion of *Limosilactobacillus* could partially elucidate the observed less loss in *Escherichia-Shigella* in gut microbiota treated with xylotetraose versus other XOS monomers, since *Limosilactobacillus* has pronounced inhibition effect against pathogenic *E. coli* [14]. The distinct prebiotic properties among XOS monomers were likely due to the different preferences of them to be utilized by specific probiotics. For instance, *Limosilactobacillus* prefers to degrade glycans with lower DP [35], which could provide a competitive advantage in the utilization of xylobiose and xylotriose over xylotetraose, probably clarifying the observed expansion of *Limosilactobacillus* by xylobiose and xylotriose rather than xylotetraose. Conversely, it seems that *Bacteroides* and *Bifidobacterium* possessing unique polysaccharide utilization loci prefer to degrade carbohydrate polymers (including xylans and XOS) with higher DP [36, 37], which could consequently explain the expansions of these probiotics mainly by treatment with xylotetraose instead of xylobiose and xylotriose. Similar results were obtained by the LEfSe analysis, which revealed that *Bacteroides* and its affiliated bacterial species *Bacteroides uniformis* represented the core bacteria enriched by xylotetraose. The above findings coincided with a previous study which discovered an ability of *Bacteroides* to secrete an unique xylanase capable of cleaving XOS with relatively high DP other than low DP [38]. However, in this study, considering the dominance of *Limosilactobacillus* among the probiotics and the prevelance of *Escherichia-Shigella* in the whole microbial communities following APEC challenge, the little efficacy in expanding *Limosilactobacillus* coupled with the low efficacy in depleting *Escherichia-Shigella* supported that the prebiotic effects of xylotetraose against APEC were inferior to those of XOS mixtures, xylobiose and xylotriose.

Microbial metabolites mediate the interactions among gut probiotics, pathogens and hosts, shaping the microbial niche that favors the growth of functional carbohydrates-degrading microbes [23, 25]. As the crucial metabolites produced through microbial fermentation of prebiotics, SCFA not only act as key energy components for enterocytes and boost intestinal defense against pathogens [16, 28], but also modulate bacterial activities in gut [15, 16]. Analogous to the study of Mikulski et al. [38], this study manifested that APEC challenge reduced the level of butyric acid instead of other SCFA in fermentation broth at both 24 and 36 h during fermentation. This might be associated with the observed action of APEC challenge to deplete *Firmicutes* and *Actinobacteria*, which encompass considerable butyric acid-producers such as *Subdoligranulum*, *Oscillibacter*, *Blautia*, *Ruminococcus torques*, *Butyricococcus* and *Eubacterium hallii* [23, 39]. In support of this view, we detected negative correlations of the above butyric acid-producers with *Escherichia-Shigella*. It has been reported that dietary XOS could stimulate certain butyric acid-producers in broiler gut [40]. Herein, we found that xylotriose and xylotetraose were effective in increasing butyric acid level in APEC-challenged gut microbiota at 24 and 36 h, respectively, during fermentation. Moreover, both XOS and their monomers sharply increased the levels of acetic and propionic acids at all time-points during fermentation. These could be responsible by the observed abilities of them to expand both *Bacteroidota* and *Firmicutes* that encompass plentiful acetic acid- and propionic acid-producers, including the aforementioned probiotics *Lactobacillus*, *Limosilactobacillus*, *Megamonas*, and *Bacteroides* [39, 41]. It was assumed that the above SCFA-producers caused obvious increases in acetic acid and propionic acid levels, conducing to inhibit APEC growth via formation of acidic microenvironment [16]. Remarkably, xylotriose and xylotetraose showed the highest efficacy in increasing acetic acid and propionic acid levels at 24 and 36 h, respectively, during fermentation. It was likely that XOS monomers with higher DP required longer duration for their complete degradation by gut microbiota [36, 42]. This could explain the observations that the production of SCFA (acetic, propionic and butyric acids) were maximized at 24 h and 36 h during microbial fermentation with xylotriose and xylotetraose, respectively. Besides the linear SCFA (acetic, propionic

and butyric acids), we also found temporal changes in the branched SCFA (isobutyric and isovaleric acids), which were reduced by both XOS and their monomers at 12 h rather than at 24–36 h when they were almost undetectable. This was similar to a previous study which revealed that XOS tended to lower isobutyric and isovaleric acids levels in broiler cecum [43].

In an attempt to further decipher the alterations in gut microbial fermentation products, we then conducted the metabolomic analysis of the fermentation supernatants that selected from CON, APEC, XA and X4 groups based on the results of 16 S rRNA sequencing. The results manifested that APEC challenge downregulated the pathways of pantothenate and CoA biosynthesis, aromatic amino acid (phenylalanine, tyrosine and tryptophan) biosynthesis, β-alanine metabolism and histidine metabolism of gut microbiota, which could endanger the nutritional linkages between gut microbiota and intestinal tissues of broilers. However, similar to a previous study where polysaccharide-based prebiotic positively regulated "gut microbiota-amino acids metabolism" network [44], this study showed that XOS treatment upregulated histidine metabolism pathway of gut microbiota under APEC challenge. This might attribute to the observed effect of XOS in enriching specific probiotics (e.g. *Limosilactobacillus* and *Lactobacillus*) [45]. Considering the critical roles of histidine metabolism in multiple physiological functions such as anti-oxidation and anti-inflammation of animals [46, 47], we deduced that XOS-induced upregulation of histidine metabolism of gut microbiota benefited intestinal health of chickens. Noticeably, relative to XOS, xylotetrose treatment played a similar role in shaping pathway enrichment profile of gut microbiota, but showed a trend to downregulate certain pathways including histidine metabolism. These results implied that xylotetrose had an inferiority to XOS, to a certain degree, in improving metabolic pathways of gut microbiota, which basically coincided with the preceding results regarding gut microbial composition.

Virulence factors assist with bacterial infection and pathogenicity for hosts [1]. Chicken cecum serves as an important reservoir for virulence factors of APEC [1]. Virulence factors expression of APEC in chicken cecum may be shaped by cecal metabolic profiles, because *E. coli* can perceive and respond to the shifts in intestinal metabolites [16]. Upon the stimulation by some metabolites such as SCFA, *E. coli* may regulate the expression of certain genes such as those involved in survival and virulence [16]. Among them, the *tnaA* gene encodes tryptophanase responsible for tryptophan synthesis, benefiting to maintain intracellular pH homeostasis of *E. coli* and protect against antibiotic stress [48]. The *glnA* gene encodes glutamine synthetase responsible for glutamine synthesis, which also aids in maintaining intracellular pH homeostasis of *E. coli* and renders a tolerance of *E. coli* to acidification and the resultant oxidative stress, subsequently favoring *E. coli* survival [49]. The *entF* gene encodes a crucial subunit of the synthetase of enterobactin, a functional peptide benefiting *E. coli* survival by combating other bacteria [50]. The *yddA* gene encodes an ABC transporter with efflux pump activity and thus contributes to drug resistance in *E. coli* [51]. The *fimH* and *csgD* genes encode an essential subunit of type I and curli fimbria, respectively, which facilitate APEC adhesion, biofilm formation and motility that conduce to establishing its infection for chickens [1]. The *luxS* gene encodes an enzyme that synthesize autoinducer-2, a key signaling molecule initiating quorum sensing, which can reinforce APEC pathogenicity by enhancing the adhesiveness, invasiveness and biofilm formation [52]. The *ompR* gene encodes a response regulator of two-component regulatory system, which impels the expression of outer membrane porins that fortify APEC invasiveness [1]. Among microbial metabolites, SCFA differentially intervene bacterial activities depending on the proportions of their non-ionized acid forms [53]. Notably, butyric acid is a much harder ionizable acid than acetic acid and propionic acid, thus being more efficiently to enter into bacterial cells in the non-ionized form and exert greater impacts on APEC physiology [54]. In this study, the supernatant of XOS fermentation by APEC-free gut microbiota increased glnA and entF expression, but reduced *yddA* expression without altering the expression of adhesion or invasion-related genes (*fimH*, *csgD*, *luxS* and *ompR*) in APEC. These might attribute to that the observed higher level of butyric acid in this fermentation supernatant elicited greater stress to APEC, which then produced complicated feedback responses to maintain its survival [48], but did not cause enhancement of its virulence. In contrast, the supernatants of both xylobiose and xylotriose fermentation by APEC-challenged gut microbiota lowered *yddA*, *fimH* and *csgD* expression, while fermentation supernatant of xylotetrose reduced ompR expression. These results suggested that xylobiose and xylotriose had different intervention effects from xylotetrose on APEC virulence, which were likely responsible by the observed similar efficacy between xylobiose and xylotriose that differed from xylotetrose in shaping the profiles of gut microbiota and their metabolites (e.g. SCFA). Strikingly, the fermentation supernatant of xylobiose caused higher ompR expression in APEC compared with that of xylotriose and xylotetrose. Overall, the above findings suggested that xylotriose could be more advantageous than xylobiose and xylotetrose in suppressing APEC virulence.

Conclusions

Both XOS mixtures and their major monomers (xylobiose, xylotriose and xylotetrose) differentially improved gut microbial structure under APEC challenge by enriching several beneficial bacteria (e.g. *Firmicutes*, *Bacteroidetes*, *Lactobacillus*, *Bacteroids*, *Limosilactobacillus*, and *Megamonas*) and subsequently improving the production of metabolites especially SCFA. These actions could thus inhibit APEC growth and cause depletion of certain harmful bacteria (Proteobacteria and *Escherichia-Shigella*), with XOS mixtures, xylobiose and xylotriose being basically efficacious but better than xylotetrose. The metabolites of xylobiose, xylotriose and xylotetrose fermented by gut microbiota differentially suppressed virulence factors expression of APEC, with xylotriose exhibiting a superiority over xylobiose and xylotetrose. Taken together, xylotriose had the best effects among the major monomers of XOS in suppressing APEC growth and virulence. The findings in this study emphasized the role of single DP in influencing the prebiotic actions of XOS against APEC, thereby providing a basis for the rational application of XOS in diets to combat bacterial challenge.

Abbreviations

APEC	Avian pathogenic Escherichia coli
CON	control
DP	Degree of polymerization
LDA	Linear discriminant analysis
LefSe	Linear discriminant analysis combined effect size measurements
NMDS	Non-metric multidimensional scaling
PcoA	Principal coordinates analysis
SCFA	Short-chain fatty acids
XOS	Xylooligosaccharides

Supplementary Information

The online version contains supplementary material available at https://doi.org/10.1186/s12866-025-03948-y.

Supplementary Material 1

Acknowledgements

Not applicable.

Author contributions

L.L.R. wrote the manuscript and performed the experiments. Q.Y.C. conduced to experimental design and sample determination. H.Y. conducted microbial analysis. Z.M.D. performed real-time PCR analysis. C.M.Z. conducted metabolomic analysis. F.Y. drew the graphs. Y.P.Z. assisted with data analysis. H.Y.Z. participated in project administration. J.J.Z. performed data curation and supervised the research. W.W.W. designed the study and revised the manuscript. All authors have read and approved the manuscript.

Funding

This study was financially supported by the National Natural Science Foundation of China (No. 32102584), the Natural Science Foundation of Guangdong Province (No. 2023A1515011112), the Key Research and Development Plan Project of Guangzhou City (No. SL2023B03J01234) and the Specific university discipline construction project (No. 2023B10564003).

Data availability

The data that support the findings of this study are available from the corresponding authors as the corresponding author, upon reasonable request. Besides, the raw sequencing data have been deposited in the Genome Sequence Archive (GSA number: CRA022271) in National Genomics Data Center (https://ngdc.cncb.ac.cn/gsa/search?searchTerm=CRA022271), China National Center for Bioinformation/Beijing Institute of Genomics.

Declarations

Ethics approval and consent to participate

The experimental animal protocols of this study were approved by the Animal Care and Use Committee of the South China Agricultural University (Approval number: 2024f217).

Consent for publication

Not applicable.

Competing interests

The authors declare no competing interests.

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Received: 26 January 2025 / Accepted: 3 April 2025
Published online: 22 April 2025

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