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Yeast cell-wall polysaccharides improve immunity and attenuate inflammatory response via modulating gut microbiota in LPS-challenged laying hens

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ABSTRACT

Effects of dietary supplementation of yeast cell-wall polysaccharides (YCWP) on production performance, ileal microbial composition, immunomodulatory and anti-inflammatory effects in LPS-challenged laying hens, were evaluated. A total of 288 35-week-old Hy-Line Brown layers were randomly assigned into 4 dietary treatments: 0, 250, 500 and 1000 mg/kg YCWP, respectively. After a 12-week feeding period, a total of 32 birds were selected from the control (n = 16) and 1000 mg/kg YCWP group (n = 16). For each group, half (n = 8) received *Escherichia coli* LPS and half (n = 8) received PBS at 1 mg/kg body weight, intravenously. Results showed that YCWP enhanced feed efficiency and egg production linearly, with optimal laying performance notable in the 1000 mg/kg YCWP group. Dietary YCWP enhanced serum IgM and expression of ileal avian β-defensin, alleviated the LPS-induced elevated levels of serum IL-6 and IL-1β and the up-regulated expression of IL-1β, TNF-α, IFN-γ, and IL-6 in spleen and/or ileal mucosa. Furthermore, anti-inflammatory and immunomodulatory effects of YCWP were linked with its enhancement effect on microbial diversity, proliferation of *Bifidobacteriaceae*, *Lactocillus*, *Candidatus_Arthromitus*, *Streptomyces*, *Bacillaceae*, and *Desulfovibrio*, and reduced abundance of *Shigella*. Therefore, YCWP has the potentials to be utilized as safe prebiotics and gut enhancer in laying hens.

1. Introduction

In modern poultry production practices, laying hens are often confronted with multiple challenges, such as immunological stress, inadequate housing conditions, pathogen infection and so on. These stressors often distort the immune systems and destabilize the physiological response of birds, thereby increasing the risk of immunosuppressive diseases. Immunosuppressive diseases further affect the performance and products quality of the poultry by increasing the susceptibility of chickens to secondary infections [1,2]. In as much as poultry meat and eggs are the main sources of protein to human nutrition, product quality must be maintained as unsafe products from diseased animals pose a threat to consumers health. In order to reduce incidence of diseases in poultry production, antibiotics was widely used as effective therapeutic agents in the past two decades. However, the use of antibiotics and medicinal products in poultry has been severely restricted or totally banned in many countries due to appearance of antibiotic-resistant bacteria and egg drug residue [3]. Therefore, it is necessary to explore safe, effective and cost affordable alternatives to antibiotics to fortify layers immunity and ameliorate inflammatory stress-challenge related problems.

Nutritional interventions for alleviation of stressors and enhancement of immunity have been advocated for in poultry production. Dietary immune-modulators supplementation has been suggested as one of safe, effective, and eco-friendly strategies for antibiotics substitution via modulating cellular and humoral immunity, enhancing resistance against infections, and counteracting immunosuppressed state in poultry [4]. Yeast (*Saccharomyces cerevisiae*) cell-wall polysaccharides (YCWP), isolated from yeast cell-wall consists mainly of β-glucan and mannan (known as mannoprotein), which are the main active ingredients of yeast cell-wall [5]. Studies showed that YCWP possessed prebiotic properties with efficacy on gut microbial modulation and

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immunity fortification [6–9] and consequently could be a potential alternative to antibiotics. In vitro, yeast β-glucan could enhance the phagocytic and bactericidal capacities of chicken macrophages [10], splenocytes [11] and heterophils [12]. In vivo, studies demonstrated that dietary YCWP enhanced humoral and/or cell immunity of laying hens [2] and broilers [13–15]. The main components of YCWP can adhere to pathogenic bacteria in the gut and modulate gut microbiota and intestinal integrity [7]. The immune-enhancing effect of YWCP may be linked with enhanced synthesis of immunoglobulins, cognizance and binding of specific antigens [2]. The aforementioned findings may partly explain the possibilities of YCWP to reduce gut pathogen colonization and suppress expression of inflammatory cytokines. However, most researches on the response of laying hens to dietary YCWP was conducted among birds with normal physiological status and reared under normal conditions. It becomes expedient to investigate the alleviating and regulatory effects of YCWP in laying hens exposed to immune inflammatory stress.

Lipopolysaccharides (LPS), a component of the cell wall of negative bacteria, has been widely used to model inflammation and stress in poultry and livestock [16–19]. *Escherichia coli* LPS interacted with the transmembrane signal transducer toll-like receptor 4 (TLR4), can lead to a series of inflammatory responses, including but not limited to the expression of pro-inflammatory cytokines and the production of inflammatory mediators [20,21]. Besides inducing inflammatory response, LPS exerted detrimental effect on the intestinal barrier function by disrupting intestinal morphology, and mRNA profile of tight junction proteins [24]. Furthermore, LPS challenge can also result in a disturbance of gut microbiota in chickens [19]. Thus, *Escherichia coli* LPS was adopted in this trial as a stress model to mimic a bacterial infection and induce inflammatory response in laying hens.

Intestinal microenvironmental homeostasis plays a functional role in inhibiting the colonization of pathogenic organisms, maintaining intestinal epithelial integrity and modulating mucosal and innate immunity in the host [22]. This homeostasis is attributed to the maintenance of a certain proportion of microbiota and their remarkable influence on genetic regulation [23]. Numerous studies have shown that genetic regulation is an important way for gut microbial metabolites to affect host health and immunity [24–26]. On the other hand, gut microbiota could also alter their gene expression to adapt to the changed conditions or diets [23]. Hence, deciphering gut microbial composition may provide useful insights into the potential impacts of its manipulation by dietary intervention on the immune status and intestinal health of the host.

There are evidences that support the important roles of gut microbiota in the therapeutic efficacy of polysaccharides [27,28]. Both yeast β-glucan and mannan have been proven to modulate gut microbiota in diversity or composition in human [29,30], which may be due to the capability of these YCWP components to attach to receptors of pathogens and with a consequent suppression of pathogen colonization in the gut. However, it is largely unknown whether YCWP addition can modulate gut microbiota dysbiosis in laying hens, ultimately benefiting immunity enhancement, intestinal integrity and anti-inflammatory response. In keeping with this, the current study was designed to investigate the immunomodulatory and anti-inflammatory effects of dietary YCWP in laying hens with or without LPS challenge, and its possible microbial mechanism.

2. Materials and methods

2.1. Materials and reagents

YCWP, derived from a unique *Saccharomyces cerevisiae*, after a specific extraction and purification process, was produced and provided by Angel Yeast Co., Ltd. (Yichang, China). *Escherichia coli* LPS (serotype O55:B5) and all standard monosaccharides were procured from Sigma Aldrich (St. Louis, USA. LPS, no. L2880; Mannose, no. M2069; Ribose, no. R7500; Rhamnose, no. R3875; Glucuronic acid, no. G5269; Galacturonic acid, no. 48280; *N*-acetyl-glucosamine, no. A4106; Glucose, no. G8270; *N*-acetyl-galactosamine, no. A2795; Galactose, no. G0750; Xylose, no. X1500; Arabinose, no. V900920; Fucose, no. F2252). The commercial assay kits for immunoglobulin A (IgA), immunoglobulin G (IgG), immunoglobulin M (IgM), interleukin 6 (IL-6), interleukin 1beta (IL-1β), and interleukin 10 (IL-10) were purchased from Shanghai Enzyme-linked Biotechnology Co., Ltd. (Shanghai, China. IgA, no. ml002792; IgG, no. ml042771; IgM, no. ml05107; IL-6, no. ml059839; IL-1β, no. ml059835; IL-10, no. ml059830). All other regents and chemicals used in the study were of analytical grade.

2.2. Characterization of YCWP

2.2.1. Determination of molecular weight (Mw)

The Mw and Mw distribution of YCWP were measured by gel permeation chromatography (GPC) according to the method described before [31] on a Shimadzu LC-20AT pump coupled to a RID-20 refractive index detector. The mobile phase was aqueous buffer containing 0.1 M $NaNO_3$ + 0.05 % (*w*/w) NaN_3, with a flow rate of 0.6 mL/min. All samples were diluted in the mobile phase, and filtered through a 0.45 μm filter before analysis. The polyethylene glycol (Tosho Co., Ltd., Tokyo, Japan) was used as the standard and the software of Shimadzu Lab Solutions GPC V5.93 was employed to determine the molecular weight of YCWP.

2.2.2. Monosaccharide composition

Monosaccharide composition of YCWP was determined using a Shimadzu LC-20AD high performance liquid chromatography (HPLC) (Tokyo, Japan). YCWP samples were acid-hydrolyzed and derivatized before analysis according to the method of Zhen et al. [2] with slight modification. The measurement parameters were as follows: chromatographic column: Xtimate C18 column (4.6 mm × 200 mm, 5.0 μm); mobile phase: 0.05 mol/L of phosphate buffer solution (pH 6.70): acetonitrile = 83:17 (*v*/v); flow rate: 1.0 mL/min; column temperature: 30 °C; sample volume: 20 μL; wavelength: 250 nm. The following monosaccharides were used as references: mannose, ribose, rhamnose, glucuronic acid, galacturonic acid, *N*-acetyl-glucosamine, glucose, *N*-acetyl-galactosamine, galactose, xylose, arabinose, fucose.

2.2.3. Infrared spectroscopy (FT-IR) and nuclear magnetic resonance (NMR)

Fourier-transform infrared spectra of the YCWP were tested on a Nicolet IS10 FT-IR spectrometer (Thermo Fisher Scientific, Madison, WI, USA) in the wavenumber range of 4000–400 cm^{-1} using the KBr disk method [2]. YCWP samples were dried prior to tableting with KBr power.

Nuclear magnetic resonance of YCWP was performed as described before [31]. YCWP was dissolved in D_2O (99.96 %). Then, the ^1H and ^{13}C spectra were analyzed by a Bruker AVANCE 600 M spectrometer (Bruker, Germany) at 25 °C. The experimental results were obtained by using MestReNova 6.1.0 software analysis.

2.2.4. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM)

The surface morphology of YCWP was observed by SEM (FEI Quanta 600, Thermo Fisher Scientific Ltd., Portland, OR). As described by a former report [32], the dried YCWP power was placed on a metal stub and sputter-coated with gold. Sample images were observed at magnifications of 200, 500 and 2000 times at 5.0 kV under high vacuum conditions.

The TEM observation of YCWP was conducted as the method described before [33]. Briefly, the YCWP was dissolved in distilled water at a concentration of 0.5 mg/mL and coated on electron microscope copper grids. Then samples were examined by TEM (FEI Tecnai G3 F30, FEI, USA), and the images were taken at accelerating voltages of 120 kV.

2.3. Animal experiment

2.3.1. Birds and experimental design

The animal protocols in this study were approved by the Animal Care and Use Committee of the Feed Research Institute of the Chinese Academy of Agricultural Sciences (ACE-CAAS-20210415). Animal experimental design is illustrated in Fig. 1. A total of 288 35-week-old Hy-Line Brown layers were randomly assigned into 4 dietary groups supplemented with YCWP at 0, 250, 500, and 1000 mg/kg. Each group contained 6 replicates with 12 birds each. Prior to the onset of the feeding trial, a 7-day adaptation period was observed. The laying hens that were randomly allocated to dietary groups had similar initial body weight and egg laying rate across all the replicates. Layers were allocated to 3-tier battery cages of 3 birds each (cage size: 40 cm × 40 cm × 35 cm) and exposed to 16 h of light/day with an intensity of 20 lx. Room temperature was maintained between 22 and 26 °C throughout the experiment. Diets and water were offered ad libitum in mash form and by nipple drinkers, respectively. The basal diet formulated according to National Research Council (National Research Council, 1994) is shown in Table 1. All hens remained in good health during the whole feeding period.

2.3.2. LPS injection and sampling

At the end of the feeding period, 16 birds were randomly selected from each of the control and $YCWP_{1000}$ (birds in this group had optimal laying performance) groups. For each group, half of the selected birds ($n = 8$) received *Escherichia coli* LPS and the remaining half ($n = 8$) received phosphate buffer solution (PBS) at the dose of 1 mg/kg body weight, intravenously via the wing vein. This is in line with the method adopted by Wu et al. [34]. At the end of 6 h after the injection, blood and tissue samples were collected from these hens. Blood samples were taken from the wing vein on the other side, and centrifuged at 3500 rpm for 10 min to harvest serum. Serum samples were stored at −20 °C until analysis. After blood collection, birds were slaughtered rapidly. Segments about 2 cm long in the middle of jejunum and ileum were collected into 10 % neutral-buffered formalin for intestinal morphology analysis. The mucosa of ileum and uterus were scraped aseptically by sterile glass slides, frozen in liquid nitrogen along with a patch of spleen. Spleen tissue, ileal and uterine mucosa were transferred to −80 °C for the quantification of gene expression. Ileal contents were also collected in sterile containers, frozen by liquid nitrogen, followed by the storage at −80 °C until further analysis.

Table 1
Composition and nutrient levels of the basal diet (as-fed basis, %).

	Content (%)
Ingredient	
Corn	59.00
Wheat	10.00
Soybean meal (44.8 % CP)	10.17
Cottonseed meal (60 % CP)	9.00
Wheat bran	1.15
Salt	0.30
Dicalcium phosphate	0.90
Calcium carbonate	8.90
DL-Methionine (99 %)	0.12
Lysine-HCl (78 %)	0.13
L-Threonine (98 %)	0.05
Choline chloride (50 %)	0.10
Premix[a]	0.13
Phytase	0.03
Xylanase	0.02
Total	100
Nutrient level[b]	
Metabolizable energy (MJ/kg)	11.26
Crude protein	16.49 (16.25)
Nonphytate phosphorus	0.33 (0.35)
Calcium	3.50 (4.01)
Lysine	0.75
Methionine	0.36
Methionine + cysteine	0.65
Threonine	0.55

[a] Premix supplied per kilogram of diet: vitamin A, 12,500 IU; vitamin D_3, 4125 IU; vitamin E, 15 IU; vitamin K_3, 2 mg; thiamine, 1 mg; riboflavin, 8.5 mg; pyridoxine, 8 mg; vitamin B_{12}, 0.04 mg; biotin, 0.1 mg; folic acid, 1.25 mg; Ca-pantothenate, 50 mg; niacin, 32.5 mg; Cu, 8 mg; Zn, 65 mg; Fe, 60 mg; Mn, 65 mg; Se, 0.3 mg; I, 1 mg.

[b] The values in parenthesis indicate analyzed values. Others are calculated values.

2.3.3. Laying performance

Daily egg number, total egg weight and biweekly feed consumption were recorded. Hen-day egg production, average egg weight, average daily feed intake, and feed-to-egg ratio were calculated out based on the periods of week 1–4, week 5–8, week 9–12, and week 1–12.

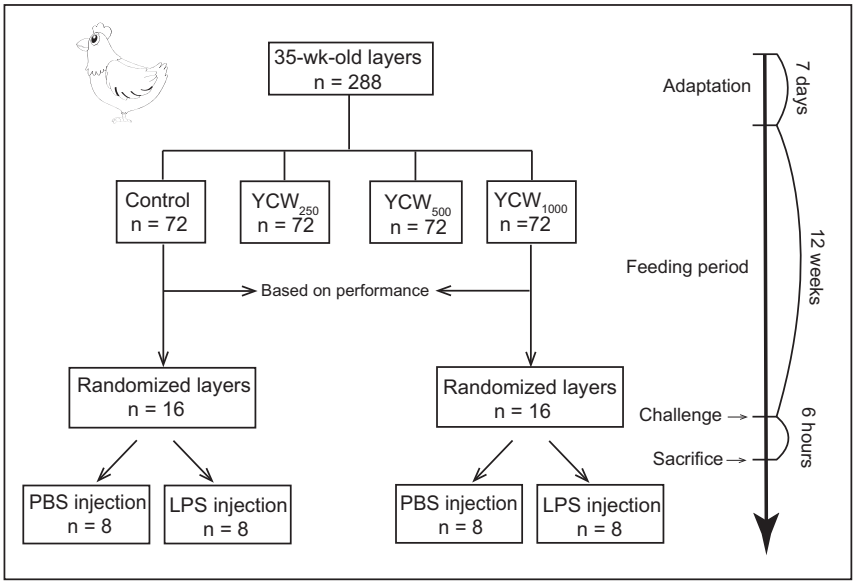


Fig. 1. Experimental design and animal groups.

2.3.4. Contents of immunoglobulins and inflammatory cytokines in serum

Serum immunoglobulins (IgA, IgG, IgM), and inflammatory cytokines (IL-6, IL-1β, IL-10) were separately determined using assay kits for chickens following manufacturer's instructions.

2.3.5. Intestinal morphology analysis

Intestinal morphology was determined as the method described before [3]. Jejunal and ileal segments were washed, dehydrated, clarified, and embedded in paraffin. Sections were cut at 5 μm thickness, dewaxed in xylene, rehydrated, stained with hematoxylin and eosin (H and E), fixed with neutral balsam, and examined by light microscopy. For each sample, three intact villi-crypt units were selected for morphometric indices evaluation, including villus height (VH, the height from the tip of the villus to the villus-crypt junction), crypt depth (CD, the depth from the base up to the crypt-villus transition region) and the villus height-to-crypt depth ratio (VH/CD).

2.3.6. RNA isolation and real-time quantitative PCR

Total RNA of the spleen, ileal and uterus mucosa were extracted using EasyPure RNA kit (TransGen Biotech Co., Ltd., Beijing, China) according to the manufacturer's instructions. The purity and concentration of total RNA were determined using Epoch Microplate Spectrophotometer (BioTek Instruments, Inc., VT, USA). Reverse transcription reactions were immediately performed using the First-Strand cDNA Synthesis SuperMix (TransGen Biotech Co., Ltd., Beijing, China). Real-time PCR for measuring gene expression was conducted in duplicate in a CFX-96 real-time PCR detection systems (Bio-Rad Laboratories, Hercules, CA, USA). The protocol for all genes was as follows: 95 °C for 15 min; 40 cycles of 95 °C for 10 s, 60 °C for 30 s. The relative gene expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method [35]. Avian β-actin was used as reference gene. The primer sequences for the target genes (IL-1β, IL-6, tumor necrosis factor-alpha (TNF-α), interferon-gamma (IFN-γ), avian β-defensin 10 (AvBD10), TLR4, Claudin-1, Occludin, zonula occludens-1 (ZO-1)) and β-actin are listed in Table 2.

Table 2
Sequences of real-time PCR primers.

Gene[a]	Primer sequence (5′ → 3′)	Accession no.
IL-1β	F: CAGCCTCAGCGAAGAGACCTT R: ACTGTGGTGTGCTCAGAATCC	NM_204524.1
IL-6	F: CAAGGTGACGGAGGAGGAC R: ACTGTGGTGTGCTCAGAATCC	NM_204628.1
TNF-α	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	NM_204267.2
IFN-γ	F: AGCTGACGGTGGACCTATTATT R: GGCTTTGCGCTGGATTC	NM_205427.1
AvBD10	F: CAAGGTGACGGAGGAGGAC R: ACTGTGGTGTGCTCAGAATCC	NM_001001609.3
TLR4	F: GAGCGTTGACTTGGCTGTC R: AAGCAACAACCAGCTATGCAC	NM_001030693.2
Claudin-1	F: AGCTGACGGTGGACCTATTATT R: GGCTTTGCGCTGGATTC	NM_001013611.2
Occludin	F: CCACTGCCTTGAGGAGCTGATTG R: ACCCAAAGTGACTGAGCATCCAAAG	NM_205128.1
ZO-1	F: CATCCCAACCCAACCACAGTAGC R: GACCGAGCAATGTCAAACTCAAAGC	NM_001301025.3
β-Actin	F: AAGTGCATGGAGGATGACCA R: GCCACTCTGTTGCCATACCA	NM_205518.1
	F: TCATCGCCTCCATCGTCTAC R: TCTTACTGCGCGTCTTCTGG	
	F: TATGAAGATCGTGCGCCTCC R: GAGGTCTGCCATCGTAGCTC	
	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	

[a] IL-1β, interleukin 1beta; IL-6, interleukin 6; TNF-α, tumor necrosis factor-alpha; IFN-γ, interferon-gamma; AvBD10, avian β-defensin 10; TLR4, toll-like receptor 4; ZO-1, zonula occludens-1.

2.3.7. Sequencing of ileal microbiota

Total microbial DNA was extracted from ileal content samples using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) following manufacturer's instructions. The quality of DNA samples was assessed by gel electrophoresis. Bacterial 16S rDNA sequences spanning the hypervariable regions v3-v4 were amplified using the primer pair 338F/806R (5′-ACT CCT ACG GGA GGC AGC A-3′ and 5′-GGA CTA CHV GGG TWT CTA AT-3′). The PCR reaction protocol was: 2 min at 95 °C (denaturation), 25 cycles at 95 °C for 30 s (denaturation), 30 s for annealing at 55 °C, 30 s of extension at 72 °C, with a final extension at 72 °C for 5 min. PCR products were extracted from a 2 % agarose gel and purified using the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, USA) according to the manufacturer's instructions. Purified amplicons were qualified and paired-end sequenced (2 × 300 bp) using Illumina MiSeq platform (Illumina, Sn Diego, USA). The row reads were deposited into NCBI Sequence Read Archive (SRA) database (Accession Number: PRJNA846975).

Sequencing and bioinformatics were performed using QIIME 2 (Ver. 2019.4) and the sequencing results were analyzed based on amplicon sequence variant (ASVs). Two obvious outliers of each group were excluded to avoid their interference with the subsequent analyses. Alpha diversity metrics inclusive of Chao 1 estimator, Observed species, Shannon index and Simpson index were employed to assess microbial richness and evenness. Principal coordinate analysis (PCoA) based on the unweighted UniFrac distance were conducted to compare the bacterial community profiles across all treatments to establish beta diversity. Taxa composition at phylum and genus levels were compared among groups. Linear discriminant analysis (LDA) effect size (LEfSe) measurements combined Kruskal-Wallis test was employed to identify the biological differential taxa among groups. The threshold for the $\log_{10}$LDA score was set as 2.0. Spearman's correlation analysis was performed for the correlations between phylotypes and phenotypes.

2.4. Statistical analysis

Statistical analyses were performed using SAS Ver. 9.2 (SAS Institute Inc., Cary, NC, USA). Data of laying performance was analyzed using one-way ANOVA and differences were compared using Duncan's Multiple Range Test. The linear and quadratic effects of YCWP doses on laying performance were evaluated by regression analysis. For results in other indices (serum indices, intestinal morphology, and gene expression), the main effects of diet, challenge, and their interaction were subjected to two-way ANOVA using the GLM procedure. Data are expressed as means with their pool standard error (SEM), and statistical significance is defined as $P < 0.05$.

3. Results

3.1. Characterization of YCWP

GPC analysis of YCWP detected three peaks, corresponding to 15.0, 17.11 and 18.35 min of retention time (Table 3 and Fig. 2A). The number-, weight-, and z-average Mw of the main peak were 18.0, 51.04, and 108.86 kDa, respectively. The Mw distribution result showed that approximately 90.75 % of YCWP had a Mw under 100 kDa, and those Mw between 10 and 100 kDa accounted for 53.14 %.

As shown in Fig. 2B and Table S1, HPLC analysis revealed the YCWP was composed of mannose, glucose, glucuronic acid, ribose, arabinose, fucose, xylose, galacturonic acid, and rhamnose with a molar ratio of 24.65: 21.62: 0.84: 0.54: 0.33: 0.09: 0.08: 0.02: 0.02. Among them, mannose and glucose were major monosaccharides of YCWP, accounting for 48.83 % and 47.59 % of the total sugar contents, respectively.

The FT-IR spectrum of YCWP in the ranges of 4000–400 cmm^{-1} are illustrated in Fig. 2C, which showed typical absorption peaks of a polysaccharide. The broad peak at 3283.93 cm^{-1} was attributed to O—H stretching vibration. The absorption peaks at 2922.53 cm^{-1} and

Table 3
The molecular weight determination of yeast cell-wall polysaccharides.

Item[a]	Peak 1	Peak 2	Peak 3
Molecular weight			
Average retention time, min	15.00	17.11	18.35
Mn, kDa	18.00	1.42	0.25
Mw, kDa	51.04	1.58	0.37
Mz, kDa	108.86	1.74	0.57
Mw distribution, %			
≤0.1 kDa	1.02		
0.1–1 kDa	18.10		
1–10 kDa	18.49		
10–100 kDa	53.14		
>100 kDa	9.25		

[a] Mn, number-average molecular weight; Mw, weight-average molecular weight; Mz, z-average molecular weight.

1628.64 cm^{-1} represented C—H and C—O stretching vibration, respectively. An absorption peak at 1369.63 cm^{-1} was caused by the bending vibration of the C—H bond. The strong peak at 1021.06 cm^{-1} was typical for the C—O—C skeletal vibration caused by dissymmetrical and symmetrical vibration of pyranose. Finally, the weak absorption peak at 810.42 cm^{-1} was confirmed as characteristic of a pyranose α-type C—H variable angle vibration.

YCWP was also characterized by ^{1}H (Fig. 2D_1) and ^{13}C (Fig. 2D_2) NMR spectra. The ^{1}H NMR spectrum showed mainly characteristic signals of saccharides (between δ 3.3 and δ 5.6 ppm), including the anomeric proton signals in the region of δ 4.5–δ 5.3 ppm and the remaining proton signals in the region of δ 3.3–δ 4.5 ppm. Except δ 4.69 ppm (hydrogen signal of water), there were two main anomeric hydrogen at δ 5.24 and δ 5.00 ppm, indicating that YCWP was mainly composed by one α-configuration and one β-configuration monosaccharides. The ^{13}C NMR spectrum of YCWP showed four signals at δ 103.06, δ 102.21, δ 100.62, and δ 98.27 ppm that can be attributed to anomeric carbons (signals between δ 95 and δ 105 ppm). These anomeric carbons signals indicated that monomers of YCWP should be pyran rings, as furan ring signals are around δ 107 - δ 109 ppm.

The SEM and TEM determination of YCWP were illustrated in Fig. 3. SEM images showed that the particles structure of YCWP was mainly irregular spheres morphology with squamous surface, and the particle size distributed between 5–50 μm. TEM result showed that YCWP grains were elliptical structure in the aqueous suspension and mostly remain as small aggregates. These grains sizes ranged from about 500–1000 nm.

3.2. Laying performance

The effects of dietary supplemental YCWP on performance of layers (35 to 47 weeks of age) are presented in Table 4. No difference in average egg weight and average daily feed intake were observed among all treatments during the whole feeding period ($P > 0.05$). Dietary YCWP addition linearly increased the egg production ($P < 0.05$) and decreased feed-to-egg ratio ($P < 0.05$) from week 9 to 12, and week 1 to 12. Birds received 500 and 1000 mg/kg YCWP presented significantly higher egg production than those in the control ($P < 0.05$). In comparison, birds in 1000 mg/kg YCWP group got an optimal performance. Thus, the follow-up investigation about LPS challenge was only conducted in birds selected from this group and the control.

3.3. Serum immunoglobulins and inflammatory cytokines

The results of the serum immunoglobulins and inflammatory cytokines of laying hens are provided in Table 5. Layers challenged with LPS showed higher serum IgA, IgG, IgM and pro-inflammatory cytokine IL-6 than those injected with PBS ($P < 0.05$). Dietary supplemental 1000 mg/kg YCWP significantly increased serum IgM and decreased IL-6 content ($P < 0.05$). There were no significant interactions for the serum IgA, IgG, IgM and IL-6 contents between LPS and YCWP ($P > 0.05$). However, a significant interactive effect between LPS and YCWP was observed for serum IL-1β content ($P < 0.05$). Birds that received the control diet and challenged with LPS exhibited a significantly higher serum IL-1β ($P < 0.05$), while birds fed diets with 1000 mg/kg YCWP showed no change after LPS challenge ($P > 0.05$).

3.4. Intestinal morphology

As shown in Table 6 and Fig. 4, the jejunal and ileal morphology were affected by LPS injection but not by YCWP addition. LPS challenge significantly deepened CD and lowered VH/CD in both jejunum and ileum of laying hens when compared with PBS injection ($P < 0.05$). There was no significant interaction between LPS and YCWP in intestinal morphology of laying hens ($P > 0.05$).

3.5. Gene expression of inflammatory cytokines in spleen, ileal and uterine mucosa

Fig. 5 shows the relative gene expression of inflammatory cytokines in spleen (A), ileal mucosa (B), and uterine mucosa (C) of laying hens. The LPS challenge significantly influenced the cytokines gene expression of laying hens via up-regulating the mRNA expressions of IL-1β, TNF-α, IFN-γ, and IL-6 in spleen, IL-1β, TNF-α, IL-6 in ileal mucosa, and TNF-α in uterine mucosa ($P < 0.05$). Dietary supplemental YCWP significantly down-regulated ileal mucosal IL-1β expression ($P < 0.05$), and had a significant interaction with LPS injection ($P < 0.05$). Furthermore, the LPS-induced the elevation of splenic IL-1β, TNF-α, IFN-γ, and ileal mucosal TNF-α and IL-6 expressions, were alleviated by YCWP ($P < 0.05$).

3.6. Gene expression of ileal mucosal AvBD10, TLR4, and tight junction proteins

The relative mRNA expressions of AvBD10, TLR4 and tight junction proteins in ileal mucosa are shown in Fig. 5D and E. The mRNA expression of AvBD10 in the ileal mucosa was significantly higher in birds offered YCWP ($P < 0.05$), while the ileal TLR4 expression was significantly up-regulated by LPS injection ($P < 0.05$) but not affected by YCWP treatment ($P > 0.05$). In addition, ileal mucosal Claudin-1 and ZO-1 expressions in LPS challenged layers were significantly down-regulated ($P < 0.05$). There were no significant interactions between LPS and YCWP for the relative expressions of ileal AvBD10, TLR4 and tight junction proteins ($P > 0.05$).

3.7. Diversity and composition of ileal microbiota

Ileal microbial alpha diversity including Chao 1, Observed species, Shannon and Simpson metrics were lowered ($P < 0.05$) by LPS challenge in the birds fed the control diet (Fig. 6A and Table S2). However, when birds received the YCWP diet, no change occurred ($P > 0.05$) in the alpha diversity of ileal microbiota in response to LPS challenge. PCoA plots revealed that LPS challenge obviously changed ileal microbial communities of laying hens fed diets with or without YCWP addition (Fig. 6B). There was also a trend of separation between the control and $YCWP_{1000}$ groups among the non-challenged birds. However, when birds challenged with LPS, the occupied positions of samples were partially overlapped between the control and $YCWP_{1000}$ groups.

As illustrated in Fig. 7A, the dominant phylum in non-challenged groups was *Firmicutes*, contributing >80 % to the whole phyla. In laying hens challenged with LPS, a higher abundance of *Proteobacteria* with a lower abundance of *Firmicutes* were observed, whereas the trend was reversed to an extent by dietary YCWP addition. At genus level, *Lactocillus*, belonging to *Firmicutes*, accounted for the largest proportion of the ileal microbial community (Fig. 7B). LPS challenge led to a

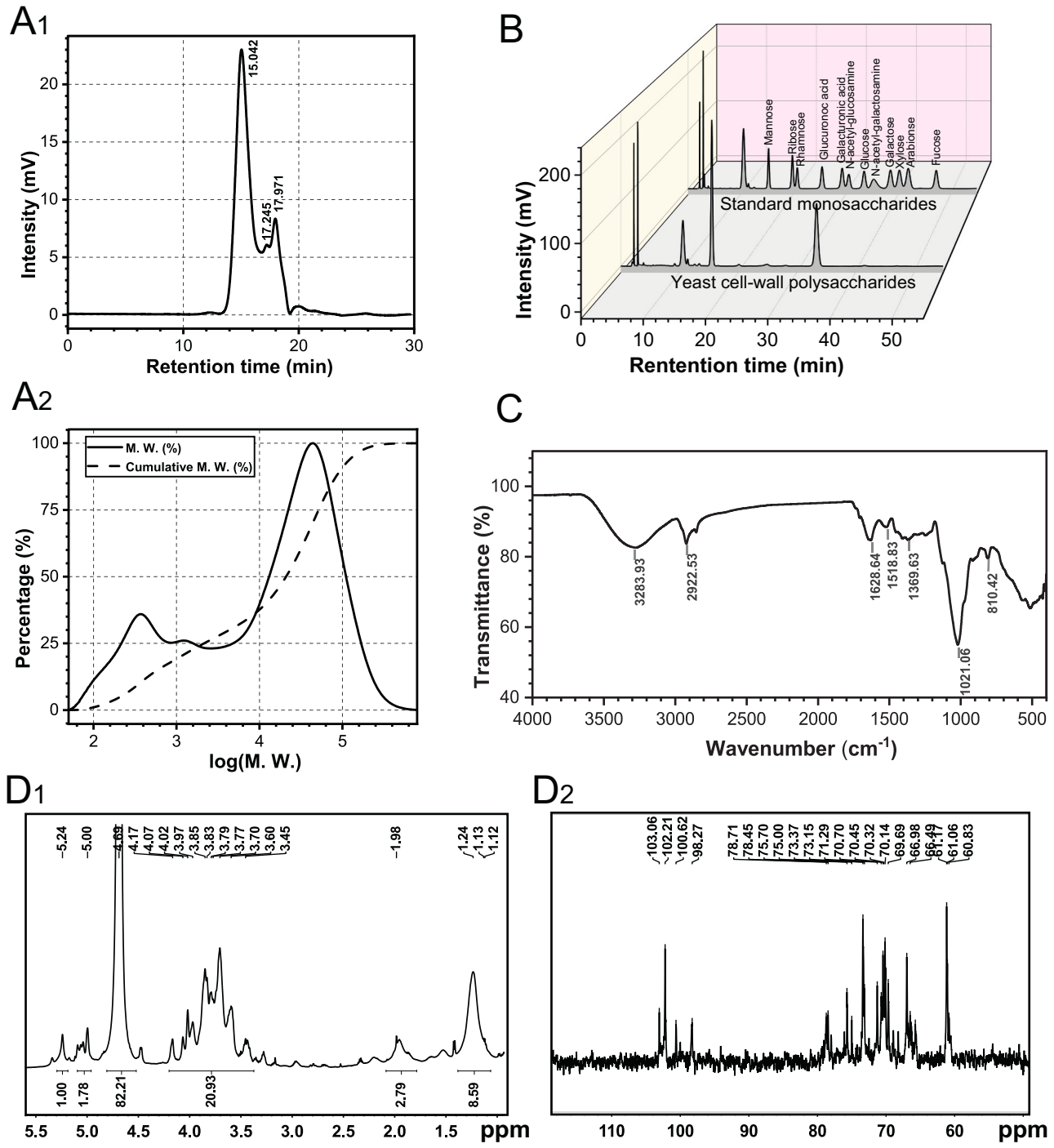


Fig. 2. Characterization of yeast cell-wall polysaccharides. (A) Chromatogram curve and molecular weight distribution curve tested by gel permeating chromatography (GPC); (B) Monosaccharide composition tested by high performance liquid chromatography (HPLC); (C) Fourier-transform infrared spectra (FT-IR) determination; (D) ^{1}H and ^{13}C nuclear magnetic resonance (NMR) analysis of YCWP.

reduced abundance of *Lactocillus* with an increased abundance of *Shigella*. The increased abundance of *Shigella* was relieved by YCWP addition, which also induced an increase in abundance of *Candidatus_Arthromitus* compared to the LPS challenged bird fed the control diet.

LEfSe analysis was applied to identify the significant differentially abundant ASVs for entire microbiota at levels from phylum to genus ($P < 0.05$; LDA > 2.0). As shown in Fig. 7C, in non-challenged LPS birds, higher abundance of family *Faecalibacterium* was notable in the ileum of birds that received the control diet, while those fed YCWP supplemented diet exhibited an increased abundance of *Bifidobacteriales* (*Bifidobacteriaceae*). After LPS challenge, ileal microbiota was enriched with *Sphingomonadaceae* (*Sphingomonas*) and *Enterobacteriaceae* (*Shigella*) in laying hens fed the control diet ($P < 0.05$). For the group of YCWP treatment coupled with LPS injection, LEfSe highlights substantial bacterial members enriched in the ileum, including class *Erysipelotrichi* (*Erysipelotrichales*, *Erysipelotrichaceae*), order *Actinomycetales* and its derivatives *Microbacteriaceae*, *Corynebacteriaceae* (*Corynebacterium*), and *Streptomycetaceae* (*Streptomyces*), families *Bacillaceae* and *Staphylococcaceae* (*Staphylococcus*) in addition to genera *Stenotrophomonas* and *Desulfovibrio*.

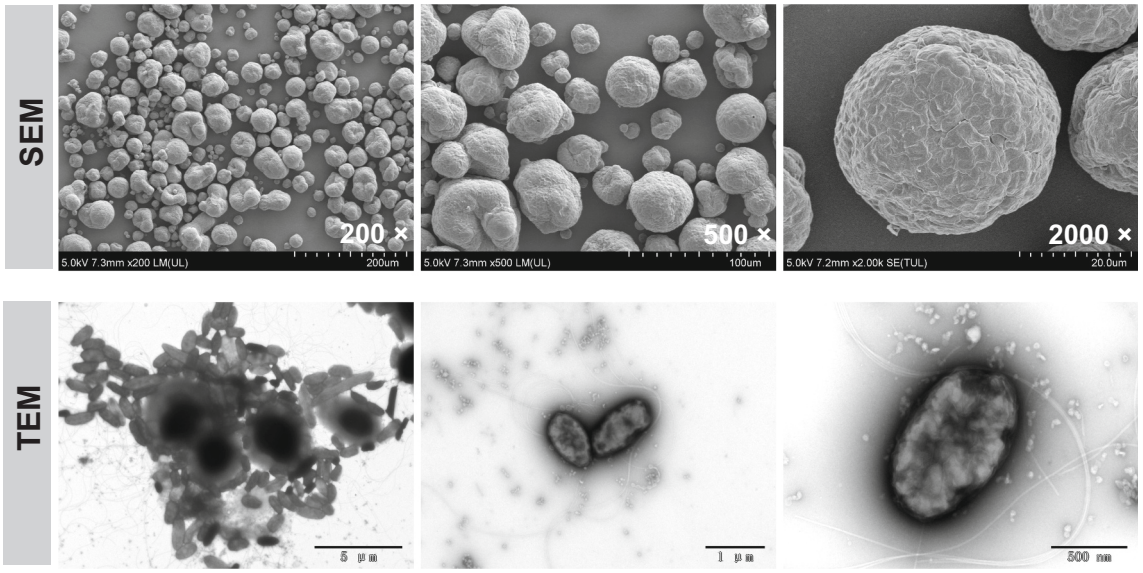


Fig. 3. Scanning electron microscopy (SEM) and transmission electron microscopy (TEM) images of yeast cell-wall polysaccharides.

A Spearman's correlation analysis was conducted to evaluate the associations between the altered bacterial members with the differential expressions of ileal cytokines and tight junctions (Fig. 8). The abundances of phylum *Firmicutes*, families *Lactobacillaceae* and *Bifidobacteriaceae*, and genus *Lactobacillus* showed negative correlations with TLR4 and some or all of pro-inflammatory cytokines expression, but positively associated with ileal tight junctions expression ($P < 0.05$). Conversely, the abundances of phylum *Proteobacteria* and its derivatives *Enterobacteriazeae* (*Shigella*) elicited positive correlations with TLR4 and some of pro-inflammatory cytokines, but negative relationships with tight junctions ($P < 0.05$). In addition, *Corynebacteriaceae* (*Corynebacterium*) was positively linked with the expressions of TLR4 and IL-6, and negatively associated with ZO-1 expression ($P < 0.05$).

4. Discussion

Yeast cell-wall consists of β-glucan, mannan, protein, lipid, and chitin, among which β-glucan and mannan are main effective components and account for 29 % ~ 64 % and 31 %, respectively [7]. The YCWP used in this study was obtained from yeast (*Saccharomyces cerevisiae*) cell-wall by modern separation technology and consists mainly of β-glucan and mannan [36]. It was consistent with the HPLC result that mannose and glucose were major monosaccharides of YCWP after acid hydrolysis, accounting for 48.83 % and 47.59 % of the total sugar contents, respectively. Thus, it can be reasonably presumed that the two main signals observed at δ 5.24 and δ 5.00 ppm in ^{1}H NMR spectrum respectively corresponded to the anomeric protons of α-mannose and β-glucose. Moreover, all the absorption peaks obtained from FT-IR spectrum agreed with the typical peaks of polysaccharides described before [27,31]. Also, the three peaks detected by GPC analysis suggested the heterogeneity of the polysaccharides, which implied the presence of more than one type of polysaccharides in the YCWP. The ^{13}C NMR spectrum analysis confirmed that monomers of YCWP were pyran rings, which coincided with the strong peaks observed in FT-IR spectrum that caused by dissymmetrical and symmetrical vibration of pyranose. Mw as a vital physical property of polysaccharides is related to their biological activities [7,37]. The Mw distribution of YCWP covered a wide range, varying from <0.1 kDa to >100 kDa, the most abundant fractions were those with the Mw between 10 kDa and 100 kDa. SEM images illustrated irregular spheres (approximately 5–50 μm) morphology of YCWP in the air while TEM images showed an elliptical structure of YCWP grains (approximately 500–1000 nm) in the aqueous suspension. The aforementioned findings lend evidence on the validity of the structure and potency of the YCWP in exerting biological activities. The unique composition and structure of YCWP may account for its beneficial effects in promoting performance, mitigating inflammatory stress and modulation of gut microbiota of laying hens.

Previous researches have demonstrated the efficacy of yeast cell-wall and its derivatives in exerting beneficial effects on the production performance of laying hens [38,39]. Similarly, this study revealed a better performance of layers in response to YCWP addition, reflected by the linear improvements in egg production and feed efficiency. The enhanced performance could be attributed to the various biological functions of YCWP components: protective effect on intestinal morphology and barrier, stimulation of innate and acquired immunity, anti-inflammatory and antioxidant effects [2,10,36,40]. Therefore, enhanced performance may be a function of stable gut health and physiological response.

Maintenance of gut health via improving intestinal morphology and up-regulation of integral membrane proteins including Claudin-1 and ZO-1 which are found at tight junctions [41], are crucial to gut integrity and invariably nutrient utilization. Therefore, assessment of mRNA profile of tight junctions as well as villi morphometrics of normal and LPS-challenged birds could reflect the integrity of the intestinal mucosal barrier. In this study, inflammatory responses and possible intestinal injury were very notable in laying hens exposed to *Escherichia coli* LPS. In the same lieu, an increased CD and a reduced VH/CD in the jejunum and ileum of layers, along with a down-regulation of mRNA expressions in ileal Claudin-1 and ZO-1 were observed. These findings pinpoint the impairment of intestinal morphology and epithelial barrier caused by LPS challenge, which are consistent with previous reports [16,18]. However, dietary supplementation of YCWP exerted no significant changes in the intestinal indices of birds from both control and LPS-challenged birds. The non-significant effect on intestinal indices implies that the enhancement effect of YCWP on production performance of the laying hens is not adducible to protective effect of YCWP on intestinal integrity or epithelial mucosa barrier. Immunomodulatory activity is one of the most common activities of polysaccharides [32], thus changes in immune and inflammatory status of the layers were further analyzed.

Immunoglobulins produced by lymphocytes are viewed crucial for humoral immunity. In the current trial, the increased serum IgA, IgG and IgM contents in response to LPS injection gave support to the previous viewpoint that LPS could stimulate the production of immunoglobulins

Table 4
Effect of dietary yeast cell-wall polysaccharides on performance of laying hens[1].

Item[2]	Egg production, %				Average egg weight, g				Average daily feed intake, g				Feed-to-egg ratio, g/g			
Period, wk	1–4	5–8	9–12	1–12	1–4	5–8	9–12	1–12	1–4	5–8	9–12	1–12	1–4	5–8	9–12	1–12
Treatments																
Control	90.08	86.98	85.35^{b}	87.41^{b}	59.57	59.28	59.10	59.31	111.40	108.35	105.18	108.31	2.07	2.14	2.09	2.09
$YCWP_{250}$	89.34	85.87	87.64^{ab}	87.61^{b}	59.63	58.78	58.68	59.03	110.97	107.57	104.51	107.68	2.08	2.13	2.02	2.08
$YCWP_{500}$	92.86	87.36	88.93^{a}	89.72^{a}	60.01	58.98	58.62	59.20	111.57	108.76	104.34	108.49	2.00	2.13	2.04	2.05
$YCWP_{1000}$	92.86	89.88	90.17^{a}	90.97^{a}	60.11	59.32	59.07	59.50	110.47	109.25	105.05	108.46	1.98	2.09	1.99	2.02
SEM	0.767	0.779	0.566	0.592	0.181	0.201	0.241	0.192	0.275	0.284	0.283	0.134	0.019	0.017	0.015	0.013
P-value																
ANOVA	0.234	0.312	0.009	0.089	0.672	0.789	0.863	0.871	0.523	0.196	0.697	0.108	0.109	0.780	0.111	0.238
Linear	0.099	0.096	0.001	0.013	0.238	0.721	0.929	0.597	0.302	0.109	0.996	0.297	0.023	0.309	0.033	0.036
Quadratic	0.255	0.185	0.003	0.047	0.493	0.652	0.686	0.734	0.491	0.234	0.481	0.504	0.077	0.123	0.055	0.118

a, b: Within a column, means with no common letters differ significantly ($P < 0.05$).
[1] $n = 6$ replicates per treatment.
[2] Control, hens received a basal diet; $YCWP_{250}$, $YCWP_{500}$ and $YCWP_{1000}$, hens received a basal diet supplemented with 250, 500 or 1000 mg/kg yeast cell-wall polysaccharides.

[42,43]. Notably, YCWP addition improved serum IgM level as well, indicating an enhancement in humoral immunity exerted by dietary YCWP. Similar results were also described by a previous study, in which birds offered yeast cell-wall exhibited higher serum IgA and IgG regardless of necrotic enteritis challenge [15].

TLR4 is a predominant transmembrane signal transducer to recognize *Escherichia coli* LPS and activate the intracellular pathways downstream of it [20], subsequently triggering the production of pro-inflammatory cytokines [21,44]. As predicted, we observed an up-regulated expression of ileal mucosal TLR4 in laying hens subjected to LPS challenge, accompanied with the increased expressions of pro-inflammatory cytokines in spleen, ileal and uterine mucosa. These coincided with the concurrently reduced expressions of ileal mucosal Claudin-1 and ZO-1. It thus suggests that the overproductions of IL-1β, IL-6, TNF-α and IFN-γ in LPS-challenged birds induce an impairment of intestinal epithelial tight junctions and has been documented previously in literature [45,46]. Besides, in concert with a previous study [1], LPS challenge triggered an elevation of serum IL-6 and IL-1β contents but exerted non-significant change on serum IL-10. There are evidences in literature that the inflammatory stress induced by LPS challenge could be mitigated with dietary interventions: yeast or yeast derived products due to their anti-inflammatory effects on animals exposed to different inflammatory stress-challenge conditions [15,47–49].

A kind of yeast (*Saccharomyces cerevisiae boulardii*) was validated to reduce serum IL-6 and IL-1β contents of pigs challenged with LPS [50]. Further on, in vivo and in vitro studies revealed that dietary yeast (*Saccharomyces cerevisiae*) cell-wall β-glucan attenuated LPS-induced increases of IL-6 and TNF-α in plasma of weaned piglets and in culture medium for pig lymphocytes [51]. Broilers offered yeast (*Saccharomyces cerevisiae*) cell-wall β-glucan presented a down-regulated IFN-γ expression [14]. Similar result was also described in mice that yeast (*Saccharomyces cerevisiae boulardii*) cell-wall components mitigated pathogen-induced transcription of pro-inflammatory cytokines [6]. Herein, dietary supplemental YCWP diminished LPS-induced increases in serum IL-6 and IL-1β contents, as well as the expressions of IL-1β, TNF-α, IFN-γ and IL-6 in spleen and/or ileal mucosa, confirming a lower inflammatory status in YCWP-treated layers in response to LPS. The aforementioned findings suggests that YCWP may have suppressed the synthesis of pro-inflammatory cytokines and deactivated the TLR4 signal.

Avian β-defensins (AvBD) are small cationic antimicrobial peptides involved in the innate immune system, which are believed to kill various microorganisms inclusive of gram-positive and -negative bacteria, fungi and viruses [52]. It was reported that LPS recognized by TLR4 could stimulate the IL-1β production, and IL-1β up-regulated the AvBD expression in the infected tissues [53]. However, the up-regulated ileal TLR4 and IL-1β expressions induced by LPS did not lead to an increased AvBD10 expression in this study. One of the possibilities is that the amount or biological activity of IL-1β synthesized in ileum was not sufficient to provoke AvBD10 expression. Whereas, the expression of ileal AvBD10 was indeed up-regulated by YCWP addition, further proving the immune-enhancing effect. The immunomodulatory activity of the YCWP is probably due to the capacity of its components to interact with receptors on the wall of animal gastrointestinal tract and that on the membranes of pathogens [54]. Furthermore, the enhanced immunity would at least partially account for the improved production performance of YCWP-fed layers. Not only does the immunomodulatory capacity of yeast cell-wall account for its mitigating effect in LPS challenged animals, gut microbiota has been demonstrated to play a role in the LPS-mediated inflammatory environment of intestine [17,18]. Therefore, we investigated the ileal microbial diversity and composition to ascertain the mechanism underlying the anti-inflammatory and immune-enhancing response of dietary YCWP, when fed to laying hens under LPS-induced disrupted inflammatory status.

A higher bacterial diversity in the intestinal tract generally means a more stable microbial community, thus benefiting to host health status via inhibition of pathogen colonization and maintenance of immune

Table 5
Effect of dietary yeast cell-wall polysaccharides on serum immunoglobulins and inflammatory cytokines of laying hens challenged with *Escherichia coli* LPS[1].

Item[2]		IgA, μg/mL	IgG, μg/mL	IgM, μg/mL	IL-6, pg/mL	IL-1β, pg/mL	IL-10, pg/mL
Control	−LPC	48.08	647.27	113.28	3.78	88.27^{b}	11.29
	+LPS	53.42	672.69	124.20	4.29	95.69^{a}	10.90
$YCWP_{1000}$	−LPS	50.51	652.14	118.78	3.61	89.09^{b}	10.98
	+LPS	53.07	681.49	128.38	3.73	91.80^{ab}	11.39
SEM		0.652	5.513	1.424	0.070	0.673	0.087
Main effect means							
Diet	Control	50.74	659.98	118.74^{b}	4.04^{a}	91.98	11.09
	$YCWP_{1000}$	51.79	669.25	123.58^{a}	3.67^{b}	90.44	11.19
LPS	−	49.28^{b}	652.14^{b}	116.03^{b}	3.70^{b}	88.68^{b}	11.14
	+	53.24^{a}	677.09^{a}	126.29^{a}	4.01^{a}	93.75^{a}	11.15
P-value							
Diet		0.347	0.383	0.028	0.002	0.098	0.582
LPS		0.001	0.024	<0.001	0.007	<0.001	0.952
Diet × LPS		0.214	0.964	0.755	0.073	0.014	0.074

a, b: Within a column, means with no common letters differ significantly ($P < 0.05$).
[1] $n = 8$ replicates per treatment.
[2] IgA, immunoglobulin A; IgG, immunoglobulin G; IgM, immunoglobulin M; IL-6, interleukin 6; IL-1β, interleukin 1β; IL-10, interleukin 10; Control, hens received a basal diet; $YCWP_{1000}$, hens received a basal diet supplemented with 1000 mg/kg yeast cell-wall polysaccharides.

Table 6
Effect of dietary yeast cell-wall polysaccharides on intestinal morphology of laying hens challenged with *Escherichia coli* LPS[1].

Item[2]		Jejunum			Ileum		
		VH, μm	CD, μm	VH/CD	VH, μm	CD, μm	VH/CD
Control	−LPC	1006.54	188.04	5.56	726.25	135.25	5.34
	+LPS	916.24	283.85	3.39	842.63	212.47	4.10
$YCWP_{1000}$	−LPS	1084.87	184.42	6.01	734.06	139.60	5.15
	+LPS	950.33	252.53	4.02	791.77	214.56	3.83
SEM		32.564	10.652	0.252	22.71	8.303	0.200
Main effect means							
Diet	Control	961.39	235.94	4.47	788.32	173.86	4.72
	$YCWP_{1000}$	1017.60	218.47	5.02	764.84	177.08	4.49
LPS	−	1045.70	186.23^{b}	5.78^{a}	730.15	137.42^{b}	5.24^{a}
	+	933.28	268.19^{a}	3.70^{b}	817.20	213.52^{a}	3.97^{b}
P-value							
Diet		0.388	0.274	0.120	0.631	0.748	0.505
LPS		0.091	<0.001	<0.001	0.060	<0.001	0.001
Diet × LPS		0.733	0.384	0.801	0.598	0.910	0.915

a, b: Within a column, means with no common letters differ significantly ($P < 0.05$).
[1] $n = 8$ replicates per treatment.
[2] VH, villus height; CD, crypt depth; VH/CD, villus height-to-crypt depth ratio; Control, hens received a basal diet; $YCWP_{1000}$, hens received a basal diet supplemented with 1000 mg/kg yeast cell-wall polysaccharides.

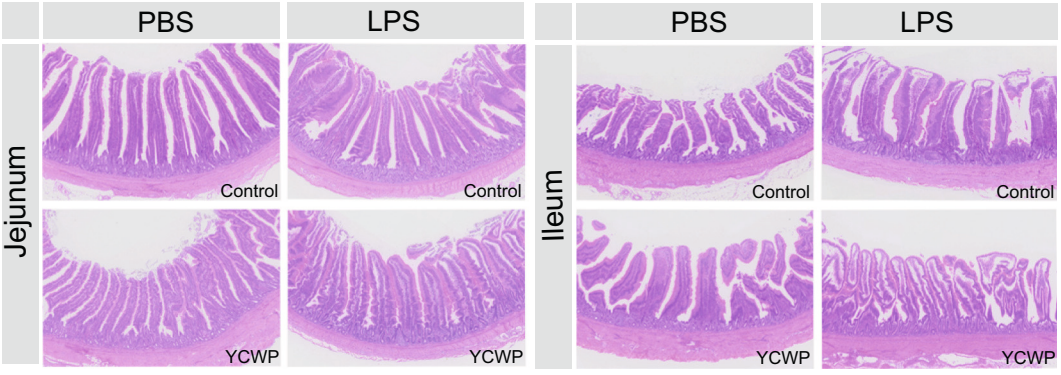


Fig. 4. Effect of dietary yeast cell-wall polysaccharides (1000 mg/kg, $YCWP_{1000}$) on the morphology of jejunum and ileum of laying hens challenged with *Escherichia coli* LPS. Hematoxylin and erosion (H&E) staining, 40× magnification.

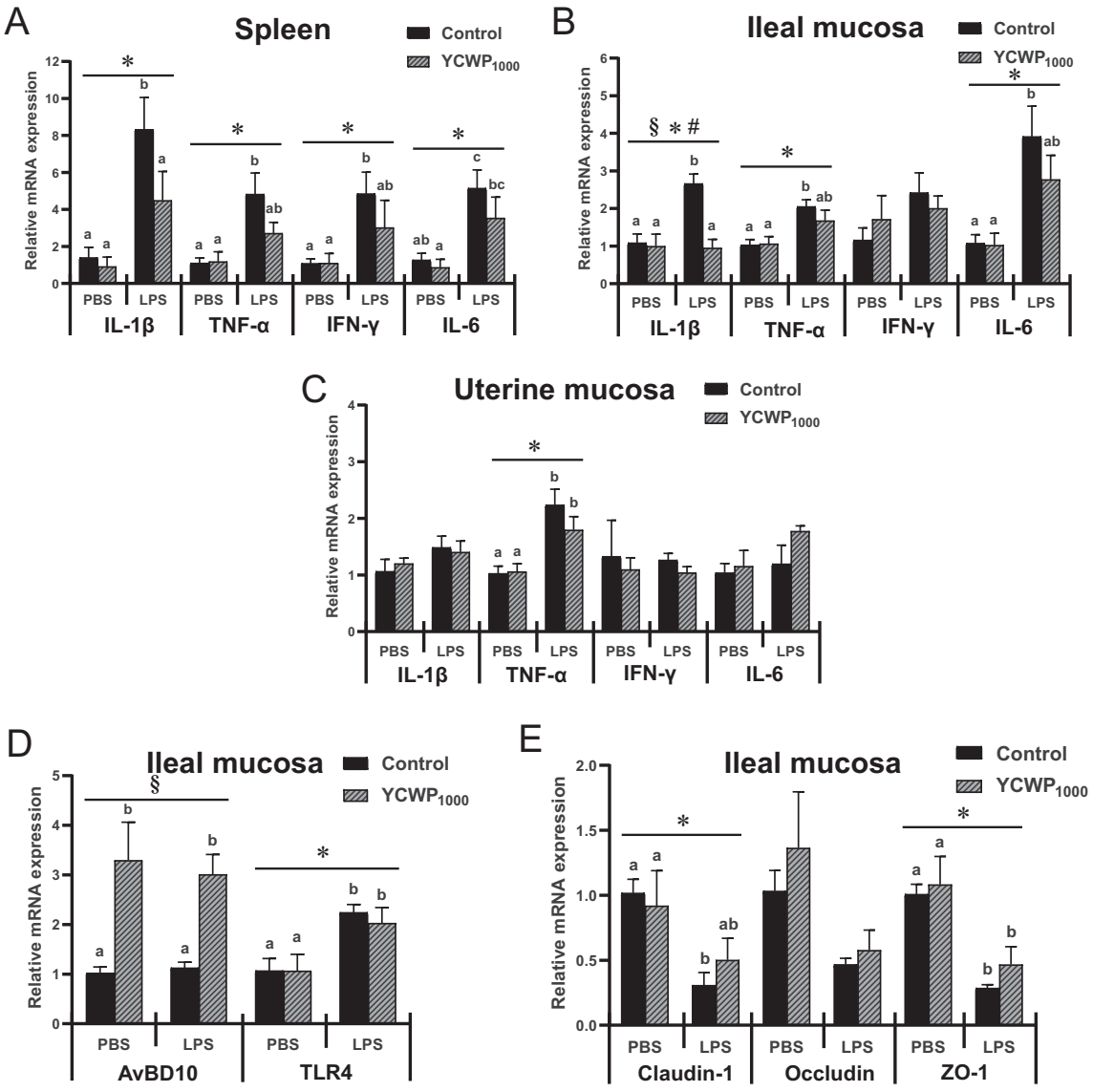
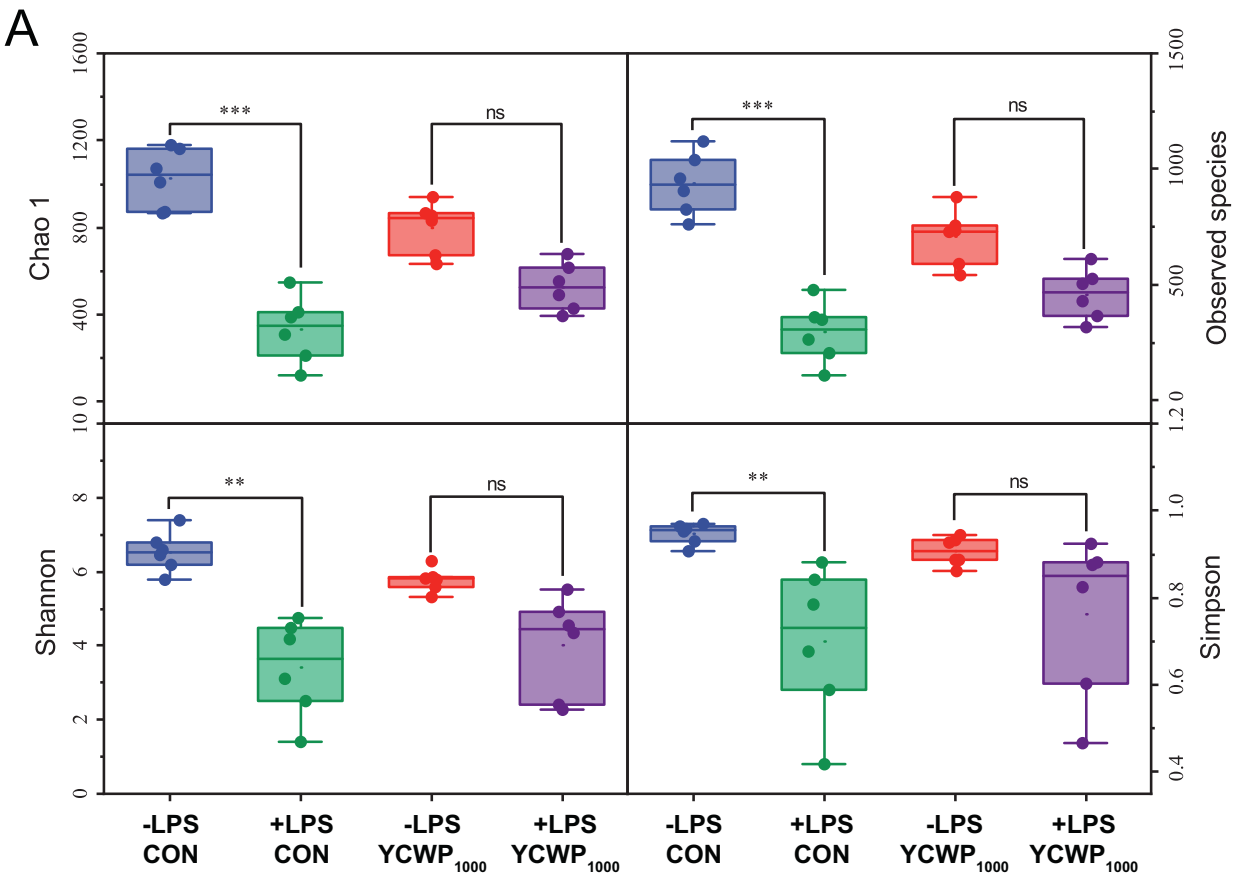


Fig. 5. Effect of dietary yeast cell-wall polysaccharides (1000 mg/kg, $YCWP_{1000}$) on the relative mRNA expression of laying hens challenged with *Escherichia coli* LPS. (A), (B) and (C) respectively represent the mRNA expression of pro-inflammatory cytokines in spleen, ileal mucosa and uterine mucosa; (C) The mRNA expression of AvBD10, TLR4 in ileal mucosa; (D) The mRNA expression of tight junction proteins in ileal mucosa. Data are represented with the means ± SE ($n = 8$). Bars with no common letters differ significantly ($P < 0.05$). § and * respectively represent significant main effects of diet and LPS challenge ($P < 0.05$), and # represent significant interactive effect between diet and challenge ($P < 0.05$).

homeostasis [55,56]. In this trial, ileal microbial richness and evenness were reduced by LPS challenge in layers which received the control diet, and were consistent with a previous study [19]. However, there was an unchanged alpha diversity in YCWP-treated birds following LPS injection, indicating that dietary YCWP effectively prevented the reduction of bacterial richness and evenness caused by LPS. Results in beta diversity analysis revealed an obviously altered microbial community structure by LPS administration regardless of the diets, whereas a separation in response to YCWP treatment only occurred in non-challenged birds. This suggested that not only could dietary YCWP modulate gut microbiota of non-challenged birds, but it could exert an interactive effect with LPS on bacterial community structure. To validate this speculation, changes in microbial composition and specific taxa were further analyzed.

Data showed that the ileal microbial composition of birds was shifted by LPS, favoring *Proteobacteria* at the expense of *Firmicutes*, which was mainly attributed to the increased *Shigella* and the reduced *Lactocillus* at genus level. As predominant members, *Firmicutes* imparts key functions to the host, such as energy metabolism and immunologic properties, while *Proteobacteria* contains a wide variety of pathogenic genera that can instigate intestinal inflammation [57,58]. Belonging to *Firmicutes*, *Lactocillus* is a common probiotic and can improve host immunity against gastrointestinal infections [59]. Conversely, *Shigella* is a gram-negative invasive pathogen, causing intense inflammation and destruction of intestine [60]. It could therefore be notable that in response to YCWP dietary treatment, the abundance of *Proteobacteria* (*Shigella*) was reduced while that of *Firmicutes* was increased. These reversed microbial abundances due to YCWP supplementation in LPS-challenged birds lend more evidence that the changes in ileal microbiota may mediate LPS-induced inflammation and impairment of intestinal barrier, as well as subsequent alleviation of inflammatory response. This was further supported by the results of correlation analysis which showed that the regulated expression of TLR4 and inflammatory cytokines were positively associated with *Proteobacteria* (*Shigella*), and negatively linked with *Firmicutes* (*Lactocillus*) in this trial. Notably, the restoration of *Firmicutes* was not due to a rebound of *Lactocillus*, but rather the increase in *Candidatus_Arthromitus* following



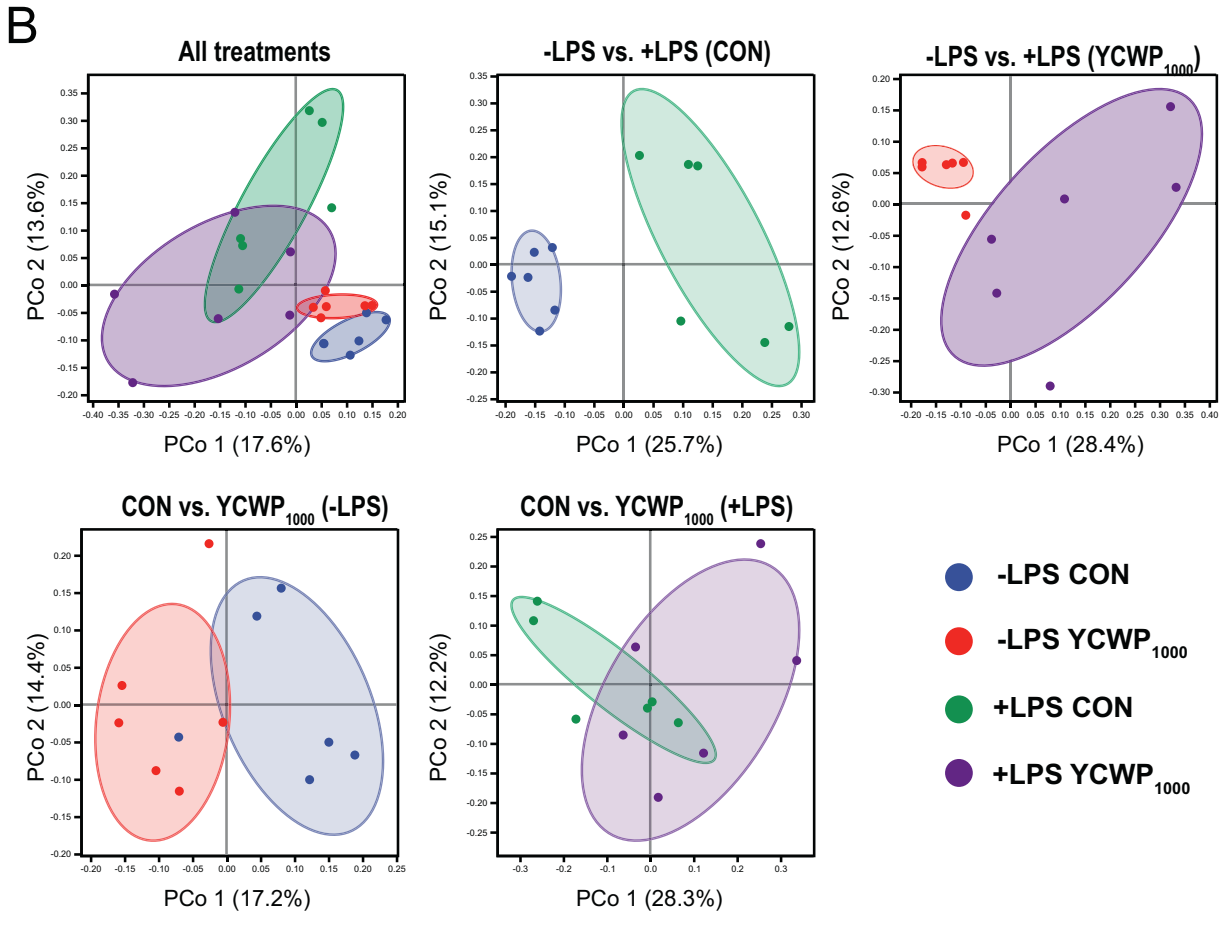


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Fig. 6. Effect of dietary yeast cell-wall polysaccharides (YCWP) on ileal bacterial diversity of laying hens challenged with *Escherichia coli* LPS. (A) Alpha diversity inclusive of Chao 1, Observed species, Simpson and Shannon indices; (B) Beta diversity: principal coordinate analysis (PCoA) based on the unweighted UniFrac distance. -LPS, hens injected with PBS; +LPS, hens challenged with LPS; CON, hens received a basal diet; $YCWP_{1000}$, hens received a basal diet supplemented with 1000 mg/kg YCWP.

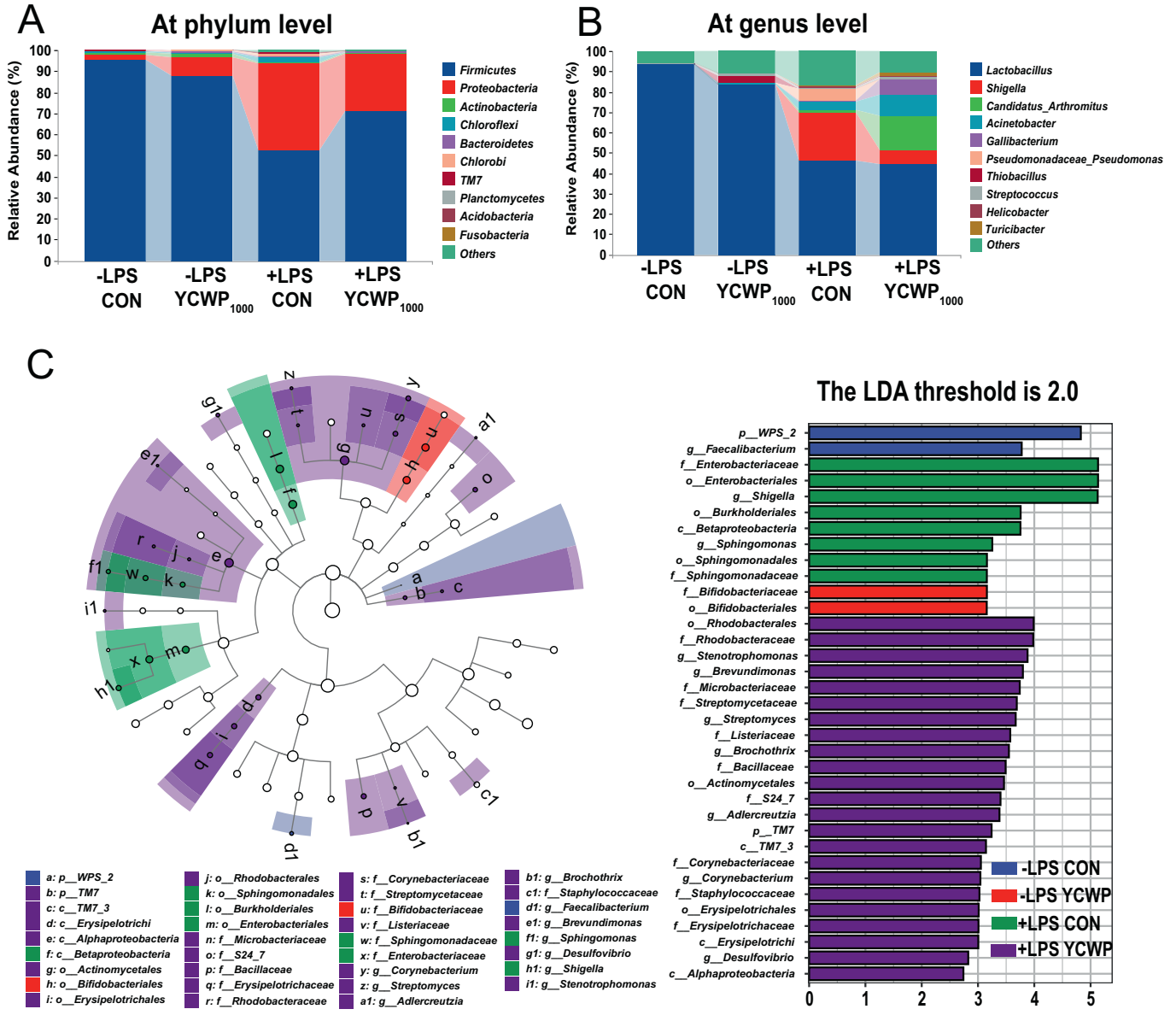

Fig. 7. Ileal microbial composition at phylum (A) and genus (B) levels and biomarkers found by linear discriminant analysis effect size (LEfSe, C) of laying hens challenged with *Escherichia coli* LPS. Species with significant difference that have an LDA score >2 are presented. -LPS, hens injected with PBS; +LPS, hens challenged with LPS; CON, hens received a basal diet; $YCWP_{1000}$, hens received a basal diet supplemented with 1000 mg/kg YCWP.

YCWP addition, which could directly modulate host immunity by T cell responses, including the T helper cell differentiation, intestinal sIgA secretion, and induction of IgA plasma cells [61,62].

Besides the dominant bacteria, LEfSe analysis identified some representative species as biomarkers to distinguish gut microbial community among groups. In non-challenged layers, dietary supplementation of YCWP significantly enriched *Bifidobacteriales* (*Bifidobacteriaceae*). *Bifidobacteriaceae* as a key probiotic in the gut, plays important roles in pathogen exclusion and intestinal barrier function [63]. Moreover, similar to the role of *Candidatus_Arthromitus* in the ileum, *Bifidobacteriaceae* is also beneficial for dendritic cell maturation and the regulation of T cell and T helper cell development [64,65]. Therefore, the increased *Bifidobacteriaceae* might exert a key role in YCWP enhancing the immunity of non-challenged birds, further benefiting to the production performance. In LPS challenged birds, LEfSe highlights the greater differential abundances of several opportunistic pathogens, including *Corynebacteriaceae* (*Corynebacterium*), *Staphylococcaceae* (*Staphylococcus*), *Erysipelotrichaceae*, *Brevundimonas* and *Stenotrophomonas*. All of them have been documented to be associated with inflammatory infections [66–70]. In the current study, correlation analysis revealed positive associations between *Corynebacteriaceae* (*Corynebacterium*) and *Staphylococcaceae* (*Staphylococcus*) with ileal TLR4 and/or IL-6 expressions. However, different from birds challenged LPS without YCWP treatment, YCWP-fed birds (injected with LPS) also enriched some

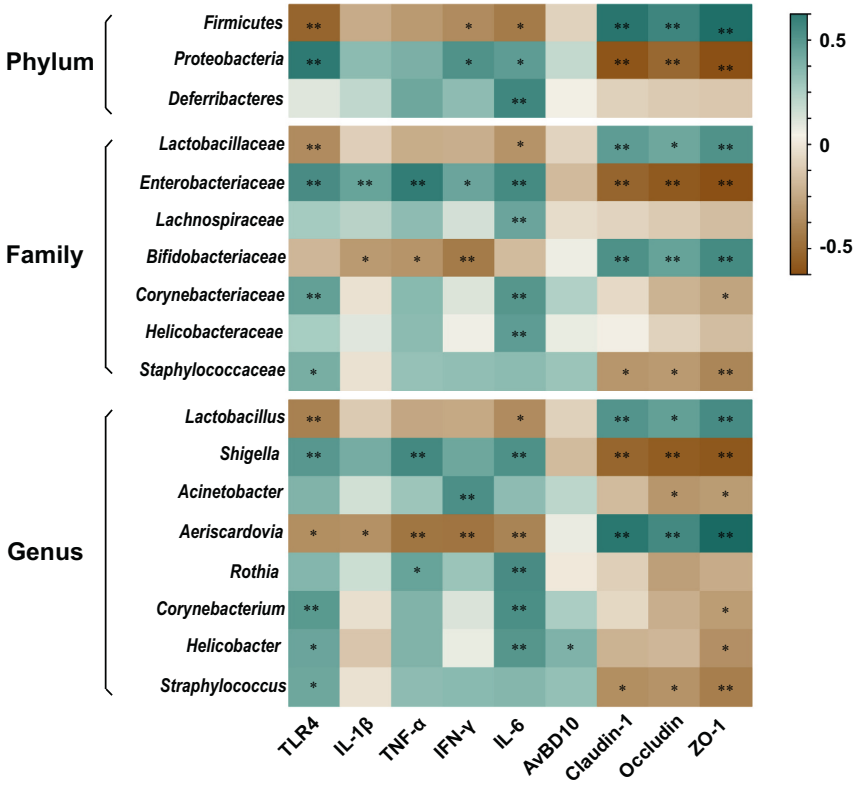

Fig. 8. Heatmap of Spearman's correlation between gut microbiota and ileal expressions of inflammatory cytokines and tight junctions. The intensity of the colors ranging from brown to jasper represented the magnitude of correlation. Significant correlation was noted by * $0.01 \leq P < 0.05$, ** $P < 0.01$. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

beneficial bacteria, such as *Streptomycetaceae* (*Streptomyces*), *Bacillaceae*, and *Desulfovibrio*. The positive effects of these beneficial gut microbes in metabolic demands and physiological response have been documented in previous literature. *Streptomyces* as a probiotic has been demonstrated to be positively correlated with egg production in laying hens [71]. *Bacillaceae* (mainly composed of *Bacillus*) could stimulate chickens' humoral and cell-mediate immunity [72]. *Desulfovibrio* was one of the main propionic acid producers that implicated in the alleviation of gut inflammation [73]. An indication that YCWP could modulate the gut microbiota in favor of microbes that could promote physiological response of laying hens to immune and inflammatory stress.

Taken together, gut microbial composition of birds exposed to LPS challenge or non-LPS challenge, and fed with or without YCWP were altered. LPS injection indeed caused a dysbiosis of ileal microbiota characterized by increased pathogens and decreased beneficial bacteria. Dietary supplemental YCWP elevated microbial diversity and *Bifidobacteriaceae* abundance in non-challenged birds, and remodeled gut microbiota composition in LPS-exposed laying hens, thereby modulating the microenvironmental homeostasis in ileum in the positive direction.

5. Conclusion

Animal welfare must be prioritized in order to enhance production efficiency, product quality and profitability. Antibiotics, which are often used as main intestinal health enhancers, have been abrogated in laying hens due to safety and health concerns. Alternative feed additive such as yeast cell-wall polysaccharides (YCWP) have been proven once again to be safe and possess the capacity to mitigate immune and inflammatory stress response in *Escherichia coli* LPS-challenged laying hens as well as birds under normal conditions. The immunomodulatory and anti-inflammatory effects of YCWP could be in part attributed to its capacity to modulate gut microbial composition, by enhancing the proliferation of beneficial microbes and suppression of pathogens colonization in the gut. The aforementioned findings in the current study support the notion that dietary interventions could be used to alleviate immune and inflammatory stress response in laying hens without causing an adverse effect on production performance.

CRediT authorship contribution statement

Zhou Jianmin: Investigation, Data Curation, Writing - Original Editing. Fu Yu: Investigation. Zhang Haijun: Writing - review & editing, Project administration. Qi Guanghai: Writing - review & editing, Supervision, Funding acquisition. Wang Jing: Writing - review & editing. Wu Shugeng: Supervision, Funding acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

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