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APPLIED MICROBIAL AND CELL PHYSIOLOGY

Fermented *Yupingfeng* polysaccharides enhance immunity by improving the foregut microflora and intestinal barrier in weaning rex rabbits

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Abstract *Yupingfeng* (YPF) is a kind of *Astragali radix*-based ancient Chinese herbal supplemented with *Atractylodis Macrocephalae Rhizoma* and *Radix Saposhnikoviae*. Increasing evidence has proven the beneficial immunomodulating activity of YPF. However, the action mechanism(s) of it is not known. Here, we explored the immunomodulatory activity of unfermented *Yupingfeng* polysaccharides (UYP) and fermented *Yupingfeng* polysaccharides (FYP) obtained using *Rhizopus oligosporus* SH in weaning Rex rabbits. The results showed that both UYP and FYP exhibited notable growth-promoting and immune-enhancing activities, improvement of the intestinal flora homeostasis, and maintenance of intestinal barrier integrity and functionality. Notably, compared with UYP, FYP effectively enhanced average daily gain, organ indices, interleukin-2 (IL-2), IL-4, IL-10, tumor necrosis factor-alpha (TNF-α), TLR2, and TLR4 mRNA levels in spleen, IL-1, IL-2, IL-4, IL-6, IL-10, IL-12, TNF-α, and IFN-γ protein concentrations in serum, and TLR2 and TLR4 mRNA expressions in the gastrointestinal tract (GIT). Moreover, FYP exhibited greater beneficial effects in improving the intestinal flora, including augment flora diversity and the abundance of cellulolytic bacteria, reduction the abundance of *Streptococcus* spp. and *Enterococcus* spp. in the GIT, particularly the foregut and maintaining the intestinal barrier integrity and functionality by upregulating zonula occludens 1, claudin, polymeric immunoglobulin receptor, trefoil factor, and epidermal growth factor mRNA levels in the jejunum and ileum. Our results indicated the immunoenhancement effect of FYP is superior over that of UYP, which is probably related with the amelioration of the intestinal microflora and intestinal barrier in the foregut.

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Introduction

A considerable number of traditional Chinese medicines (TCMs) have a long history of being used in Asian countries for the prevention and treatment of diseases and improvement of immunity (Ma et al. 2013), because TCMs cause few side effects and exhibit low toxicity (Li et al. 2008). In recent years, the interest in TCMs is growing not only commercially but also academically, due to a broad spectrum of immune

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regulatory activities of TCMs (Burns et al. 2010). *Yupingfeng* (YPF) is a kind of *Astragali radix* (AR)-based TCMs supplemented with *Rhizoma Atractylodis Macrocephalae* (RAM) and *Radix Saposhnikoviae* and has been clinically prescribed for hundreds of years to prevent or treat colds, flu, and inflammation-associated diseases (Hou and Xin 2000), including asthma (Chen et al. 2014), allergic rhinitis (Makino et al. 2004), and allergic conjunctivitis (Chen 2013). Modern pharmacological studies have shown that the YPF polysaccharides are one of the main active components of this formula (Jiang et al. 2010), which can wield bidirectional immunomodulatory effects (Du et al. 2013). Early study verified that the total polysaccharides of YPF could produce beneficial immunomodulatory effects by promoting the proliferation of splenocytes and the secretion of IL-2 (Yang et al. 2015). Moreover, *Rhizopus oligosporus* has been widely used to produce a variety of bean-fermented foods, such as fermented bean curd, fermented soya beans, soy sauce, and tempeh, which has a powerful enzyme production system, and *R. oligosporus*-fermented *canavalia* has been reported to improve the bioactive potential (Niveditha and Sridhar 2012; Vedavyas et al. 2014). TCMs fermented by probiotic exerted a greater immune effect (Bose et al. 2012a), including the biotransformation of YPF (Wang et al. 2010; Xu et al. 2006).

Increasing evidence indicated that the commensal microbiota is critical for the development and maintenance of immune and intestinal homeostasis through stimulating immune system and regulating the intestinal mucosal barrier functions in animals (Walsh et al. 2014). It has been shown that immune response is positively regulated by intestinal microbiota via highly conserved pattern recognition receptors (PRRs) including Toll-like receptors (TLRs), which recognize microbe-associated molecular patterns (MAMPs) (Goto and Kiyono 2012), resulting in specific immune responses, such as defensins and cytokine release (Netea and Meer 2011). Moreover, symbiotic bacteria also can strengthen intestinal barrier function by promoting the development and functional maturation of intestinal epithelial cells and intestinal architecture (Round and Mazmanian 2009), imparting stimuli for continuing repair and restoration of intestinal barrier (Sharma 2010). However, a reduced biodiversity and a disorder of gut flora are associated with dysregulation of immune responses and impairment of intestinal barrier (Round and Mazmanian 2009), which lead to hyperresponsiveness immune response and increased intestinal permeability, and eventually contribute to the development of inflammatory bowel disease (Dupont 2016). Conspicuously, substantial studies have revealed that probiotics, prebiotics, synbiotics, and TCMs (Chen et al. 2016) can exert beneficial effects on immunity via affection of intestinal microbiota homeostasis and regulation of intestinal barrier.

The fermentation with *R. oligosporus* has been discovered to increase active ingredients, and the fermentation process of TCMs has been uncovered to improve their pharmaceutical effects (Alolga et al. 2015). We hypothesized that fermented *Yupingfeng* polysaccharides (FYP) with *R. oligosporus* SH may be more successful than unfermented *Yupingfeng* polysaccharides (UYP) in enhancing the immune responses via improving the microflora homeostasis and intestinal barrier integrity and functionality. In the present study, employing weaning rex rabbit with an underdeveloped immune and digestive system, we undertaken to evaluate whether the beneficial immunomodulatory effects of UYP and FYP are related to ameliorate intestinal microbiota and barrier function, and whether FYP with *R. oligosporus* SH may wield a stronger helpful immunomodulatory impacts than UYP.

Materials and methods

Materials

Yupingfeng polysaccharides (YP) were provided by the Guangzhou Foshan Institute of Technology. The carbohydrate and protein concentrations of YP were 70.09 and 16.00 %, respectively. *R. oligosporus* SH (CCTCC M2015360) was screened and identified by the Microecosystem Engineering Laboratory, College of Veterinary Medicine, Sichuan Agricultural University, Chengdu, China (NCBI upload sequence ID: KP340799.1).

Preparation of FYP

YP was placed in flasks and dissolved in the ddH$_2$O (0.1 g mL^{-1}). The FYP was inoculated with 3 % *R. oligosporus* SH, whereas the UYP was inoculated with saline; both samples were fermented at 200 rpm, 37 °C for 2 days. The broths were subjected to low-speed centrifugation to sediment the particles, and the supernatants were stored at 4 °C. Endotoxin quantity of the final UYP and FYP broth was 4.46 ± 0.02 and 4.55 ± 0.05 EU/mL ($P > 0.05$), respectively.

Experimental design and sampling

The experiment was performed in the breeding center of rex rabbit research institution (Chengdu, China). After 7 days of acclimation in separated cages under the same temperature (25 ± 2 °C controlled by automatic heating and ventilation devices) with access to customized fodder without antibiotics free and water ad libitum, 45, weaning (day 35) rex rabbits (Sichuan White Rex Rabbit) were randomly divided into normal control (NC) group, UYP group and FYP group. During the entire experimental period of 4 weeks, rex rabbits were fed with customized fodder (Table S1) with saline, UYP, and FYP (10 mL/kg) in the NC group, UYP group, and FYP group, respectively. There were not illness and death during the

experimental period. At the end of the fourth week, rex rabbits were weighed, and 10 rex rabbits were randomly selected and sacrificed in each group. Blood was collected from the ear vein under anesthesia, and serum was separated. The rex rabbits were then sacrificed according to the institutional animal care guidelines. The spleen and liver were quickly excised and weighed, then stored in liquid nitrogen. Fresh fecal and intestinal tissue samples were collected from the jejunum, ileum, and cecum, which were immediately transferred into liquid nitrogen for temporary storage before they were sent to the laboratory where the samples were stored at −80 °C until further analysis.

Determination of serum levels of cytokines

The separated serums from each group were used for the cytokines assay. The levels of interleukin-1 (IL-1), IL-2, IL-4, IL-6, IL-10, IL-12, tumor necrosis factor-alpha (TNF-α), and interferon-gamma (INF-γ) in serum were measured using the ELISA kits (R&D, USA) according to the instructions, and the regression curves of various cytokines were presented in Table S2.

DNA extraction

Genomic DNA was isolated from the fecal samples (200 mg each) by using the TIANamp stool DNA kit (TIANGEN, Beijing, China) according to the instruction. For better extract of gram-positive bacteria DNA, the second incubation at 95 °C for 10 min was executed after the initial incubation at 70 °C for 5 min and the Inhibit EX was used to adsorb other impurities in the protocol. DNA quality was analyzed by 2 % (w/v) agarose gel electrophoresis. Finally, DNA was stored at −80 °C before further analysis.

PCR-DGGE analysis

The V3 region of the bacterial 16S rDNA of total bacterial DNA was amplified by the universal bacterial primer with a GC-clamp (314f-GC, 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG GCC TAC GGG AGG CAG CAG-3′ and 518r, 5′-ATT ACC GCG GCT GCT GG-3′) (Wang et al. 2015). PCR reaction was performed on MyCycler™ Thermal Cycler (Bio-Rad, CA, USA) with the following condition: 95 °C for 4 min; 35 cycles of 94 °C for 30 s, 58 °C for 30 s, and 72 °C for 1 min; and 72 °C for 8 min. The size and quality of PCR products were detected by 1.5 % agarose gel electrophoresis. Subsequently, denaturing gradient gel electrophoresis (DGGE) analysis of the mixtures (10 μL PCR product and 10 μL loading buffer) was performed using DCode™ Universal Mutation Detection System (Bio-Rad, CA, USA) as follows: polyacrylamide gel with 35–65 % linear gradients of denaturant (100 %

denaturant equivalents to 7 Murea and 40 % (v/v) formamide) in 1× Tris acetate-EDTA buffer (TAE). DGGE was performed at 60 °C for 5 min at 200 V and subsequent at 60 °C for 16 h at 100 V. After electrophoresis of the mixture, the polyacrylamide gel was stained with $AgNO_3$ and imaged using a BIO-RAD Gel Doc XR+ (Bio-Rad, CA, USA).

Quantitative PCR quantification of microbial

A CFX Connect™ Real-Time system (Bio-Rad, CA, USA) system and SYBR® Premix Ex Taq™ II (TaKaRa, Dalian, China) were used to perform quantitative PCR (Q-PCR), to estimate the abundance of well-known beneficial bacteria, harmful bacteria, and major cellulolytic bacteria in samples. Primers for Q-PCR of microflora are listed in Table 1. The reaction mixture (25 μL) included 9.5 μL of sterile deionized water, 12.5 μL SYBR® Premix Ex TaqTM II, 1 μL of forward, and reverse primer and 1 μL template DNA. The protocols were as follows: 95 °C for 1 min, subsequently, 40 cycles of 94 °C for 15 s, annealing at the optimal temperatures for 30 s, 72 °C for 30 s and subsequently, the melting curves were produced to supervise specificity of the PCR primers.

In order to build the standard curves, the amplified products were cloned and sequenced as described above. Positive clones were then cultured to extract the plasmids using A E.Z.N.A.™ plasmid mini kit (Omega Bio-Tek, USA), and the Nano Drop spectrophotometer was subsequently used to determine the concentration and quality of plasmid DNA, which include goal gene sequence. The standard curves were built using triplicate tenfold serial dilutions of the plasmid DNA. Copy numbers of the target bacterial phylum or genus for samples were calculated according to the standard curves.

Determination of mRNA expression levels by Q-PCR

Total RNA was isolated by RNAiso Plus reagent (Takara, Dalian, China) according to the protocol, and the RNA concentration was determined by a Nano Drop spectrophotometer. Then RNA was reverse transcribed into cDNA using PrimeScript™ RT reagent Kit (Takara, Dalian, China) according to the manufacturer's instructions. Finally, cDNA was stored at −80 °C before analysis.

Q-PCR was performed using SYBR® Premix Ex Tap™ II and ROX reference dye II following the manufacturer's instructions to estimate the relative expression of TLR2 and TLR4 in the spleen, liver, and intestinal tissue, and zonula occludens protein 1 (ZO-1), claudin, occludin, immunoglobulin A (IgA), polymeric immunoglobulin receptor (PIGR), β-defensin, trefoil factor 1-like (TFF), and epidermal growth factor (EGF) in intestinal tissue. The information of primers was listed in Table 2. The SYBR signal was detected by CFX Connect™ Real-Time

Table 1 Primer information and standard curves of microflora for Q-PCR

Bacterial species	Primer sequence (5 → 3)	Regression curve/Tm/size	Reference
Total bacteria	F: CGGYCCAGACTCCTACGGG R: TTACCGCGGCTGCTGGCAC	$y = 14.354–0.2607X$ $R^2 = 0.997$ Tm = 60 °C 130 bp	Guo et al. (2008)
Firmicutes	F: GGAGYATGTGGTTTAATTCGAAGCA R: AGCTGACGACAACCATGCAC	$y = 13.073–0.2985X$ $R^2 = 0.998$ Tm = 60 °C 126 bp	
Bacteroidetes	F: GGARCATGTGGTTTAATTCGATGAT R: AGCTGACGACAACCATGCAG	$y = 14.390–0.2807X$ $R^2 = 0.998$ Tm = 58 °C 126 bp	
Fibrobacter succinogenes	F: GGTATGGGATGAGCTTGC R: GCCTGCCCCTGAACTATC	$y = 13.098–0.1725X$ $R^2 = 0.996$ Tm = 56 °C 445 bp	Koike and Kobayashi (2001)
Ruminococcus flavefaciens	F: TCTGGAAACGGATGGTA R: CCTTTAAGACAGGAGTTTACAA	$y = 14.211–0.2739X$ $R^2 = 0.998$ Tm = 57 °C 295 bp	
Prevotella prophyromonas	F: GGTGTCGGCTTAAGTGCCAT R: CGGAYGTAAGGGCCGTGC	$y = 10.508–0.2952X$ $R^2 = 0.993$ Tm = 58 °C 140 bp	Rinttila et al. (2004)
Bifidobacterium	F: TCGCGTCYGGTGTGAAAG R: CCACATCCAGCRTCCAC	$y = 13.837–0.2045X$ $R^2 = 0.998$ Tm = 62 °C 243 bp	
Clostridium cluster I	F: ATGCAAGTCGAGCGAKG R: TATGCGGTATTAATCTYCCTTT	$y = 13.376–0.2266X$ $R^2 = 0.998$ Tm = 60 °C 120 bp	
Enterococcus spp.	F: CCCTTATTGTTAGTTGCCATCATT R: ACTCGTTGTACTTCCCATTGT	$y = 14.356–0.2542X$ $R^2 = 0.998$ Tm = 52 °C 144 bp	
Lactobacillus	F: AGCAGTAGGGAATCTTCCA R: CACCGCTACACATGGAG	$y = 14.899–0.2863X$ $R^2 = 0.999$ Tm = 58 °C 341 bp	Walter et al. (2001)
Butyrivibrio fibrisolvens	F: CGCATGATGCAGTGTGAAAAGCTC R: CCTCCCGACACCTATTATTCATGG	$y = 14.290–0.2907X$ $R^2 = 0.994$ Tm = 60 °C 240 bp	Fernando et al. (2010)
Clostridium cluster IV	F: GCACAAGCAGTGGAGT R: CTTCCTCCGTTTTGTCAA	$y = 14.288–0.2472X$ $R^2 = 0.992$ Tm = 60 °C 230 bp	Matsuki et al. (2004)
Clostridium cluster XIVa	F: AAATGACGGTACCTGACTAA R: CTTTGAGTTTCATTCTTGCGAA	$y = 14.748–0.2615X$ $R^2 = 0.997$ Tm = 60 °C 440 bp	Matsuki et al. (2002)
Enterobacteriaceae	F: CATTGACGTTACCCGCAGAAGAAGC R: CTCTACGAGACTCAAGCTTGC	$y = 13.870–0.1927X$ $R^2 = 0.995$ Tm = 52 °C 195 bp	Bartosch et al. (2004)
Streptococcus spp.	F: AGAGTTTGATCCTGGCTCAG R: GTTAGCCGTCCCTTTCTGG	$y = 14.086–0.2630X$ $R^2 = 0.996$ Tm = 60 °C 485 bp	Franks et al. (1998)

system. Amplification was performed as follows: predenaturation at 95 °C for 4 min, followed by 40 cycles of denaturation at 94 °C for 15 s, annealing for 15 s at suitable temperature, extension at 72 °C for 15 s, and another extension at 72 °C for 8 min. The melting curve was acquired after amplification phase, and 5 μL of the PCR products was drawn for electrophoresis by using 1.2 % agarose gel to confirm the specific amplicon. The amplification efficiency of the abovementioned genes was controlled between 98 and 103 %. Relative expression of the genes was analyzed using the $2^{-\Delta\Delta CT}$ method; mRNA levels were presented as fold changes after normalization to the housekeeping gene (GAPDH) and relative to a calibrator.

Statistical analysis

NTSYS 2.10 package (Exeter software) and SPSS 19.0 were used to construct a phylogenetic tree and principal component analysis (PCA) of DGGE profiles, respectively. Biodiversity index of DGGE profiles, Shannon-Wiener index, species evenness index, and species richness index

were calculated as previously described (Gupta et al. 2015). Data were expressed as mean ± SD and analyzed using SPSS version 19.0. The difference was evaluated by one-way ANOVA and considered significant if $P < 0.05$ or $P < 0.01$.

Results

The average daily gain and organ index of rex rabbit

The average daily gain (ADG) and organ indices are presented in Fig. 1. Treatment with UYP ($P < 0.05$) and FYP ($P < 0.01$) significantly increased the ADG of rex rabbit, and the ADG in the FYP group was significantly higher than that in the UYP group ($P < 0.01$, Fig. 1a). The spleen index of the UYP and FYP groups showed higher values than that of the NC group ($P < 0.01$), and in the FYP group, the spleen index was significantly higher than that in the UYP group ($P < 0.05$, Fig. 1b), while UYP and FYP have less effect on the liver index ($P > 0.05$).

Table 2 Primer information of genes for Q-PCR

Gene name	Primer sequence (5 → 3)	Tm (°C)/ size (bp)	Accession
IL-2	F: TGAAACATCTTCAGTGTCTAGAAG	60/203	Z36904.1
	R: CCATTTGTTCAGAAATTCTACAATG		
IL-4	F: GTCACTCTGCTCTGCCTCCT	60/302	AF169170.1
	R: GCAGAGGTTCCTGTCGAGTCC		
IL-10	F: GAGAACCACAGTCCAGCCAT	60/179	D84217.1
	R: CATGGCTTTGTAGACGACGTT		
IFN-γ	F: CGGTGGATGATCGGCTGGTCC	60/124	NM_001081991.1
	R: CGCCGACCTCGAAACAGCGT		
TNF-α	F: CCTGCTGGCCAACGGCATGA	60/120	AB128153.1
	R: AGGAGCACGTAGGAGCGGCA		
TLR2	F: TGCCTCCTTGTTACCTATGC	57/153	NM_001082781
	R: AGATGAAGTTGTTCCCTCCG		
TLR4	F: GAGCACCTGGACCTTTCAAATAAC	57/235	NM_001082732
	R: GAACTTCTAAACCACTCAGCCCTTG		
Claudin	F: GGAGCAAAAGATGCGGATGG	60/93	NM_001089316.1
	R: AATTGACAGGGGTCAAAGGGT		
Occludin	F: GCAAGAGGCCGTATCCAGAG	60/207	XM_008262320.1
	R: AGTCCGTCTCGTAGTGGTCT		
ZO-1	F: CCTGCGAAACCCACCAAAG	60/293	XM_008269782.1
	R: ATGCTGTCAGAAAGGTCAGGG		
IgA	F: GCTTTCTACGGCTTGGACCT	60/128	AF090361.1
	R: ACGGTCGCTGAAGTGTCTTT		
PIGR	F: ACCATCACCTGCCCTTTCAC	60/286	NM_001171045.1
	R: GACCAGGAGTTAGCAGTCGG		
β-defensin	F: CAGGGGCCGAAAATCTTGCT	60/104	XM_008256301.1
	R: TGCAGCAGTAGTTGCCGTTT		
TFF	F: TGTTTTAACCGCTCCATCCCC	60/84	XM_002724403.2
	R: GAACGGGCACTCCTCTTCT		
EGF	F: CTGGGTCCTGCACCTTTCTT	60/164	XM_008267485.1
	R: TGCCTTTCCAAATCCACCCA		
GAPDH	F: TGTTTGTGATGGGCGTGAA	56/129	NC_013676.1
	R: CCTCCACAATGCCGAAGT		

Effects of UYP and FYP on cytokine expression in the spleen

The mRNA expressions of inflammatory (IL-2, TNF-α, and IFN-γ) and anti-inflammatory (IL-4 and IL-10) cytokines were raised after administration of UYP and FYP (Fig. 2). Compared to NC group, FYP significantly increased the expressions of IL-2 and TNF-α, and UYP appreciably enlarged the expression of IFN-γ ($P < 0.05$) (Fig. 2a). However, only FYP significantly upregulated the expressions of IL-10 ($P < 0.05$) and IL-4 (Fig. 2b). Meanwhile, there were no significant differences between the UYP and FYP in the expression of cytokines ($P > 0.05$).

Effects of UYP and FYP on cytokine secretion in serum

As shown in Fig. 3, UYP and FYP increased the concentrations of IL-2, IL-4, IL-12, TNF-α, and IFN-γ when compared with NC group in the serum, which indicated that UYP and FYP could promote the secretion of some cytokines and maintain a low level and controllable of immune activation. However, treatment with only FYP effectively improved the concentrations of cytokines in serum and the levels of IL-2, IL-6, and TNF-α, and IL-10 (Fig. 3b, c, e, and h) in FYP group were significantly higher than those in UYP group ($P < 0.05$ or $P < 0.01$).

Effects of UYP and FYP on TLR2 and TLR4 expressions

The mRNA expressions of TLR2 and TLR4 were significantly elevated by treatment with UYP and FYP (Fig. 4). Treatment with UYP increased the mRNA expression of TLR2 in the jejunum ($P < 0.05$), ileum, and spleen ($P < 0.01$) (Fig. 4a), and improved TLR4 mRNA in the ileum and spleen ($P < 0.01$) (Fig. 4b). Treatment with FYP resulted in significantly augmented TLR2 mRNA expression in the jejunum, ileum, and spleen ($P < 0.01$) (Fig. 4a), and declined the TLR2 mRNA expression in the cecum and enlarged TLR4 mRNA in the jejunum, ileum, and cecum ($P < 0.01$) (Fig. 4b) compared with the NC group. Furthermore, compared to UYP, FYP significantly upregulated TLR2 mRNA expression in the jejunum ($P < 0.05$) and ileum ($P < 0.01$) (Fig. 4a), TLR4 mRNA expression in the jejunum ($P < 0.05$) and spleen ($P < 0.01$) (Fig. 4b), TLR2 expression in the spleen, and

Fig. 1 The ADG and organ indices of rex rabbits in each group. **a** ADG ($n = 15$). **b** Spleen index. **c** Liver index ($n = 10$). * and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with NC group and UYP group, respectively
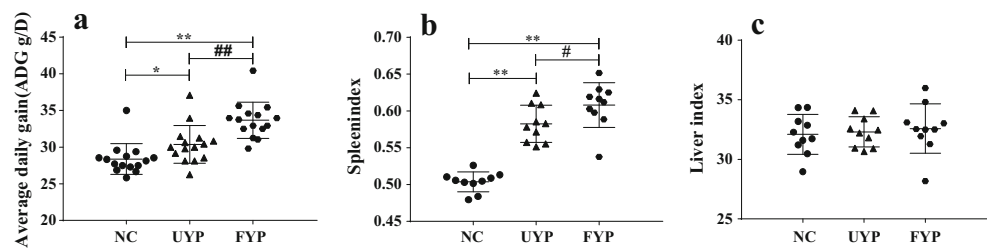
TLR4 in the ileum and cecum. However, there was no significant effect on TLR2 mRNA expression in the cecum ($P > 0.05$).

Intestinal microbiota community was revealed by PCR-DGGE

In this study, complicated and analogous DGGE fingerprints of total bacterial community were presented significantly different among the test groups in the gastrointestinal tract (GIT) (Fig. 5). The DGGE patterns found in feces from between UYP group and FYP group seemed to be more similar than those from the NC group in the jejunum and ileum (Fig. 5a and b), whereas slight difference appeared among the three groups in the cecum. The discernable bands of the DGGE profiles were descending along the cecum, ileum, and jejunum. Some bands appeared in all groups, whereas some bands only appeared in NC group, UYP group, or FYP group. Meanwhile, the strengths of some bands changed considerably. And the banding patterns in the UYP or FYP group seemed to be more diverse than those obtained from the NC group in jejunum and ileum (Fig. 5a and b). Additionally, the discernable bands of samples from FYP seemed to be the most diverse than those observed from the other groups in the GIT (Fig. 5).

Treatment with UYP or FYP caused a notable alteration in the bacterial community, as also revealed by the unweighted

Pair Group Method with Arithmetic mean algorithm (UPGMA) dendrogram and by the principal component analysis (PCA) based on the unweighted UniFrac distance matrixes derived from DGGE profiles. More specifically, the dendrograms from the total samples illustrated that each group was clustered into a cluster. The similarity coefficients of the banding patterns from the different groups were significant difference from 44, 39, and 71 % to 100 % in the jejunum, ileum, and cecum, respectively. Thus, the similarity coefficient of the samples from the cecum was the highest. The samples in the FYP and NC groups were clustered into a large branch in the cecum (about 78 % similarity, Fig. 5c), whereas the FYP and UYP were clustered into a large branch in the jejunum and ileum (both possessed about 72 % similarity, Fig. 5a and b). Meanwhile, PCA plots were consistent with the above dendrograms, which revealed the relationships between the community structures of the rex rabbit gut microbiota, and demonstrated that treatment groups separately gathered together in different locations in different GIT (Fig. 5d, e, and f). The first and second principal components explained 58.14 and 21.45 %, 61.41 and 19.66 %, and 36.40 and 25.23 % of the variance in the jejunum, ileum, and cecum, respectively. Thus, the flora community structures of UYP group and FYP group showed higher relatedness in the jejunum and ileum (Fig. 5d and e), while the higher relatedness of the flora community structures appeared in between the NC group and FYP group in the cecum (Fig. 5f).
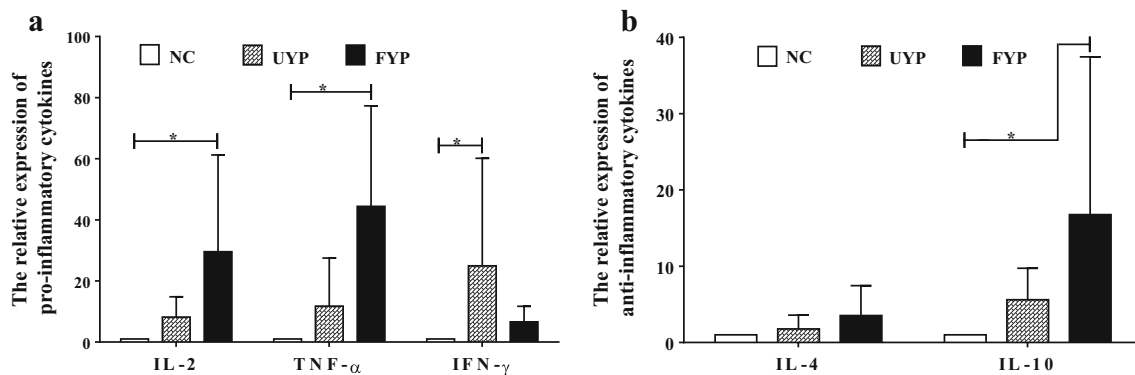

Fig. 2 The mRNA relative expressions of cytokines in the spleen. **a**, **b** Inflammatory and anti-inflammatory cytokines, respectively. The mRNA quantification data were normalized to the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH). Gene expression level was expressed as values relative to the NC group, and presented with the means ± standard deviation ($n = 6$). *and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with NC group and UYP group, respectively. *IL-2* interleukin-2, *IL-4* interleukin-4, *IL-10* interleukin-10, *TNF-α* tumor necrosis factor-α, *IFN-γ* interferon-γ
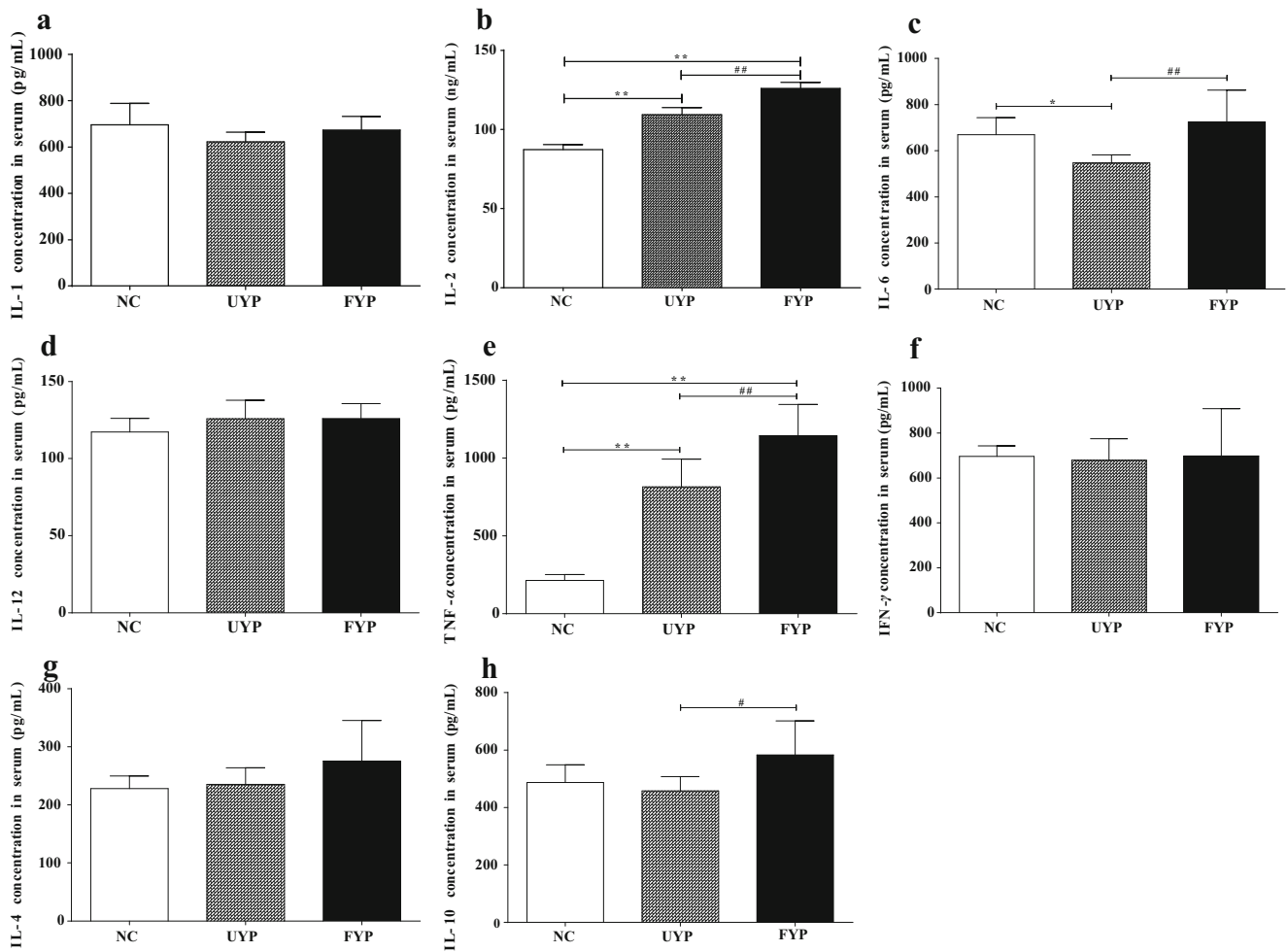
Fig. 3 Effects of UYP and FYP on cytokine concentration in serum. **a–h** Concentration of IL-1, IL-2, IL-6, IL-12, TNF-α, IFN-γ, and IL-4 and IL-10, respectively. Data were presented with the means ± standard deviation ($n = 6$). *and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with NC group and UYP group, respectively. *IL-1* interleukin-1, *IL-2* interleukin-2, *IL-4* interleukin-4, *IL-6* interleukin-6, *IL-10* interleukin-10, *IL-12* interleukin-12, *TNF-α* tumor necrosis factor-α, *IFN-γ* interferon-γ
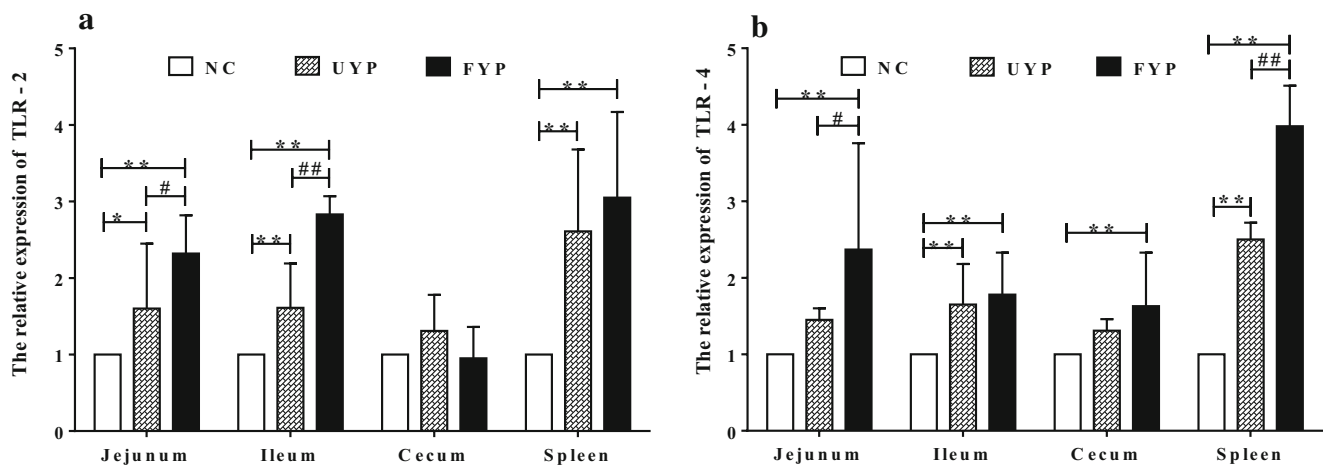
Fig. 4 Effects of UYP and FYP on TLR2 and TLR4 expressions in rex rabbit. **a, b** TLR2 and TLR4, respectively. The mRNA quantification data were normalized to GAPDH. Gene expression level was expressed as values relative to the NC group and presented with the means ± standard deviation ($n = 6$). *and # indicate $P < 0.05$, ** and ## indicates $P < 0.01$, compared with NC group and UYP group, respectively

In addition, bacterial diversity was clarified by DGGE fingerprint analysis (Fig. 5). There was no significant difference in the Shannon-Weiner diversity index (Fig. 5g), evenness index (Fig. 5h), and richness index (Fig. 5i) among the three groups in the cecum ($P > 0.05$), while these indices were significantly higher in the UYP and FYP groups when compared with the NC group in the jejunum and ileum ($P < 0.05$ or 0.01). These indices in the FYP group were consistently higher than those of UYP in the jejunum ($P < 0.05$) and ileum (Fig. 5g, h, and i). Overall, these results indicated that the different treatments caused different microbial community structures and predominant population. The intestinal microflora structures of the jejunum and ileum, but not the cecum, were greatly affected after treatment with UYP and FYP, especially in the FYP groups.

Microbial populations quantified by Q-PCR

Treatment with UYP and FYP resulted in considerable alteration in gut flora including differentially increasing the abundance of total bacteria, Firmicutes, Bacteroidetes, *Prevotella prophyromonas*, *Butyrivibrio fibrisolvens* (except for UYP in ileum), *Clostridium cluster IV*, and *Clostridium cluster XIVa* ($P < 0.01$, Fig. 6a–c, d, g, h, and j), as well as remarkably reduced the abundance of *Enterococcus* spp. ($P < 0.01$, Fig. 6n) in the jejunum and ileum, but not in the cecum. Moreover, the abundance of *Lactobacillus* was significantly enhanced in the cecum and jejunum ($P < 0.05$, Fig. 6e); the abundance of *Enterococcus* spp. in the cecum ($P < 0.05$) and the abundance of *Streptococcus* spp. in the cecum and ileum ($P < 0.01$) were significantly decreased upon treatment with UYP and FYP (Fig. 6n and o). After FYP treatment, the abundance of total bacteria, Firmicutes, Bacteroidetes, *P. prophyromonas*, *Lactobacillus*, *Bifdobacterium*, *Fibrobacter succinogenes*, *B. fibrisolvens*, *Clostridium cluster IV*, and *Clostridium cluster XIVa* remained a higher level, whereas that of Enterobacteriaceae, *Enterococcus* spp., and *Streptococcus* spp. were lower than those in the NC group in the GIT. Interestingly, after FYP treatment, the abundance of total bacteria, Firmicutes, *B. fibrisolvens* in the ileum ($P < 0.01$), *Bifidobacterium* spp. in the cecum ($P < 0.05$), and *F. succinogenes* in the jejunum ($P < 0.05$) were obviously higher, but that of Enterobacteriaceae in the ileum and *Enterococcus* spp. in the ileum and jejunum were clearly lower ($P < 0.01$) in comparison with UYP (Fig. 6). Others showed no significant difference among the three groups in the GIT. Overall, these results indicated treatment with UYP or FYP resulted in a considerable impact on the number of some vital bacteria in the jejunum and ileum, particularly in FYP group.

Effects of UYP and FYP on physical barrier in intestine

The genes expressions of tight junction, including ZO-1, claudin, and occludin in the jejunum and ileum were shown in Fig. 7a and d, respectively. FYP upregulated the mRNA expressions of ZO-1, claudin, and occludin in the jejunum and ileum, whereas only the mRNA expressions of claudin in the jejunum and ZO-1, claudin, and occludin in the ileum in UYP groups. In addition, only FYP established a higher level of mRNA expression of the genes in the GIT. The mRNA expressions of ZO-1 and claudin in the FYP group were significantly higher than that in the UYP and NC groups in the ileum ($P < 0.05$, Fig. 7d).

Effects of UYP and FYP on immunological barrier in the intestine

As shown in Fig. 7b and e, treatment with UYP and FYP markedly promoted the mRNA expressions of IgA, PIGR, and β-defensin in the jejunum and ileum. The mRNA expressions of β-defensin in the jejunum and ileum ($P < 0.05$) and PIGR in ileum ($P < 0.01$, Fig. 7e) in FYP were noticeably higher than that in NC group. Meanwhile, a higher level of mRNA expression still appeared in the FYP group; the mRNA expression of PIGR in FYP group was significantly higher than that in the UYP group in the ileum ($P < 0.01$, Fig. 7e).

Effects of UYP and FYP on chemical barrier in the intestine

The results of Fig. 7c and f showed higher mRNA levels of TFF and EGF were observed in the UYP and FYP groups when compared with the NC group. The mRNA expression of TFF was obviously facilitated in the jejunum by UYP ($P < 0.05$) and FYP ($P < 0.01$, Fig. 7c), and that in ileum by FYP ($P < 0.05$, Fig. 7f). Meanwhile, only treatment with FYP resulted in significantly increasing the mRNA expression of EGF in the jejunum ($P < 0.05$, Fig. 7c) and ileum ($P < 0.01$, Fig. 7f). In addition, the mRNA expression of EGF in FYP group was significantly higher than that in the UYP group in the jejunum ($P < 0.05$, Fig. 7c) and ileum ($P < 0.01$, Fig. 7f).

Discussion

The beneficial effects of commensal microflora, probiotics, and their fermentation products on health and immunity were well evidenced (Dupont 2016). The fermentation process has been revealed to improve the biological properties and pharmacological activities of TCMs (Alolga et al. 2015). In the study, we investigated whether FYP with *R. oligosporus* SH could produce a stronger immunomodulatory property in comparison with UYP and the mechanism of immunopotentiating effects of UYP and FYP in rex rabbit. Our results indicated UYP and FYP possessed growth promotion and immunopotentiating capability and could improve
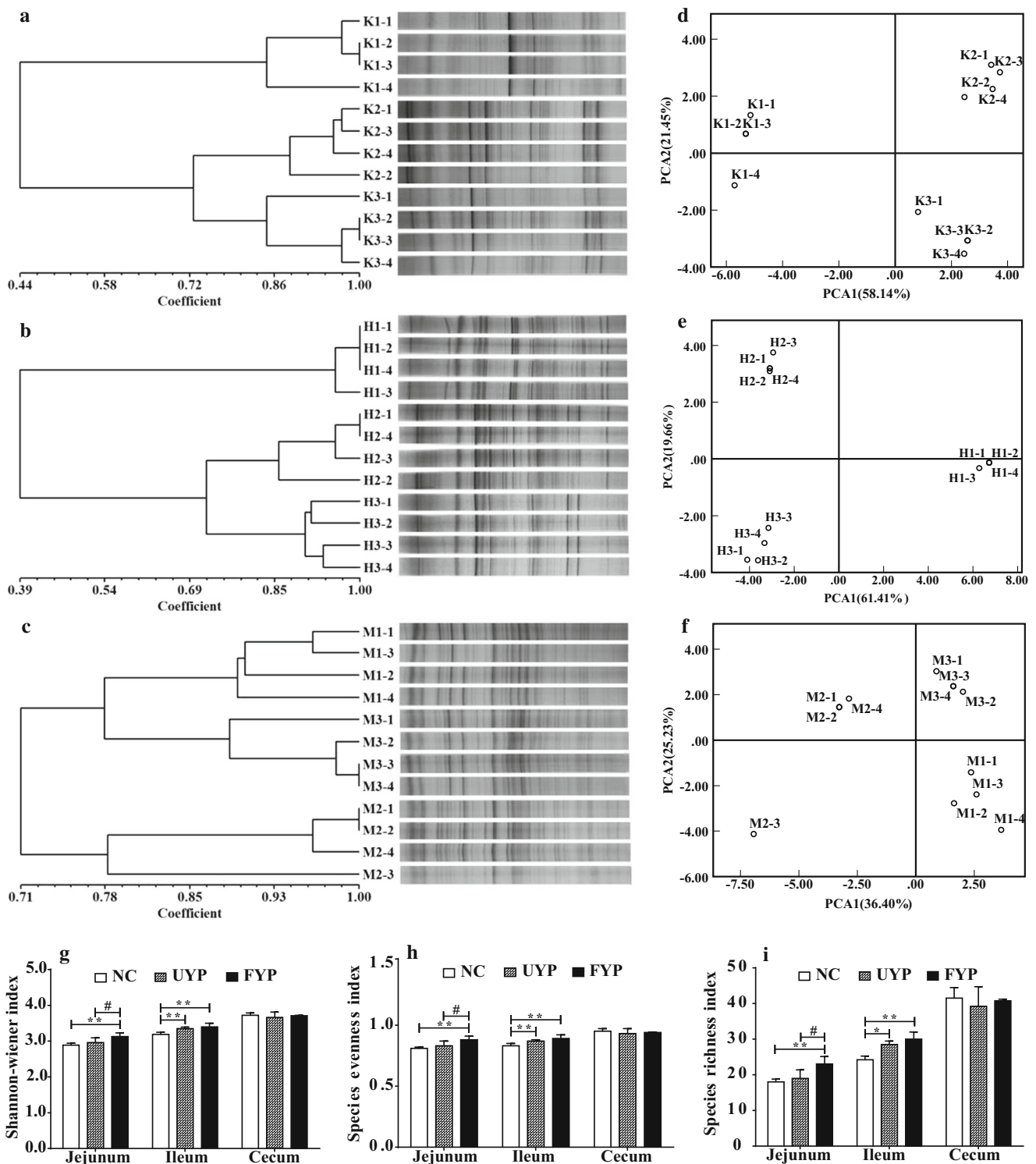
Fig. 5 Effects of UYP and FYP on the intestinal microbiota community. **a–f** UPGMA dendrogram combined with PCR-DGGE profiles, and PCA profiles of the jejunum, ileum, and cecum, respectively. **g–i** Shannon-Wiener index, evenness index, and richness index of the gastrointestinal microbiota on DGGE profiles, respectively. Data were presented with the means ± standard deviation ($n = 4$). *and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with NC group and UYP group, respectively. *K* jejunum, *H* ileum, *M* cecum. *K1*, *H1*, and *M1* NC group; *K2*, *H2*, and *M2* UYP group; *K3*, *H3*, and *M3* FYP group

the balance of intestinal flora and maintain the integrity of intestinal barrier.

The spleen is a pivotal immune organ for creatures, which is the node of development, activation, and
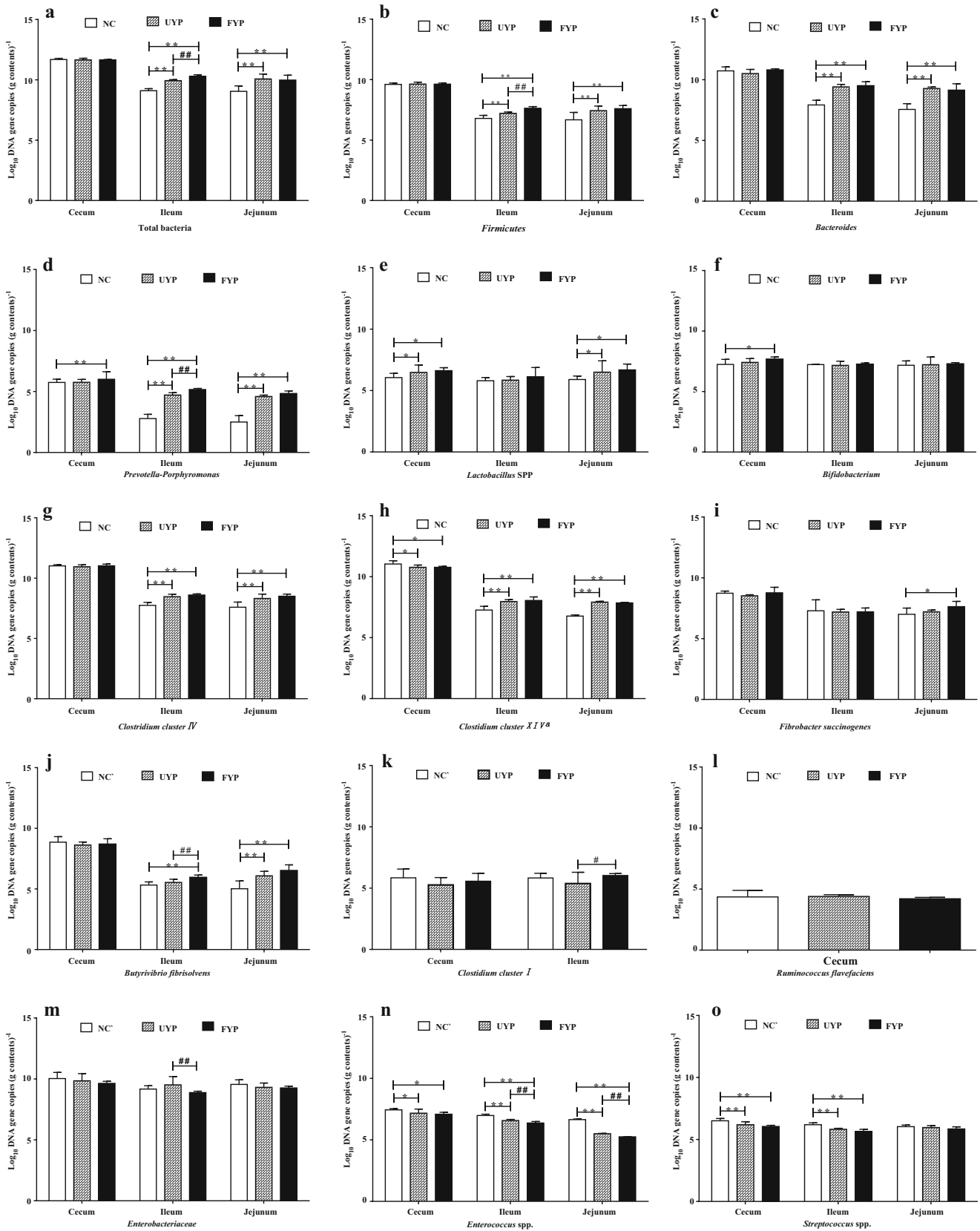
proliferation of immune cells. The development state of immune organs directly impacts immune function and the capability of disease resistance. Thus, the immune organ index is known as the preliminary indicators to reflect

Fig. 6 Effects of UYP or FYP on functional bacteria. **a–o** Log_{10}DNA gene copies of total bacteria, Firmicutes, Bacteroidetes, *Prevotella prophyromonas*, *Lactobacillus* spp., *Bifidobacterium*, *Clostridium cluster IV*, *Clostridium cluster XIVa*, *Fibrobacter succinogenes*, *Butyrivibrio fibrisolvens*, *Clostridium cluster I*, *Ruminococcus flavefaciens*, Enterobacteriaceae, *Enterococcus* spp., and *Streptococcus* spp. in the cecum, ileum, and jejunum, respectively. Log_{10}DNA gene copies quantification data were normalized to the standard curve lines and presented with the means ± standard deviation ($n = 6$). *and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with the NC group and UYP group, respectively

immune organ development and immune function. Previous study declared that the protective function of AR on the spleen of rats with obstructive jaundice by inhibiting apoptosis in the spleen (Zhang et al. 2009), and the polysaccharides of *Cyrtomium macrophyllum* (CM) could promote the development of the spleen (Ren et al. 2014). Compared with YPF, YPF-fermented product significantly increased spleen index in immunosuppressive mice (Xu et al. 2006). Similar to the results, the present experiment showed that the spleen indices in the UYP and FYP groups were higher than that in the NC group (Fig. 1b). Treatment with only FYP induced higher spleen indices (Fig. 1b). Based on these results, it is conceivable that UYP and FYP could promote the development of the spleen, and FYP may be more effective than UYP in promotion of immune organ indices.

Cytokines are soluble extracellular pleiotropic peptides or glycoproteins, which possess multiple sources, multiple targets, and multiple functions (Burns et al. 2010), such as engaging in innate and adaptive inflammatory host-protective responses, regulating the development, differentiation, activation, and function of immune cells, as well as starting repair processes aimed to maintain homeostasis (Hiscott and Ware 2011). A number of studies have reported that TCMs induced appropriate immune responses through regulation of mRNA and protein expression of cytokines genes (Burns et al. 2010). Various polysaccharides of TCMs including CM (Ren et al. 2014) have beneficial effects on cytokines expression. Moreover, the biotransformation of YPF could enhance the immune function by improving the contention of IL-2 in serum (Wang et al. 2010). Our results were consistent with these findings that treatment of UYP and FYP augmented mRNA expressions of IL-2, IL-4, IL-10, TNF-α, and IFN-γ in the spleen when compared to the NC group. The mRNA expressions of IL-2, IL-4, IL-10, and TNF-α were significantly enhanced only by FYP ($P < 0.05$, Fig. 2). Simultaneously, both UYP and FYP exhibited immune-enhancing activity were revealed by their capability to increase the concentrations of IL-2, IL-4, IL-12, TNF-α, and IFN-γ in serum of rex rabbit and to decrease the ratio of IFN-γ/IL-4, indicating that the Th1/Th2 balance toward Th2 response. FYP also disclosed a significantly higher facilitation of IL-2, IL-6, TNF-α, and IL-10

secretion compared to UYP (Fig. 3). These results suggested that both UYP and FYP could ameliorate immune responses by regulation of cytokine synthesis, and FYP is more effective than UYP. In addition, the variation trends of cytokine concentrations in serum and cytokines mRNA levels in the spleen were not all identical, indicating that UYP and FYP not only affecting the transcription of cytokines but also regulating cytokines translation.

Ligands induce TLR2 constructing a heterodimer with either TLR1 or TLR6 to regulate cytokine synthesis. TLR2/1 elevates IFN-γ secretion, while TLR2/6 stimulates IL-10 production, which indicated that TLR2 signaling possesses bidirectional immunomodulatory activity (Bryant et al. 2015). Maybe, this explains why both pro- and anti-inflammatory cytokines were improved by UYP or FYP (Figs. 2 and 3). TLR4 is other important PRRs associated with immunity and is expressed in immune cells and enterocytes, and it elicits cytokine synthesis with recruitment of innate and adaptive immune cells to improve the body's defense function via activation of NF- B (Frosali et al. 2015). TCMs and their components affected the activation status of DCs via TLRs signaling pathways (Li and Zhang 2015). The *rhubarb* polysaccharides evoked immunomodulatory activity through the TLR4 signaling pathway (Zhang et al. 2013). Our results showed that the mRNA expression of TLR2 and TLR4 were upregulated by UYP or FYP, and only FYP could more effectively upregulate TLR2 and TLR4 mRNA levels (Fig. 4). Based on these findings, it is suggested that immune-enhancing activities of UYP and FYP probably through activating TLR2 and TLR4 pathways, and the effect was better for FYP.

TCMs can affect the intestinal flora (Chen et al. 2016), for instance, the unfermented and fermented *Rhizoma coptidis* significantly augmented the population of *Bifodobacterium* and *Lactobacillus* (Bose et al. 2012b) as well as the RAM polysaccharide which could also obviously ameliorate and adjust the disordered intestinal microflora (Wang et al. 2014a). Simultaneously, microbial flora plays a crucial role in accurately regulating the immune responses, and alterations in the gut microbiota are associated with shifts in intestinal immune response (Ivanov and Dan 2011). Unfermented and fermented *Flos Lonicera* maintained immune balance through significantly improving disordered intestinal flora and intestinal permeability (Wang et al. 2014b). In the study, PCR-DGGE profiles revealed that the patterns of intestinal flora possessed noticeable differences among the three groups, and the bacterial diversities were significantly higher in the UYP and FYP groups than that in the NC group in the jejunum and ileum (Fig. 5). In addition, cluster analyses of PCR-DGGE fingerprints indicated that each group was distinctly separated cluster, and the lowest similarity coefficients of the banding patterns among the different groups were 44 and 39 % in the jejunum and ileum (Fig. 5a and b). PCA analyses indicated a close association of the UYP and FYP groups in
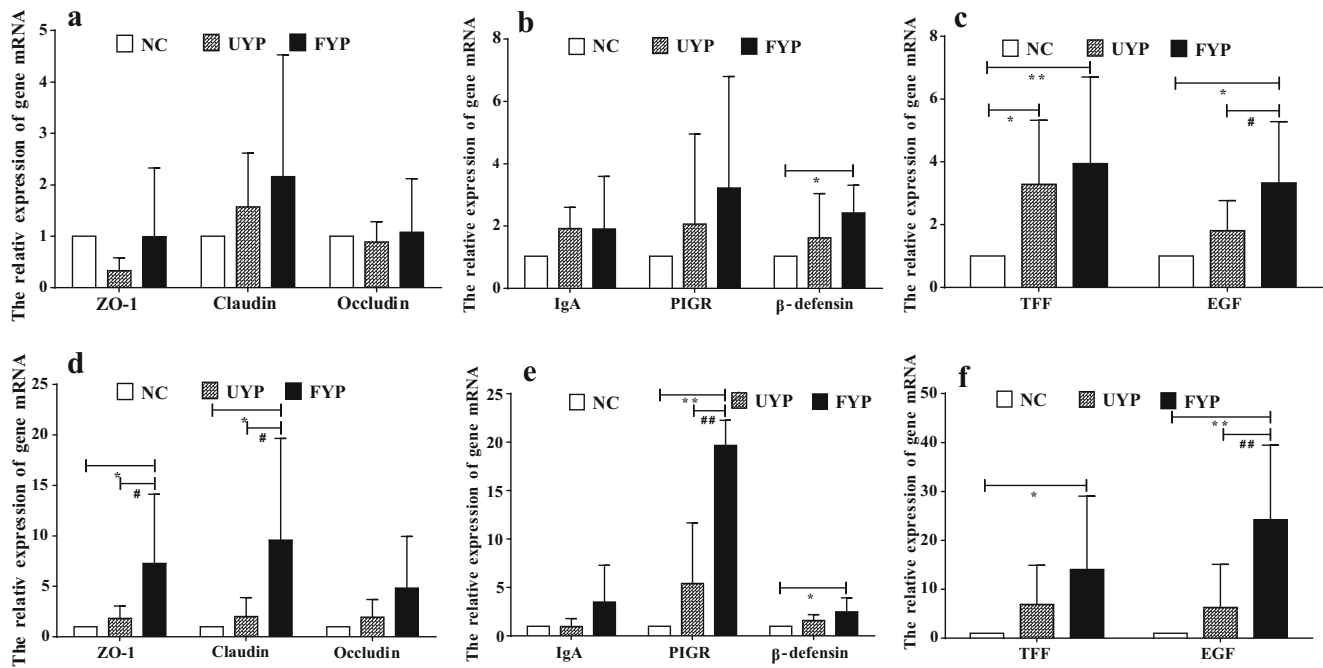
Fig. 7 Effects of UYP or FYP on intestinal barrier of rex rabbit. **a–c** Physical barrier, immunological barrier, and chemical barrier in the jejunum, **d–f** indicates those in the ileum, respectively. The mRNA quantification data were normalized to GAPDH. Gene expression level was expressed as values relative to the NC group and presented with the means ± standard deviation ($n = 6$). *and # indicate $P < 0.05$, ** and ## indicate $P < 0.01$, compared with NC group and UYP group, respectively. *ZO-1* zonula occludens protein 1, *IgA* immunoglobulin A, *PIGR* polymeric immunoglobulin receptor, *TFF* trefoil factor 1-like, *EGF* epidermal growth factor

the jejunum and ileum (Fig. 5d and e). These findings implied that treatment with UYP or FYP resulted in different microbial community structures and predominant flora in the foregut, and compared to UYP, FYP can exert a more beneficial consequence.

Moreover, in our study, there was a rise in the abundance of total bacteria, Firmicutes, Bacteroidetes, and beneficial bacteria, while the number of harmful bacteria was decreased after treatment with UYP or FYP in the GIT (Fig. 6). These results implied that microbial community structures were improved by UYP and FYP. Firmicutes and Bacteroidetes are predominant bacteria in the GIT of rex rabbits (Zeng et al. 2015). Firmicutes mainly includes multiple cellulolytic bacteria, which is closely related to bioconversion of feeds in the body (Evans et al. 2011). Bacteroidetes is known to promote the catabolism of plant cell wall (Cheryl et al. 2006), and increased abundance of Bacteroidetes might ameliorate the intestinal mucosal barrier function, ultimately enhancing the innate immune responses (Sonnenburg et al. 2005). Results of this study indicated that UYP and FYP significantly raised the abundance of Firmicutes and Bacteroidetes in the jejunum and ileum ($P < 0.01$, Fig. 6b and c), and the abundance of total bacteria, Firmicutes, and Bacteroidetes remained a higher level only in the FYP group than that in the NC group in the GIT, especially in the jejunum and ileum (Fig. 6a, b, and c). These results demonstrated that intestinal microenvironment and gut microflora composition appeared significant changes

treatment with UYP and FYP in foregut, and FYP possessed better activity.

P. prophyromonas are representative species of sugar-fermenting microbes and synergistically participate in complex carbohydrate disintegration along with cellulolytic bacteria via production of β-galactosidases (Nathani et al. 2015). *F. succinogenes* and *B. fibrisolvens* are major cellulolytic bacteria species in rumen ecosystem (Nathani et al. 2013), converting undigested carbohydrates to short-chain fatty acids (SCFAs) including acetate, propionate, and butyrate, which can improve the intestinal microbial community structure and intestinal barrier integrity and participate in immunomodulation, including controlling the regulatory T cell development and function (Hu and Hongbo 2015) and cytokine production (Shastri et al. 2016). *Clostridium cluster IV* and *Clostridium cluster XIVa* are butyrate-producing bacteria in the GIT (Suchodolski 2010). A study has revealed that *Clostridium cluster IV* and *Clostridium cluster XIVa* actively regulated the host's immune system by promoting differentiation of naive T cells toward regulatory T cells which can cause the specific increase of IL-10-producing Treg cells in the spleen (Atarashi et al. 2011). In the study, the results revealed that the amount of *P. prophyromonas*, *R. flavefaciens*, *Clostridium cluster IV*, and *Clostridium cluster XIVa* was significantly increased by UYP and FYP in the jejunum and ileum ($P < 0.01$, Fig. 6d, g, h, and j). In addition, only FYP could more effectively elevate the abundance of four species

in the GIT (Fig. 6d, g, h, and j). This result suggested that the immunoenhancement activity of UYP and FYP may be related to increasing the number of cellulolytic bacteria in the foregut.

Lactobacillus and *Bifidobacterium* are recognized as beneficial intestinal flora. Previous studies have shown that they could evoke nonspecific immune responses through enhancing production of cytokines via the activation of TLR pathways (Klein et al. 2008) and upregulate the expressions of claudin and occludin in the intestine (Wang et al. 2014c). The increased abundance of *Lactobacillus* and *Bifidobacterium* may be associated with improving immunity through activation of TLRs (Klein et al. 2008). Our results revealed that both UYP and FYP increased the number of *Lactobacillus* and *Bifidobacterium* in the GIT (Fig. 6 e and f). Enterobacteriaceae, *Enterococcus* spp., and *Streptococcus* spp. are recognized as normal parts of the digestive tract flora, which are considered as major opportunistic pathogens that could cause many diseases (Munita et al. 2012). *Bifidobacterium* can inhibit the growth of Enterobacteriaceae (Simone et al. 2014), *Streptococcus* spp. (Aloisio et al. 2014), and *Enterococcus* spp. (Itami et al. 1996). This study displayed that the number of Enterobacteriaceae, *Enterococcus* spp., and *Streptococcus* spp. were decreased by UYP and FYP in the GIT, particularly FYP in the jejunum and ileum (Fig. 6m, n and o). The results indicated that the beneficial immune effects of UYP and FYP may be due to increasing the number of probiotics to inhibit the proliferation of harmful bacteria, especially FYP. However, what kind of specific bacteria showed obvious correlation between FYP or UYP and the immunoenhancement effects that need to be studied further.

The intestinal barrier is a complex and vital system for nutrient absorption, defense against harmful substances invasion, and maintaining immune balance. It consisted of physical barrier refers to tight junctions, immunological barrier relates to IgA, PIGR and β-defensins, chemical barrier contains TFF and EGF and biological barrier consisted of commensal flora (Dai and Wang 2015). Thus, the homeostasis of the intestinal barrier integrity and function is directly related to the permeability of the GIT. ZO-1, claudin, and occludin are three key proteins for the strength of tight junction in the GIT (Halpern and Denning 2015). Unfermented or fermented *Flos Lonicera* improved intestinal barrier function by upregulating ZO-1 and claudin mRNA expressions (Wang et al. 2014b). Similarly, our study displayed UYP and FYP significantly raised the ZO-1, claudin, and occludin mRNA levels in the jejunum and ileum, and FYP showed higher activity of improving physical barrier of the GIT when compared to UYP (Fig. 7a and d).

A pivotal role for intestinal barrier surfaces is the considerable synthesis of secretory immunoglobulin A (SIgA) by plasma cells (Anand et al. 2007). Commensal flora promotes SIgA and PIGR productions to maintain host-microbial mutualism and immune homeostasis through activation of PRRs (Kaetzel 2014). β-Defensins belong to antimicrobial peptides, which are primarily produced by intestinal epithelial cells, and may strongly strengthen host defense indirectly by inducing immune responses (Halpern and Denning 2015). Researches have shown that decoction YPF could obviously raise the secretion of IgA in mucosa (Hou and Xin 2000). Similarly, our results revealed that UYP and FYP increased the mRNA expressions of IgA, PIGR, and β-defensins, and FYP more strongly facilitated their mRNA levels (Fig. 7b and e). These results implied that UYP and FYP played a beneficial role in immune barrier, and FYP exerted a greater activity than UYP.

TFF and EGF are recognized as epithelial reparative factors (Halpern and Denning 2015). TFF is a small polypeptide primarily synthesized by the intestinal goblet cells, which contributed to epithelial regeneration and maintain mucosal integrity by regulating TLRs in the GIT (Jing and Sun 2011). It has been shown that *kiwifruit* improved colonic barrier function by upregulating the expression of TLRs and TFF genes (Gunaranjan et al. 2014). EGF, a heat- and acid-stable peptide, along with the EGF-receptor accelerates proliferation and differentiation of epithelial cells, remarkably improves gut integrity, and heals damaged mucosa or renews epithelial cells (Dvorak 2010). In the study, the mRNA expressions of TFF and EGF were increased after treatment with UYP and FYP, and FYP more strongly facilitated their mRNA levels (Fig. 7). These results indicated that both UYP and FYP possessed potent beneficial effect on chemical barriers, and FYP a stronger effect.

It is demonstrated that the interplay between the MAMPs derived from microbes and PRRs including TLRs expressed on a variety of cells could maintain the intestinal homeostasis and epithelial barrier integrity (Louis and Lin 2009) via regulating immune cell differentiation and synthesizing cytokines, defensins, and immunoglobulins. Therefore, TLR pathway may be considered as an interface among intestinal barrier, microbiota, and immune system, which play a pivotal role in shaping intestinal microbiota (Frosali et al. 2015). While intestinal flora also can regulate the TLRs expression to maintain immune balance (Goto and Kiyono 2012). For example, *B. fragilis* through TLR2, stimulated cytokine production and T cell differentiation (Round et al. 2011). In addition, the gastrointestinal barrier integrity and function are closely related to TLR pathways; TLR2 and TLR4 pathways are essential for mucosal protection against acute intestinal injury via maintenance of epithelial barrier integrity (Cario 2008). In return, the intestinal barrier provides venues for intestinal flora modulating the immune responses by TLRs, especially intestinal immune, because the intestinal barrier contains many immune cells and non-immune cells, which express TLRs (Fermín et al. 2014). Therefore, the integrity of the intestinal barrier is the foundation of flora regulating immune through TLRs. In the

current study, both UYP and FYP exhibited robust immune-enhancing activity, raising the expressions of TLR2 and TLR4 and improving intestinal microbial community and intestinal barrier integrity. In addition, FYP exhibited significantly higher beneficial effects than UYP. These results indicated that the immunoenhancement activity of the two formulations, particularly FYP, perhaps is related to improve the intestinal community structure and intestinal barrier integrity and functionality by activating the TLR2 and TLR4 pathways.

In conclusion, our study highlighted that treatment with FYP resulted in more prior improvement in promoting growth and strengthening the immune than UYP. Moreover, our results also suggested the above beneficial effects of FYP are probably related to stimulate cytokines synthesis by activation of TLR2 and TLR4 pathways, ameliorate intestinal community structure, and improve intestinal barrier integrity and functionality. In future studies, we will devote to identify and isolate the compound(s) or mediator(s) in the FYP that are accounted for exerting the immunoenhancement effects of FYP.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval The experiment was permitted by the Institutional Animal Care and Use Committee of the Sichuan Agricultural University.

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