Effect of Supplementation Yeast Fermentation Products on Growth Performance and Intestinal Health of Weaned Piglets Challenged With Salmonella Typhimurium

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Research

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Abstract

Background: This study investigated the effects of piglets with dietary supplementation yeast fermentation product (YFP) on growth performance, immune status and intestinal inflammation after a *Salmonella* typhimurium challenge. Twenty-four weaned piglets were assigned to four treatments including: non-challenge control (Con); *Salmonella* typhimurium-challenged control (ST); ST + 0.2% YFP (0.2% YFP); and ST + 0.4% YFP (0.4% YFP). All piglets were challenged twice with *Salmonella* typhimurium. All of them were killed at 7^{th} day after the second challenge to obtain plasma and intestine for analysis.

Results: 0.4% YFP increased average daily gain (ADG) and duodenal villus height and villus height/ crypt depth ($P < 0.05$) and decreased feed-gain ratio ($P < 0.05$) after *Salmonella* typhimurium challenge compared with ST group. The 0.4% YFP decreased the elevating concentrations IL-1b and IL-6 ($P < 0.01$) induced by *Salmonella* typhimurium and increased the concentration of IL-10 ($P < 0.05$) in plasma compared with ST group. Furthermore, YFP influenced the apoptosis related mRNA levels of Bax, Bcl-2 and caspase 3 ($P < 0.05$) and increased intestinal occludin protein expression ($P < 0.05$). 0.4% YFP down-regulated the mRNA expressions of TLR4, MyD88, IRAK1, TRAF6 and NFkB ($P < 0.05$) and decreased the mRNA expression of IL-6, TNFa ($P < 0.05$) and increased the IL-10 ($P < 0.01$) in duodenum compared with ST group. In addition, 0.4% YFP decreased the phosphorylation levels of p38 MAPK and ERK1/2 ($P < 0.01$).

Conclusions: Dietary 0.4% yeast fermentation product supplementation had positive effects on growth performance and intestinal barrier function and reduced intestinal inflammation of weaned piglets challenged with *Salmonella* typhimurium.

1. Background

Due to the risk of emergence of resistant bacteria and resistance in humans, China has already banned the use of antibiotics in feed from 2020, though amounts of studies have reported that the use of antibiotics can improve growth performance and protect intestinal infections of weaned piglets [1–3]. Therefore, increasing attention has focused on searching for alternatives to antibiotics.

The effects of dietary supplementation with yeast fermentation products on the improvement of intestinal health could therefore be of great importance in pigs. Yeast fermentation products contain amounts of bioactive compounds including nucleotides, nutritional metabolites and cell wall polysaccharides (specifically β-glucan and mannan). Several studies have investigated the impact of yeast cell wall components on immune function of weaning piglets [4–7]. It has been reported that yeast β-glucans alleviated the elevated of pro-inflammation cytokines and enhanced the production of anti-inflammatory cytokines in LPS or *Escherichia* coli challenged piglets [8, 9]. Dietary supplementation yeast-derived mannans could improve piglets' weight gain and enhanced immune function [10]. Since

yeast fermentation products are beneficial to the growth of piglets, it has been considered to be a potential in-feed antibiotic alternative.

However, how the influence of yeast fermentation products on intestinal health on the condition of *Salmonella* typhimurium challenge is unclear and the underlying mechanism is not documented. Therefore, this study focused on indicating how yeast fermentation products improved growth performance and intestinal health for piglets challenged with *Salmonella* typhimurium. Our study will provide potentiality to find alternatives to antibiotics.

2. Materials And Methods

2.1 Animals and experimental design

A total of 24 weaned male piglets (Duroc · Yorkshire · Landrace) with an initial average body weight of were housed at Laboratory Animal Center of Nanjing Agricultural University. The piglets used did not receive *Salmonella* typhimurium vaccines, antibiotic injections or antibiotics in feed. All piglets used in this study were susceptible to *Salmonella* typhimurium. Piglets were housed in pens (pen size: 1.2 m · 1 m) in an environmental controlled nursery building. Each pen was equipped with a feeder, a nipple drinker and plastic-covered expanded metal floors. The room temperature was maintained at 25 ± 1℃ throughout the study. The piglets had free access to feed and water. The yeast fermentation products are provided by Cargill Company (XPC, Diamond V, Cedar Rapids, IA, USA).

The experiment lasted for 5 weeks, including 21 days before and 14 days after the first *Salmonella* typhimurium challenge. All piglets were randomly allotted to four dietary treatments (n = 6) based on the initial body weight included: (1) Negative control (Con): control diet, without *salmonella* typhimurium challenge; (2) Positive control (ST): control diet, with *salmonella* typhimurium challenge; (3) 0.2% YFP: control diet plus 2 g/kg feed, with *salmonella* typhimurium challenge; (4) 0.4% YFP: control diet plus 4 g/kg feed, with *salmonella* typhimurium challenge (Table 1). The experimental diets were fed to pigs through the study duration. After 21 days experimental diets feeding, the piglets of the last three groups orally inoculated with 5 mL suspension containing 10^9 CFU of *Salmonella* typhimurium. A week later, the second challenge was performed the same as the first. Body weight of per piglet was recorded at days 0, 7, 14, 21 and 35 and feed consumption per pen was recorded every day of the experiment to calculate the average daily gain (ADG), average daily feed intake (ADFI) and feed efficiency (F:G) from d 1 to 21 (pre-challenge) and d 21 to 35 (post-challenge).

Table 1
Ingredient composition of experimental diets (%, as-fed basis)

Ingredient	Percentage (%)
Corn	60.00
Soybean meal	30.00
Fish meal	6.60
Lys	0.12
$CaHPO_3$	0.80
Rock powder	0.88
Salt	0.60
1% Premix[a]	1.00
Calculated nutrient content	
DE, kcal/kg	3283
CP, %	18
Ca, %	0.80
Total P, %	0.60

[a]Premix provided these amounts of vitamins and minerals per kilogram on an as-fed basis: vitamin A: 11000–13000 IU, vitamin D3: 3000–4000 IU, vitamin E: 3000–4000 IU, vitamin K3: 6 mg, vitamin B2: 10 mg, vitamin B6: 5 mg, vitamin B12: 0.5 mg, niacin: 70 mg, pantothenic acid: 550 mg, folic acid: 40 mg, biotin: 5 mg, choline: 30 mg, Mn: 40 mg, Fe: 200 mg, Zn: 300 mg, I: 2 mg, Cu: 350 mg, Sn: 10 mg.

2.2 Sample collections

All the piglets were sacrificed by exsanguination at the end of the study. Blood samples were collected into heparinized tubes. Plasma was separated by centrifugation at 3000 rpm/min for 15 min and stored at -20 °C until analysis. Once dead, the abdomen was immediately opened, and the intestinal tract was excised. Duodenum segments (approximately 8 cm in length) were opened longitudinally and gently flushed with sterile saline to remove the contents. Duodenal mucosa samples were collected by lightly scraping with sterile glass slides on rice, immediately frozen in liquid nitrogen and stored at -80 °C for analysis. 3-cm sections from the duodenum (to about 10 cm distal to the pylorus) were collected and fixed in 4% paraformaldehyde for intestinal morphology analysis.

2.3 Intestinal morphology analysis

Duodenal segments were removed from the stationary solution and then were embedded in paraffin. Sections (3 μm) were stained with hematoxylin and eosin (HE) to investigate architecture of the

duodenum. Stained slices were scanned with the Pannoramic SCAN II and images were captured with 3DHISTECH software (3DHISTECH Ltd. Budapest, Hungary).

2.4 Plasma inflammatory cytokine concentration analysis

Plasma IL-1β, IL-6 and IL-10 concentrations were measured using the ELISA kits suitable for porcine IL-1β, IL-6 and IL-10 (Jiangsu MEIMIAN Industrial Co. LTD, China), respectively, according to the manufacture's protocol. Plasma concentrations of IL-1β, IL-6 and IL-10 were calculated from the standard curve and expressed as ng/L.

2.5 Total RNA isolation and real-time PCR

Total RNA was isolated from 30 mg frozen duodenal mucosa with 1 mL TRIzol (Sangon Biotech, Shanghai, China) and reverse-transcribed according to the manufacturer's protocol (Vazyme Biotech, Nanjing, China). Diluted cDNA (2 μL, 1:25) was used as template for real-time PCR that was performed on a real-time PCR system (Mx3000P, Stratagene, USA). Moreover, GAPDH was chosen as a reference gene to normalize the mRNA abundance of target genes. All primers were synthesized by and listed in Table 2. The $2^{-\Delta\Delta CT}$ method was used to analyze real-time PCR data.

Table 2
The primer sequences for RT-PCR.

Target genes	Primer sequences (5' to 3')	
IL-1β	F: ACATGCTGAAGGCTCTCCAC	R: CAGGGTGGGCGTGTTATCTT
IL-6	F: GCAGTCACAGAACGAGTGGA	R: CTCAGGCTGAACTGCAGGAA
IL-10	F: CGGCCCAGTGAAGAGTTTCT	R: TGCCTTCGGCATTACGTCTT
TNFα	F: GCCCTTCCACCAACGTTTTC	R: CAAGGGCTCTTGATGGCAGA
TLR4	F: CGTGCAGGTGGTTCCTAACA	R: AAAGGCTCCCAGGGCTAAAC
MyD88	F: CCATTCGAGATGACCCCCTG	R: TAGCAATGGACCAGACGCAG
NFκB	F: CGGGGACTACGACCTGAATG	R: CTTTCTGCACCTTGTCGCAC
IRAK1	F: GCAGTTGTCACGGTTTCGTC	R: GAAGTCTCCCAGTTTGGGCA
TRAF6	F: CCAGAGACCCACAATCCCAC	R: ACCCTCCCTCCGAAGACTAC
Bax	F: GACAGGGGCCCTTTTGCTTC	R: CCGCCACTCGGAAAAAGACT
Bcl-2	F: GAACTGGGGGAGGATTGTGG	R: GCCGGTTCAGGTACTCAGTC
Caspase3	F: GTGGGACTGAAGATGACA	R: ACCCGAGTAAGAATGTG

2.6 Western blot analysis

Total protein was extracted from 50 mg frozen samples as previously described [11]. Protein concentration was measured with Pierce BCA Protein Assay kit (No. 23225, Thermo Scientific) according

to manufacturer's instruction. Western blot analysis of Occludin (DF7504, Affinity, diluted 1:1000), HSP70 (AF5466, Affinity, diluted 1:1000), Bax (BS6420, Bioworld, diluted 1:1000), TLR4 (SC293072, Santa Cruz, diluted 1:200), p38 (AF6456, Affinity, diluted 1:1000), p-p38 (AF4001, Affinity, diluted 1:1000), ERK1/2 (AF0155, Affinity, diluted 1:1000), p-ERK1/2 (AF1015, Affinity, diluted 1:1000) were carried out. The β-actin (AC026, ABclonal, diluted 1;50000) was used as internal control.

2.7 Statistical analysis

All data were checked for normality using exploratory analysis. Data were analyzed using SPSS 21.0 for Windows (SPSS Inc., Chicago, IL, USA). All data were analyzed using One-Way ANOVA with diet (YFP) as a fixed factor. The piglets were recognized as a statistical unit. The values were presented as the means ± SEM with significance at $P < 0.05$.

3. Results

3.1 Growth performance

Salmonella typhimurium administration tended ($P = 0.09$) to decrease the average daily gain (ADG) compared with Con group, yet 0.4% YFP supplement was significantly ($P < 0.05$) increased compared with ST group. The ratio of feed and gain (F/G) was observed to significantly increase with *Salmonella* typhimurium challenged compared with control group and it reversed by 0.4% YFP ($P < 0.05$). No significant changes were observed in ADG or F/G between control and YFP supplement groups before *Salmonella* typhimurium treatment (Table 3).

Table 3
Growth performance

Items	Con	ST	0.2% YFP	0.4% YFP	*P* Value		
					P 1	P 2	P 3
Pre-challenge							
ADG (Kg)	0.49 ± 0.02	0.45 ± 0.03	0.45 ± 0.04	0.48 ± 0.02	0.384	0.975	0.498
ADFI (Kg)	0.73 ± 0.02	0.80 ± 0.02	0.74 ± 0.01	0.76 ± 0.03	0.053	0.104	0.219
F/G	1.51 ± 0.07	1.72 ± 0.09	1.69 ± 0.15	1.68 ± 0.06	0.131	0.808	0.738
Post-challenge							
ADG (Kg)	0.66 ± 0.03	0.51 ± 0.04	0.52 ± 0.09	0.69 ± 0.04	0.094	0.920	0.048
ADFI (Kg)	1.13 ± 0.02	1.05 ± 0.06	1.04 ± 0.12	1.15 ± 0.04	0.531	0.886	0.358
F/G	1.76 ± 0.09	2.09 ± 0.03	2.00 ± 0.13	1.81 ± 0.09	0.023	0.533	0.043
P 1: ST vs Con							
P 2: 0.2% YFP vs ST							
P 3: 0.4% YFP vs ST							
The values are presented as mean ± SEM.							

3.2 Plasma cytokines

Salmonella typhimurium-challenged had higher concentrations of plasma IL-1β ($P < 0.01$) and IL-6 ($P = 0.001$) and lower concentration of plasma IL-10 ($P < 0.05$) than the Con piglets. Compared with ST piglets, the concentrations of plasma IL-1β ($P < 0.01$) and IL-6 ($P < 0.01$) was lower in 0.4% YFP piglets, meanwhile the concentration of IL-10 was higher ($P < 0.05$) in plasma (Fig. 1).

3.3 Intestinal morphology

Compared with the Con, the duodenum villus height and villus height/crypt depth were significantly lower ($P < 0.01$) in the ST group; however, the crypt depth was not affected by *Salmonella* typhimurium. The villus height and villus height/crypt depth were reversed to the control levels by 0.2% or 0.4% YFP exposure (Fig. 2).

3.4 Intestinal occludin and heat shock protein 70

Salmonella typhimurium challenge affected occludin ($P < 0.05$) (Fig. 3A) and HSP70 ($P < 0.05$) (Fig. 3B) protein expression in duodenum compared with Con group. Piglets fed 0.4% YFP had higher occludin ($P < 0.05$) protein expression, while YFP did not alleviate the increased HSP70 protein expression.

3.5 Intestinal apoptosis-related gene abundances and protein expression

The relative mRNA abundances of Bcl2-associated X protein (Bax) ($P < 0.01$) and cysteinyl aspartate specific protease 3 (caspase 3) ($P < 0.001$) were up-regulated by *Salmonella* typhimurium treatments; however, they were down-regulated by 0.4% YFP addition. B-cell lymphoma-2 (Bcl-2) ($P < 0.05$) mRNA abundance was down-regulated by *Salmonella* typhimurium treatments, 0.4% YFP addition up-regulated the mRNA abundance (Fig. 4A). Furthermore, Bax protein expression in the ST groups was significantly higher ($P < 0.05$) than the control group, but 0.2% or 0.4% YFP addition retrieved it (Fig. 4B). These results deduced that YFP could alleviate the intestine mucous apoptosis induced by *Salmonella* typhimurium.

3.6 Intestinal TLR4 signaling pathway expression

Piglets challenged with *Salmonella* typhimurium had higher mRNA abundance of TLR4 ($P < 0.01$), IL-1 receptor-associated kinase 1 (IRAK 1) ($P < 0.01$), NFκB ($P < 0.05$) and TNF-α receptor-associated factor 6 (TRAF 6) ($P < 0.01$) compared with the Con. 0.4% YFP supplementation significantly decreased mRNA abundance of TLR4, MyD88, IRAK1, NFκB and TRAF6 ($P < 0.05$) (Fig. 5A), the same as TLR4 protein expression ($P < 0.05$) (Fig. 5B).

3.7 Intestinal inflammation cytokines mRNA expression

Salmonella typhimurium-challenged significantly increased the mRNA expression of IL-1β, IL-6 and TNFα ($P < 0.05$) and significantly decreased IL-10 ($P < 0.01$) mRNA expression compared with the Con and 0.4% YFP addition could decrease IL-6 and TNFα mRNA expression and increase IL-10 mRNA expression. (Fig. 6).

3.8 Intestinal MAPK signaling pathway expression

Compared with the Con piglets, the phosphorylation levels of ERK (Fig. 7A) and p38 (Fig. 7B) were significantly increased ($P < 0.01$) in the *Salmonella* typhimurium-challenged piglets. Meanwhile, 0.2 % and 0.4% YFP all significantly inhibited the activation of the key proteins in the MAPK pathway compared with ST group.

4. Discussion

Yeast fermentation product is used widely in the livestock industry. The potential mechanisms of action of yeast products that maintains an advantageous intestinal environment [12] by regulating microbial ecology and elevates intestinal immunity through preventing pathogenic bacteria from intestinal epithelial cells, consequently improving growth performance have been reported [13, 14]. In this study, during the pre-challenge period, supplementation of 0.2% and 0.4% YFP had no significant effect on growth performance of piglets compared with the control. However, during the *Salmonella* typhimurium challenge period, 0.4% YFP significantly enhanced ADG compared with ST group. In general, the present study indicates that the relatively high dose of YFP offered protection against *Salmonella* typhimurium

and that is beneficial to enhance growth performance of piglets during *Salmonella* typhimurium infection. The following discussion was focused on the 0.4% YFP effects after *Salmonella* typhimurium infection.

Intestinal morphology can be used as an indicator for intestinal health [15]. To response inflammation induced by pathogens, deeper crypts exhibited faster cellular turnover allowing renewal of villus as needed [16]. In the current study, dietary YFP supplementation improved intestinal histomorphology in the infectious piglets. *Salmonella* typhimurium challenge induced significantly deeper crypt and shorter villus in the duodenum, the same observations reported challenged with *Salmonella* typhimurium in piglets. In the challenged piglets, however, the dietary YFP supplementation remarkably increased villus height and decreased crypt depth, which contributed significantly to enhance V/C ratio, indicating the effective nutrient absorption capability and the decreased F/G.

Tight junctions among intestinal epithelial cells, which protect the body from intestinal pathogens, influence intestinal mucosal barrier function to a great extent [17]. Occludin, as the major integral proteins, form the continuous tight junction strands [18]. In the current study, YFP supplementation significantly increased occludin expression in duodenum, which is expected to improve intestinal mucosal barrier function. Our results demonstrated that YFP kept intestinal integrity and barrier function partially by elevating intestinal tight junction protein expression.

Currently, apoptosis is derived by the expression of Bax, Bcl-2 and caspase 3 [19]. In this study, the mRNA abundances of Bax and caspase 3 were significantly up-regulated, and Bax protein expression level was also increased in the ST group, demonstrating that ST induced intestinal epithelial cell apoptosis, but both mRNA abundances and Bax protein expression were down-regulated with supplementation YFP. Our findings showed that YFP might protect intestinal barrier function by inhibiting apoptosis pathway.

LPS induces inflammation in pigs by stimulating the production of cytokines such as IL-1, IL-6 and TNFα, which are considered as endogenous mediators of inflammation [20]. IL-10 is an important anti-inflammatory cytokine which is required for protection in the regulation of intestine homeostasis during host defense [21, 22]. HSP70 also plays a significant role on intestinal inflammation response. The concentrations of plasma IL-1β, IL-6 and IL-10 were measured as indicators of systemic pro- and anti-inflammation responses, respectively. In the present study, the increased plasma IL-1β and IL-6 concentrations indicated successful establishment of the *Salmonella* typhimurium challenge model. And *Salmonella* typhimurium caused an increased of intestinal HSP70 protein expression. Piglets receiving YFP had less concentration of plasma IL-1β and IL-6 and higher concentration of IL-10 than ST group, hence, implying that YFP lowered pro-inflammation of the immune system. The overproduction of pro-inflammatory cytokines is associated with anorexia, which may explain the observed reduction in ADG in *Salmonella* typhimurium-challenged piglets in our study [23, 24]. Meanwhile, the evidence that lower mRNA expression of IL-1β, IL-6 and TNFα, and higher IL-10 expression compared to the ST piglets in duodenum indicated that dietary supplementation with YFP contributed to beneficial immunoregulatory responses.

Toll like receptor 4 (TLR4) is the first identified of TLRs family, which recognizes the LPS component of Gram negative bacteria on the cell surface [25]. The activation of TLR4 signaling pathway activates members of the MAPK family including p38 and ERK1/2, leading to numerous pro-inflammatory cytokine genes translation [26]. TLR4 and MAPK signaling are of great significance in intestinal inflammation [27, 28]. In the present study, ST challenge increased mRNA expression of TLR4 and its downstream signals IRAK1, TRAF6 and NFκB. YFP supplementation decreased TLR4, MyD88, IRAK1, TRAF6 and NFκB mRNA expression and TLR4 protein, p38 phosphorylation and ERK1/2 phosphorylation. Duan et al. [29] demonstrated that mannan oligosaccharide (a component of YFP) supplementation could enhance intestinal mucosal immune competence and suppress intestinal inflammation by decreasing the contents of pro-inflammatory cytokines. Sun et al. [30] demonstrated that *saccharomyces* cerevisiae polysaccharide (a component of YFP) could decrease the pro-inflammation mediators of IL-1β and IL-6 at protein and mRNA levels by inhibiting MAPK activity in mice with DSS-induced colitis, which were consistent with our results.

5. Conclusion

Dietary yeast fermentation products supplementation could suppress *Salmonella* typhimurium hazards. And *salmonella* typhimurium-challenged piglets fed diets supplementation with 0.4% yeast fermentation products exhibited better growth performance possibly by alleviating intestinal inflammation and apoptosis to improve intestinal health.

Declarations

Acknowledgments

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Authors' contributions

Yongsen Zhao contributed to all data analysis and drafting of the manuscript. Danping Wang and Meng Jiang were responsible for animal care, breeding and sampling. Jinglong Chen provided technical support. Xianjing Yang contributed to the experimental design, data interpretation and critical revision of the manuscript.

Availability of data and materials

The data analyzed in this study are available from the corresponding author on reasonable request.

Ethics approval and consent to participate

All procedures with animals were approved by the Animal Ethics Committee of Nanjing Agricultural University. The sampling procedures followed the "Guidelines on Ethical Treatment of Experimental

Animals" (2006) No. 398 set by the Ministry of Science and Technology, China.

Consent for publication

Not applicable

Conflict of interest

The authors declare that they have no conflict of interest.

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Figures

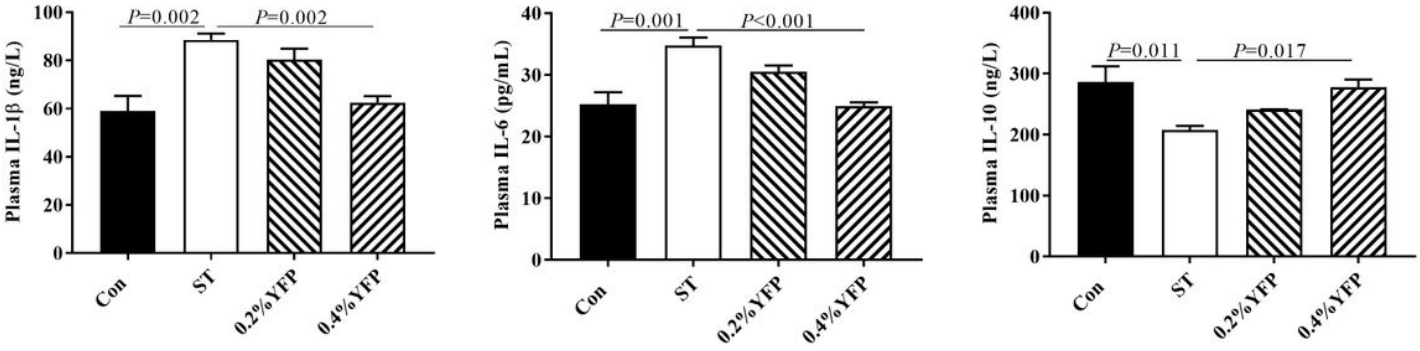

Figure 1

Effect of YFP supplementation on concentration of plasma inflammatory cytokines after Salmonella typhimurium challenge in piglets. The values are presented as mean ± SEM.

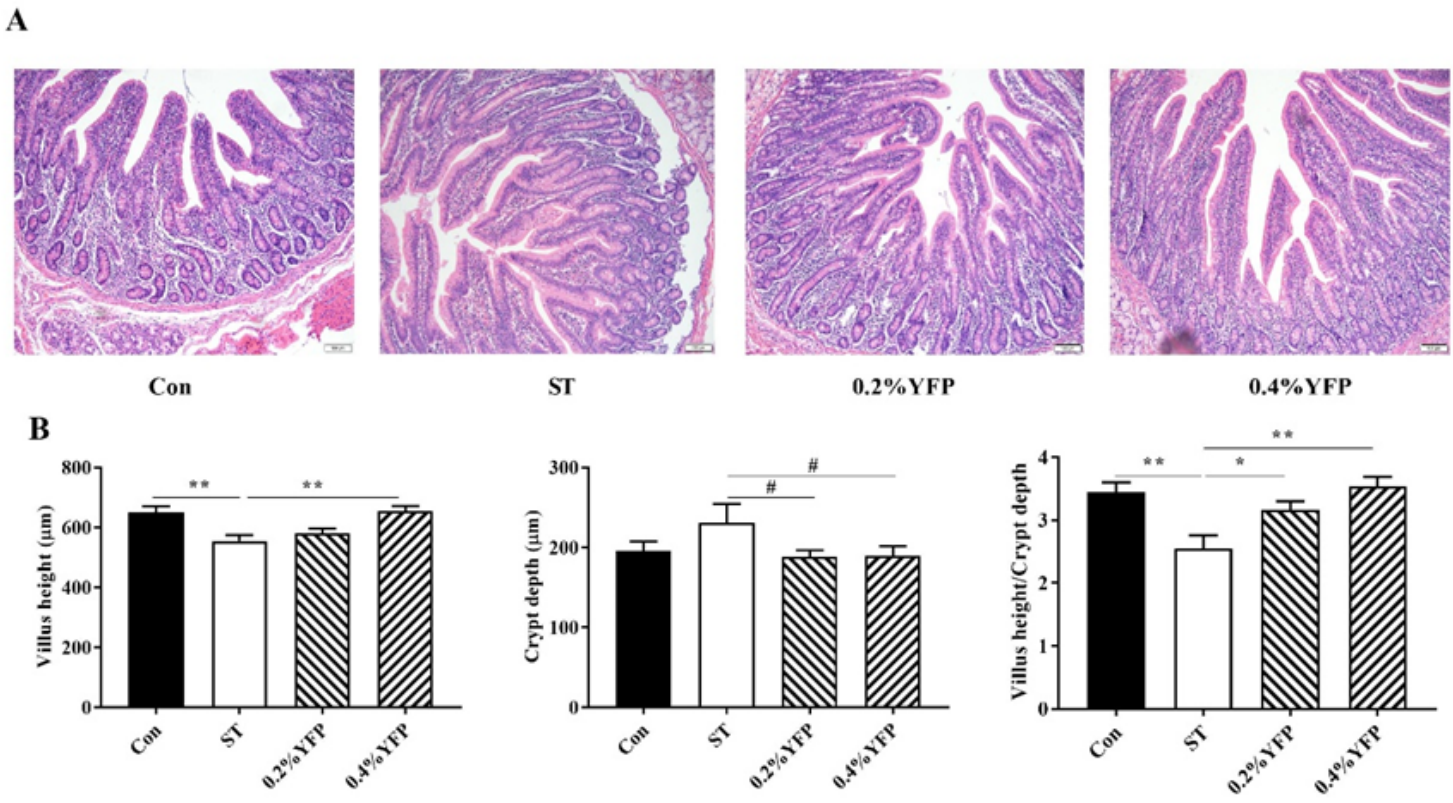

Figure 2

Effect of YFP supplementation on duodenal morphology after Salmonella typhimurium challenge in piglets. The values are presented as mean ± SEM. *P < 0.05, **P < 0.01, #P < 0.1

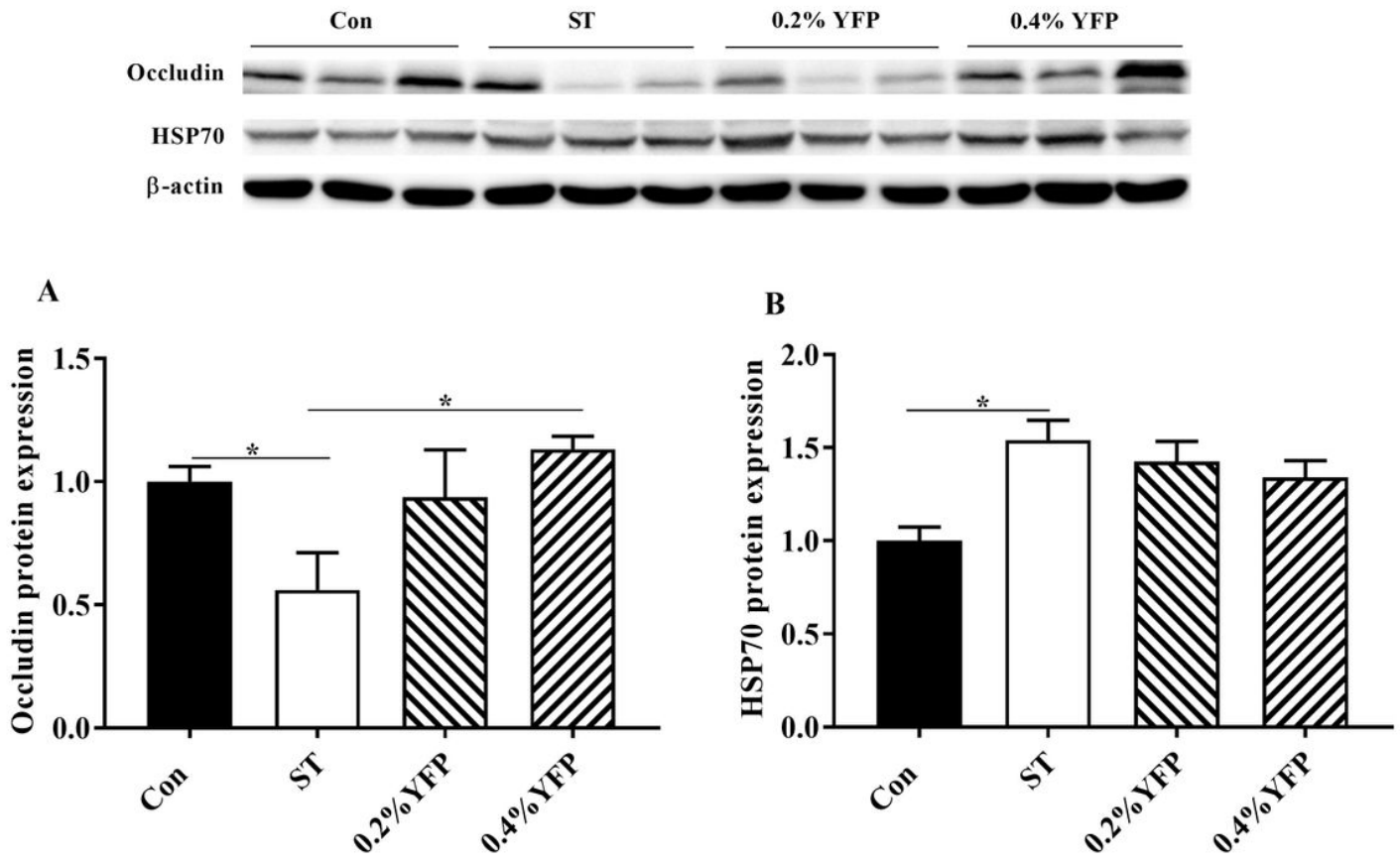

Figure 3

Effect of YFP supplementation on occludin and heat shock protein 70 (HSP 70) protein expression in duodenum after Salmonella typhimurium challenge in piglets. β-actin were used for normalization for relative occludin and HSP70 expression. The values are presented as mean ± SEM. *P < 0.05

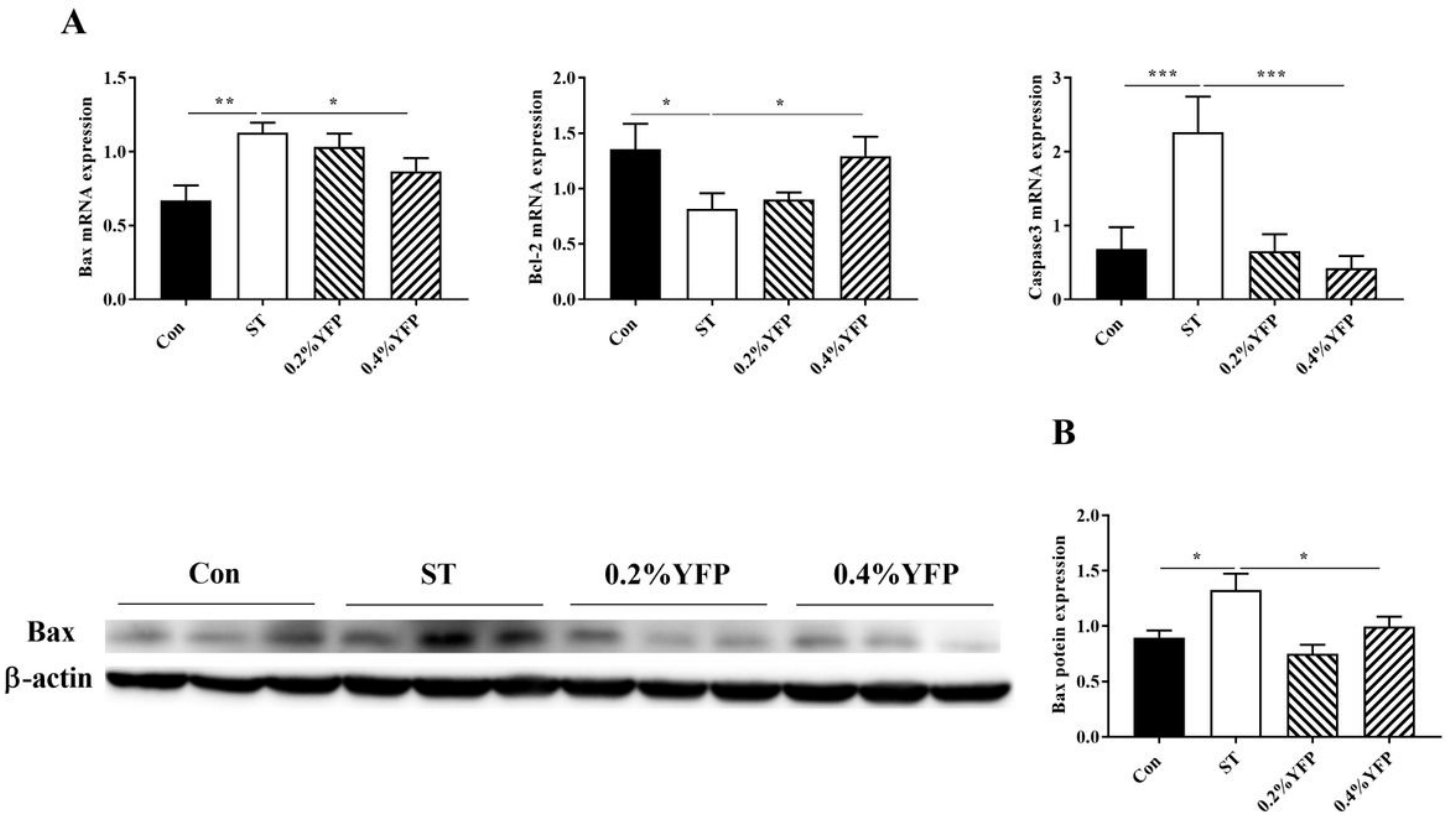

Figure 4

Effect of YFP supplementation on mRNA expression of key molecules of apoptosis signaling after Salmonella typhimurium challenge in piglets. The values are presented as mean ± SEM. *P < 0.05, **P < 0.01

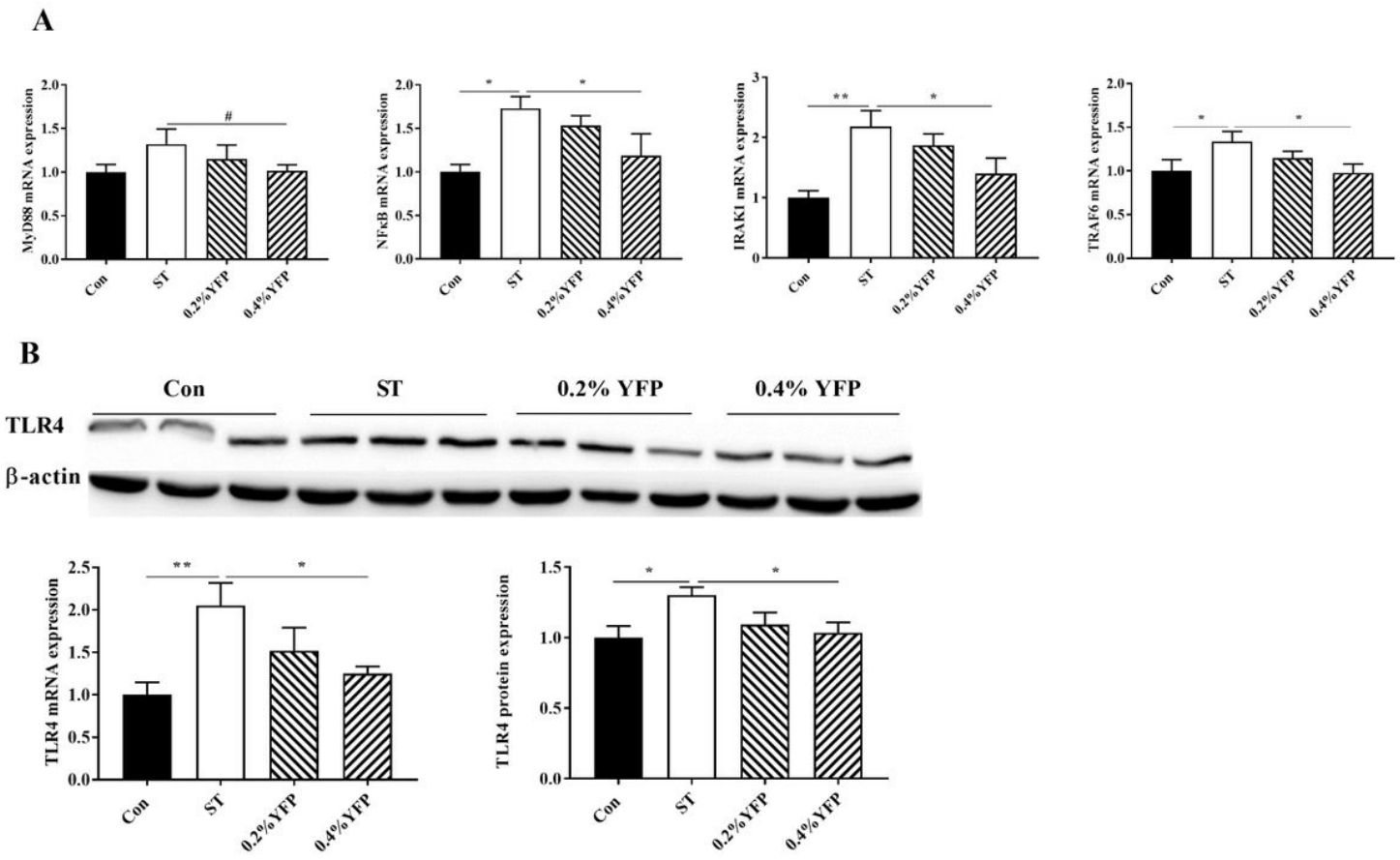

Figure 5

Effect of YFP supplementation on mRNA and protein expression of inflammation-related signaling molecules after Salmonella typhimurium challenge in piglets. β-actin were used for normalization for relative TLR4 expression. The values are presented as mean ± SEM. *P < 0.05, **P < 0.01

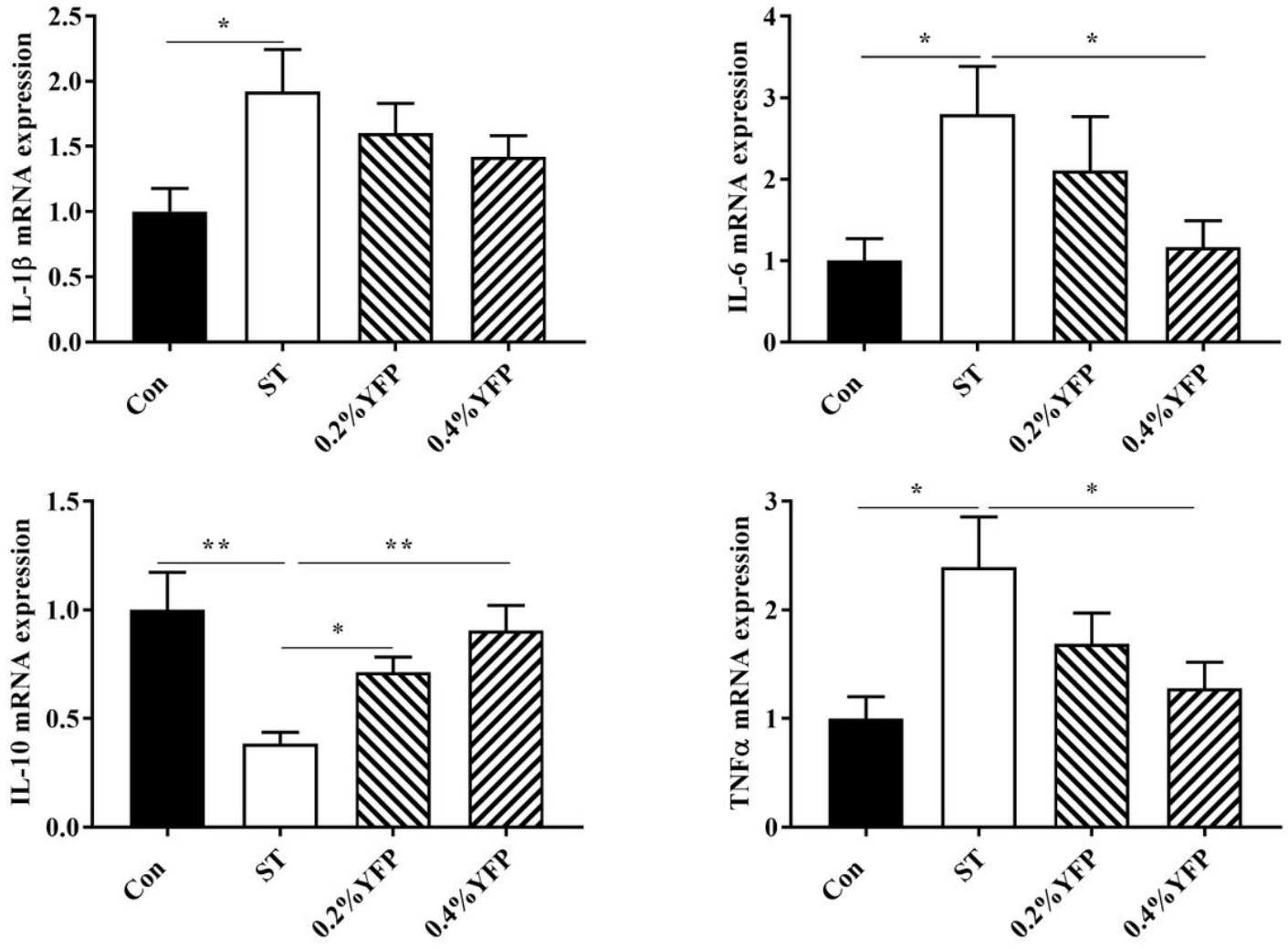

Figure 6

Effect of YFP supplementation on mRNA expression of inflammation cytokines in duodenum after Salmonella typhimurium challenge in piglets. The values are presented as mean ± SEM. *P < 0.05, **P < 0.01

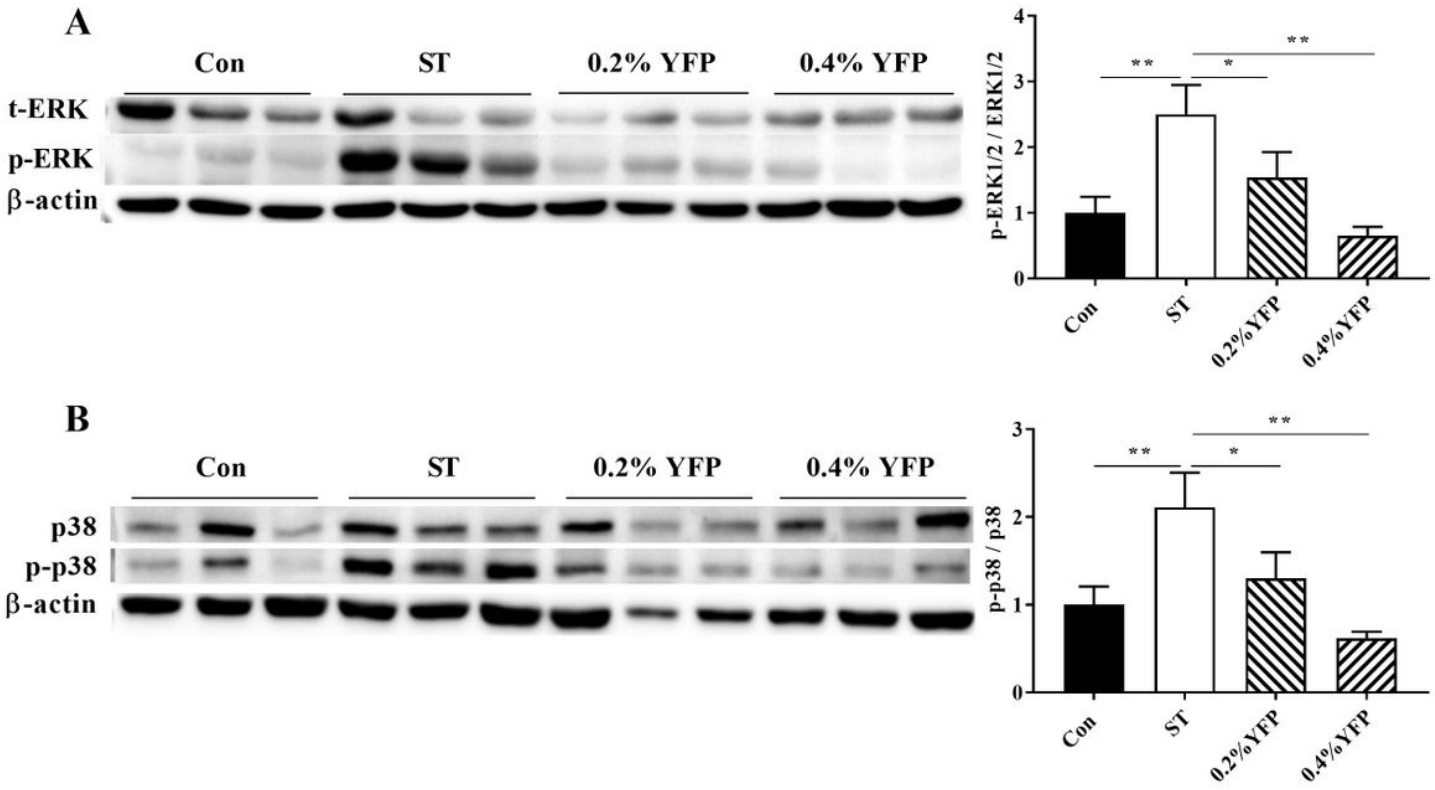

Figure 7

Effect of YFP supplementation on duodenal extracellular signal-related kinase 1/2 (ERK1/2) and p38 phosphorylation levels after Salmonella typhimurium challenge in piglets. Phosphorylated forms of ERK1/2 and p38 were normalized to the total amount of each protein. The values are presented as mean ± SEM. *P < 0.05, **P < 0.01