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Supplementation with yeast culture improves the integrity of intestinal tight junction proteins via NOD1/NF-κB P65 pathway in weaned piglets and H_2O_2-challenged IPEC-J2 cells

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ABSTRACT

This study was explored the effect of yeast culture supplementation on the intestinal tight junction (TJ) proteins of weaned piglets and porcine small intestinal epithelial (IPEC-J2) cells, and the underlying mechanism was further investigated. Yeast culture increased the growth performance of weaned piglets, reduced the diamine oxidase (DAO) activity in serum, increased the villus height and villus height/crypt depth (V/C) ratio of jejunum, and enhanced the mRNA expression and protein abundance of intestinal TJ proteins. Proinflammatory factors were decreased in serum and intestinal tissues, and the NOD1/nuclear factor κB (NF-κB) P65 pathway was inhibited. The results in vitro are consistent with those in vivo, and found that Occludin and ZO-1 correlated negatively with NOD1/NF-κB P65 pathway related factors, suggesting that Occludin and ZO-1 play key roles in the activation of NOD1-mediated pathway. These findings may provide new insights into the mechanism by which yeast culture regulates TJ proteins.

1. Introduction

As the largest barrier between the host and the external environment, the intestine is closely related to host health and disease development (He, Wang, Wang, & Wang, 2019; Weiss & Hennet, 2017; Traina, 2019). Its barrier function is very complex and comprises multiple protective mechanisms, including the tight junction (TJ) structure, the mucus layer, the microbial community, and abundant gut-associated lymphoid tissues (GALT) (Wells, Rossi, Meijerink, & van Baarlen, 2011; Soderholm & Pedicord, 2019; Antonini, Lo Conte, Sorini, & Falcone, 2019), which collectively maintain the host health, both locally and systemically. Several studies have shown that the integrity of the intestinal barrier is critical for health and disease (Turner, 2009; Camilleri, Madsen, Spiller, Greenwood-Van Meerveld, & Verne, 2012; Choi, Yeruva, & Turner, 2017), and that changes in intestinal permeability are pathophysiologically important in intestinal barrier dysfunction (Odenwald & Turner, 2013; Stevens et al., 2018; Harhaj & Antonetti, 2004). However, rapid permeability disorders involve the transcriptional regulation of the TJ proteins (Suzuki, 2013; Oshima & Miwa, 2016). Several factors, such as disease and stress, can destroy the integrity of TJ structure by different mechanisms, and host protective defense mechanisms are activated in an attempt to restore the normal state, mainly by upregulating the expression of several key TJ proteins.

Intestinal permeability is very sensitive to immune-cell signal, and some proinflammatory cytokines can impair the epithelial TJ barrier and contribute to the inflammatory process by allowing various antigens to penetrate the intestinal lumen (AL-Sadi, Boivin, & Ma, 2009). The innate immune recognition of the intestine mucosal surfaces is considered to be a critical mediator of intestinal homeostasis (Pardo-Camacho, González-Castro, Rodiño-Janeiro, Pigrau, & Vicario, 2018).

Abbreviations: TJ, tight junction; IPEC-J2, porcine small intestinal epithelial cells; H&E, hematoxylin and eosin; IBW, initial body weight; FBW, final body weight; ADG, average daily gain; DAO, diamine oxidase; GALT, gut-associated lymphoid tissues; NF-κB, nuclear transcription factor κB; MAPK, mitogen-activated protein kinases; RIPK2, receptor interacting serine/threonine kinase 2; TNF-α, tumor necrosis factor α; IL-18, interleukin 18; V/C, villus height/crypt depth; MLC, Myosin light chain; MLCK, myosin light chain kinase

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Pattern recognition receptors, such as cytoplasmic nucleotide binding oligomerization domain (NOD)-like receptors, are involved in complex signaling pathways and trigger different responses to maintain intestinal homeostasis (Motta, Soares, Sun, & Philpott, 2015). One of the main signaling pathway is through NOD1 or NOD2 signals, which activates nuclear transcription factor κB (NF-κB) and/or mitogen-activated protein kinases (MAPKs) via receptor interacting serine/threonine kinase 2 (RIPK2), causes the release of various proinflammatory cytokines and chemokines (Strober, Murray, Kitani, & Watanabe, 2006). Several studies have suggested that NOD1-mediated signaling pathway plays an important role in the integrity of intestinal barrier (Caruso, Warner, Inohara, & Núñez, 2014; Keestra-Gounder & Tsolis, 2017).

Yeast culture is mainly composed of yeast-cell metabolites, fermented variant medium and a small amount of inactive yeast cells, is a microecological preparation with multiple functions and high nutritional value (Shurson, 2018). Yeast culture is reportedly beneficial to intestinal health and has immunomodulatory capability (Park, Kim, Kim, & Moon, 2017; Ganda Mall et al., 2017). Dias et al. (2018) showed that yeast culture supplementation improved the rumen pH and microbial nitrogen synthesis. Jiang et al. (2017) reported that yeast culture increased the relative abundance of *Ruminobacter* and *Bifidobacterium* in the rumen of lactating cows. Jensen, Patterson, and Yoon (2008) showed that yeast culture had anti-inflammatory effect and specifically activated natural killer (NK) cells. Our previous studies have also confirmed that yeast culture is beneficial to the intestinal health and immunity of animal (published in Chinese Journals), but the effect of yeast culture on intestinal TJ proteins is not well understood.

In the present study, we hypothesized that supplementation with yeast culture benefits the intestinal TJ proteins and is involved in the regulation of TJ proteins via the NOD1-mediated signaling pathway. In this study, the hypothesis was tested in weaned piglets and porcine small intestinal epithelial (IPEC-J2) cells, allowing verification both in vivo and in vitro and the investigation of the underlying molecular mechanism. Our findings provide new insights into the mechanism underlying the regulation of intestinal TJ proteins by yeast culture and an important reference for future nutritional interventions in the intestinal health in piglets using yeast culture.

2. Materials and methods

2.1. Animals and experimental design

Twelve Duroc × Large White × Landrace crossbred weaned piglets (initial body weight 8.69 ± 0.46 kg, weaned at 28 days of age) were selected and allowed a 3-day acclimatization period before they were stochastically divided into two groups: control diet (basal diet) and yeast culture diet (basal diet supplemented with 5 g/kg yeast culture) (n = 6 each group). The diet composition and nutrient levels are present in Table 1. The trial lasted for 14 days, the ambient temperature was controlled at 22–28 °C, and feed and water were available ad libitum during the experimental period.

At the end of the trial, the final body weight (FBW) was recorded. Blood samples were taken from the precaval vein, serum was separated at 4 °C by centrifugation at 3000 × *g* for 20 min and then stored at −20 °C. Three piglets were selected randomly from each group and killed after intracardial injection of sodium pentobarbital (50 mg/kg bodyweight). Two samples of small intestine tissue (jejunum and ileum) were collected gently and carefully (avoiding external contact and extrusion of tissue samples to maintain intestinal mucosal integrity). One intestinal tissue sample (3–5 cm-length) was preserved in 4% formaldehyde solution for histological evaluation. The other sample was washed gently with phosphate-buffered saline (PBS) to remove the intestinal contents, and then immediately frozen in liquid nitrogen and stored at −80 °C for further analysis.

Table 1
Composition and nutrient contents of the basal diets (%w/w, as-fed basis).

Item	Amount (%)
Corn	58.0
Extruded Soybean	12.0
Soybean meal	18.7
Fish meal	3.0
Whey powder	5.0
Calcium hydrogen phosphate	1.2
Limestone	0.8
Salt	0.3
Vitamin and mineral premix[a]	1.0
Nutrient levels	
DE (digestible energy), Mcal/kg	3.37
Crude protein (%)	19.1
Calcium (%)	0.83
Total phosphorus (%)	0.65
Met (%)	0.40
Lys (%)	1.40
Thr (%)	0.85
Trp (%)	0.22

[a] Provided the following per kg of diet: Vitamin A, 2200 IU; Vitamin D_3, 300 IU; Vitamin E, 22 IU; Vitamin K_3, 0.70 mg; Vitamin B_{12}, 0.023 mg; Biotin, 0.15 mg; D-pantothenic acid, 13.5 mg; Folacin, 0.50 mg; Nicotinic acid, 22 mg; Choline, 550 mg; Riboflavin, 4.50 mg; Thiamine, 2.0 mg; Cu, 6.0 mg; Fe, 105 mg; Zn, 110 mg; Mn, 5 mg; I, 0.15 mg; Se, 0.30 mg.

2.2. Assessment of intestinal permeability

The diamine oxidase (DAO) activity (Jiancheng Bioengineering Institute, China) and D-lactata concentration (Jiancheng Bioengineering Institute, China) in the serum of piglets were measured with commercial kits.

2.3. Histological evaluation

The fixed intestinal tissues were embedded, sectioned and stained with hematoxylin and eosin (H&E) as described in the literature (Jung, Kim, Ha, Choi, & Chae, 2006). The tissue sections were examined with light microscopy.

2.4. Detection of inflammatory factors in serum

The levels of interleukin 18 (IL-18) (Xinle Biological, China) and tumor necrosis factor α (TNF-α) (Xinle Biological, China) in the serum of piglets were measured with enzyme-linked immunosorbent assays (ELISAs).

2.5. Cell culture and cell treatments

The IPEC-J2 cell line was a gift from Professor Zhanyong Wei (Henan Agricultural University, Zhengzhou, China). The cells were cultured in DME/F12 medium (Hyclone, USA) supplemented with 10% fetal bovine serum (Gibco, New Zealand), 1% penicillin-Streptomycin (Solarbio, China), 1% insulin-transferrin-sodium selenite (Sigma, USA) and 5 ng/mL epidermal growth factor (Sigma, USA) at 37 °C under 5% CO_2. Generally, cells were divided into four treatment groups: (1) control treatment, in which the cells were cultured in complete DME/F12 medium for 24 h, then washed with PBS, and cultured in DME/F12 medium for another 12 h; (2) yeast culture treatment, in which 15.625 mg/mL yeast culture was cocultured with IPEC-J2 cells in complete medium for 24 h and washed with PBS, after which then complete medium was added to culture cells for another 12 h; (3) H_2O_2 treatment, in which the cells were cultured in DME/F12 medium for 24 h, after which 0.6 mM H_2O_2 (China Pharmaceutical Group Chemical

Reagents Co., Ltd, China) was added to treat cells for 12 h. (4) yeast culture pre-protection + H_2O_2 treatment, in which the cells were co-cultured with 15.625 mg/mL yeast culture for 24 h, washed with PBS, and then incubated with 0.6 mM H_2O_2 for 12 h.

2.6. Real-time PCR analysis

Total RNA was extracted from the intestinal tissues and IPEC-J2 cells. Real-time PCR was performed with the All-in-One™ qPCR Mix (GeneCopoeia, USA) according to the manufacturer's instruction. The primers of *Occludin*, *ZO-1*, *IL-18*, *NOD1*, *RIPK2*, *NF-κB* and *β-actin* were used as previously descried (Chen et al., 2016; Luo et al., 2017; Wang et al., 2018), The primers of *MAPK* and *TNF-α* were as follows: *MAPK*, F: ACCCCAACAAACGGATCACA, R: ATGAGTTCCTTCAGCCGCTC; *TNF-α*, F: TTGAGCATCAACCCTCTGGC, R: ATTGGCATACCCACTCTGCC. Three replicates were performed for each reaction, and the results were calculated as the relative values of control group.

2.7. Western blotting analysis

The protein abundance in the tissue samples and IPEC-J2 cells were measured with western blotting, as previously described (Wang et al., 2018). The membranes were incubated with primary antibodies directed against Occludin (13409-1-AP; diluted 1:1500; Proteintech, China), ZO-1 (ARP36636-P050; diluted 1:250; AVIVA, USA), NOD1 (OAEB01838; diluted 1:1000; AVIVA, USA), RIPK2 (A13453R; diluted 1:1500; Solarbio, China), NF-κB P65 (#6956; diluted 1:1000; Cell Signaling Technology, USA), MAPK P38 (#8690; diluted 1:1000; Cell Signaling Technology, USA), and β-actin (60008-1-lg; diluted 1:10000; Proteintech, China) at 4 °C overnight, then washed 3 times with TBST (Tris-HCl buffer salt solution + Tween) for 15 min. Membranes were incubated with secondary antibodies at room temperature for 1 h. Super ECL Kit (Millipore, USA) was used for signal detection according to the manufacturer's instruction. The Image J software was used to analyze the band intensity, and all results were normalized to β-actin.

2.8. Immunofluorescence analysis

After the cells were fixed with absolute ethanol, blocked with 5% bovine serum albumin, a primary antibody directed against Occludin (13409-1-AP; diluted 1:50; Proteintech, China) or ZO-1 (21773-1-AP; diluted 1:100; Proteintech, China) was incubated overnight at 4 °C. The cells were washed with PBST (phosphate-buffered saline + Tween) and incubated with an appropriate secondary antibody at room temperature for 1 h. Nuclei were stained with 4′,6-diamidino-2-phenylindole (DAPI, Boster, China). The distribution of TJ proteins was observed under a fluorescence microscope.

2.9. Statistical analysis

Results were expressed as means ± SD. Data analysis were performed using one-way analysis of variance (ANOVA) procedure of SPSS 20.0 by *Tukey* test (data from in vitro study) or with an independent-samples *t* test (data from in vivo study). Differences were considered significant at the 0.05 or 0.01 level.

3. Results

3.1. Growth performance, intestinal permeability, intestinal morphology and expression of intestinal TJ proteins in weaned piglets

Weaning stress can lead to intestinal incompleteness (including structural and functional aspects), which affects the growth of piglets. As shown in Fig. 1, yeast culture supplementation increased the FBW ($p < 0.05$) and average daily gain (ADG) ($p < 0.05$) of weaned piglets (Fig. 1B), the diamine oxidase (DAO) activity ($p < 0.05$) (Fig. 1C) in the serum was reduced while there was no significant difference in D-lactate concentration ($p > 0.05$) (Fig. 1D) compared with control. Histological evaluation showed that the jejunum villi in the yeast culture group were arranged neatly and were less broken (Fig. 1E), and the villus height and villus height/crypt depth (V/C) ratio were increased ($p < 0.05$) (Fig. 1F) compared with control. No significant differences were observed in the ileum of the two groups ($p > 0.05$) (Fig. 1G, H).

Yeast culture supplementation increased the mRNA level and protein abundance of ZO-1 in the jejunum ($p < 0.05$) and ileum tissues ($p < 0.01$) (Fig. 1J, K, M), and the mRNA level of *Occludin* was increased in the ileum ($p < 0.01$) (Fig. 1I). These results indicated that yeast culture increased the expression of TJ proteins, was beneficial to intestinal health, and thus promoted the growth of weaned piglets.

3.2. Expression of NOD1-mediated signaling pathway related factors in weaned piglets

The NOD1-mediated signaling pathway is considered to contribute to the integrity of the intestinal barrier, and its activation can cause the release of inflammatory factors. Therefore, we first detected the levels of proinflammatory factors in the serum and intestinal tissues of the weaned piglets. There was no significant difference in serum IL-18 ($p > 0.05$) (Fig. 2A), whereas the level of TNF-α was reduced ($p < 0.05$) (Fig. 2B) in the yeast culture group. The mRNA expression of *TNF-α* was downregulated in the jejunum ($p < 0.05$) and ileum ($p < 0.05$) (Fig. 2D), whereas that of *IL-18* showed no significant change ($p > 0.05$) (Fig. 2C).

Based on the results for proinflammatory factors, we measured the expression of NOD1-mediated signaling pathway related factors. Compared with control, the mRNA expression of *NOD1* ($p < 0.01$), *RIPK2* ($p < 0.01$), *NF-κB* ($p < 0.05$) and *MAPK* ($p < 0.05$) were downregulated in the jejunum, *NOD1* ($p < 0.05$) and *MAPK* ($p < 0.01$) were downregulated in the ileum (Fig. 2E, F, G, H). The protein abundance of NOD1 ($p < 0.05$), RIPK2 ($p < 0.05$) and NF-κB P65 ($p < 0.05$) were downregulated in the jejunum (Fig. 2I, J, K, L). No significant differences in MAPK P38 were observed in the jejunum ($p > 0.05$) and ileum tissues ($p > 0.05$) of the two dietary treatment groups (Fig. 2I, M). This means that the NOD1/NF-κB P65 pathway was inhibited in the yeast culture group.

3.3. Expression and distribution of TJ proteins in IPEC-J2 cells

To further investigate the effect of yeast culture on TJ proteins, we used IPEC-J2 cells to verity in vitro. Compared with the control, yeast culture enhanced the mRNA expression of *Occludin* ($p < 0.01$), but not of *ZO-1* in IPEC-J2 cells ($p > 0.05$) (Fig. 3B, C). Compared with H_2O_2 group, yeast culture pre-protection increased the mRNA level of *Occludin* ($p < 0.01$) and *ZO-1* ($p < 0.01$) to resist the TJ damage caused by H_2O_2 (Fig. 3B, C). Western blotting showed that yeast culture increased the protein abundance of Occludin ($p < 0.05$), whereas that of ZO-1 ($p > 0.05$) was unaffected (Fig. 3D, E, F), and yeast culture partly alleviated H_2O_2-induced injury by enhancing the protein abundance of both Occludin ($p < 0.01$) and ZO-1 ($p < 0.01$). Immunofluorescence assay showed that yeast culture promoted the localization of Occludin to the cytoplasm (Fig. 3G). In contrast, the localization of ZO-1 was not affected by yeast culture (Fig. 3H). These results indicate that yeast culture strengthens the structural basis of TJ strands in IPEC-J2 cells, and repair the barrier damage caused by H_2O_2 by increasing the expression of TJ proteins.

3.4. Expression of NOD1-mediated signaling pathway related factors in IPEC-J2 cells

The expression of NOD1-mediated signaling pathway related factors is shown in Fig. 4. In the yeast culture-treated IPEC-J2 cells, the mRNA expression of *NOD1* ($p > 0.05$), *RIPK2* ($p > 0.05$), *NF-κB* ($p > 0.05$)

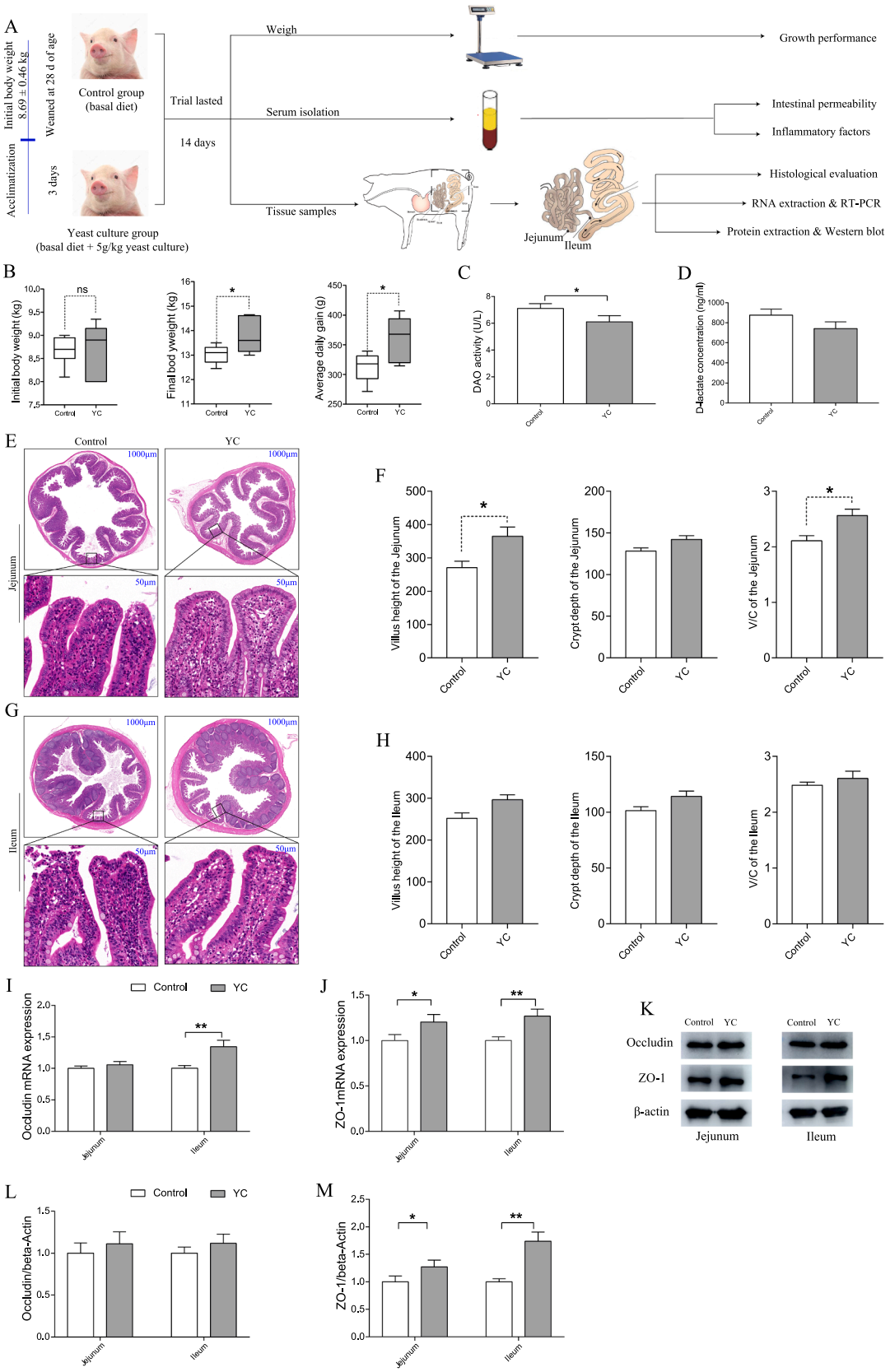

Fig. 1. Effects of yeast culture on the growth performance, intestinal permeability, intestinal morphology and expression of intestinal TJ proteins in weaned piglets. Schematic diagram of the experimental design (A). Growth performance in weaned piglets (B), (n = 6). DAO activity (C) and D-lactate concentration (D) in serum, (n = 3). Histological evaluation of jejunum (E, F) and ileum (G, H), (n = 3). Relative mRNA expression of *Occludin* (I) and *ZO-1* (J) in the jejunum and ileum, (n = 3). Protein abundance of Occludin and ZO-1 (K), (n = 3). Intensity analysis of Occludin (L) and ZO-1 (M). YC = yeast culture group; DAO, diamine oxidase. Bars with asterisk(s) (* or **) indicates statistically significant difference at $p < 0.05$ or $p < 0.01$ level, respectively.

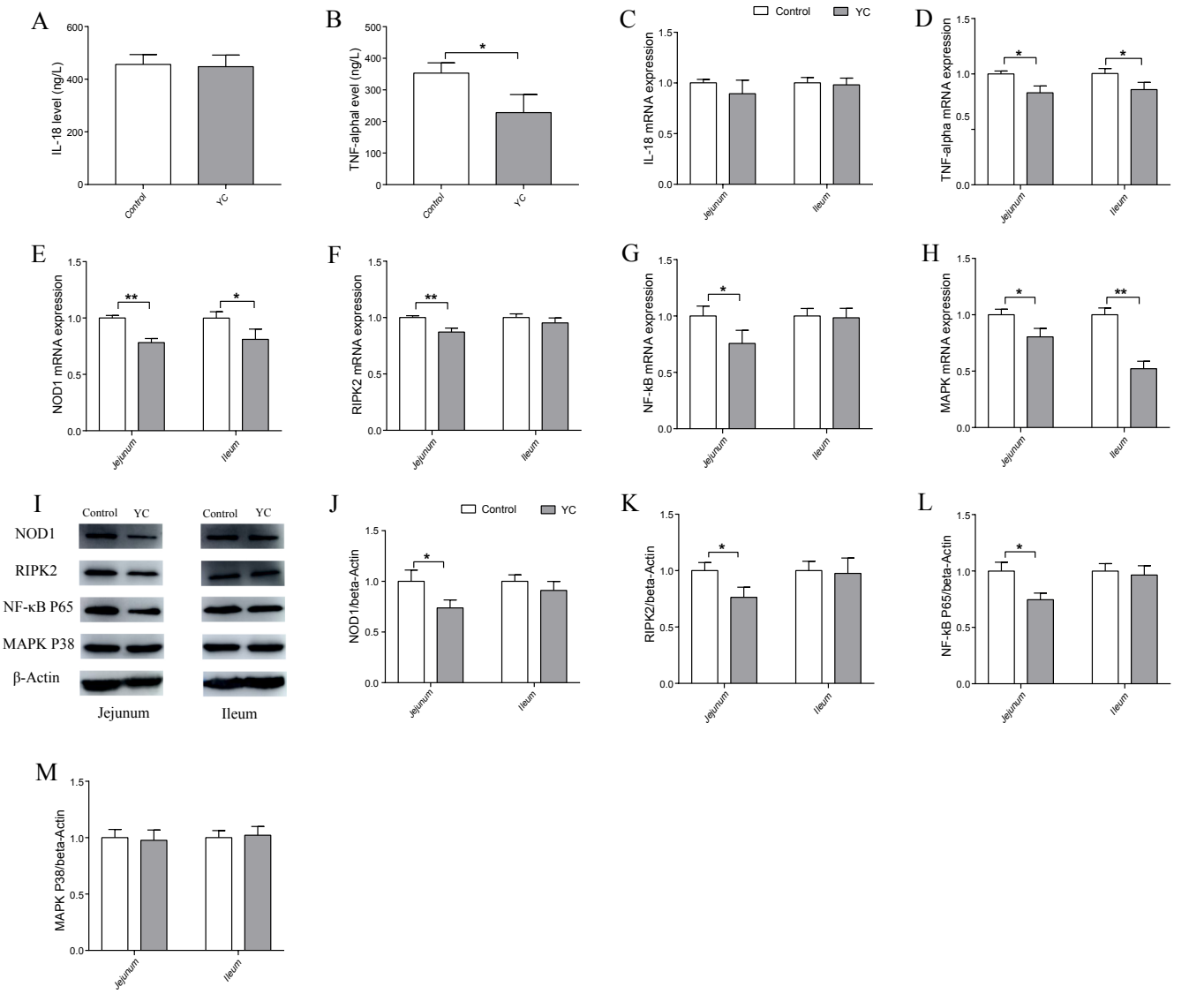

Fig. 2. Effect of yeast culture on the NOD1-mediated signaling pathway related factors in weaned piglets. Serum levels of IL-18 (A) and TNF-α (B), (n = 3). Relative mRNA expression of *IL-18* (C), *TNF-α* (D), *NOD1* (E), *RIPK2* (F), *NF-κB* (G) and *MAPK* (H) in the jejunum and ileum, (n = 3). Protein abundance of NOD1, RIPK2, NF-κB P65 and MAPK P38 (I), (n = 3). Intensity analysis of NOD1 (J), RIPK2 (K), NF-κB P65 (L) and MAPK P38 (M). YC = yeast culture group; IL-18: interleukin 18; TNF-α: tumor necrosis factor α; RIPK2: receptor interacting serine/threonine kinase 2; NF-κB: nuclear transcription factor κB; MAPK: mitogen-activated protein kinases. Bars with asterisk(s) (* or **) indicates statistically significant difference at $p < 0.05$ or $p < 0.01$ level, respectively.

and *MAPK* ($p > 0.05$) did not differ significantly from that in the control (Fig. 4A, B, C, D). However, yeast culture pre-protection reduced the expression of *NOD1* ($p < 0.05$), *RIPK2* ($p < 0.05$), *NF-κB* ($p < 0.05$) and *MAPK* ($p < 0.01$) when the cells were stimulated with H_2O_2. Compared with control group, there was no significant difference in protein abundance of NOD1 ($p > 0.05$), RIPK2 ($p > 0.05$), NF-κB P65 ($p > 0.05$) and MAPK P38 ($p > 0.05$) in yeast culture-treated group (Fig. 4E, F, G, H, I). Yeast culture pre-protection reduced the protein abundance of NOD1($p < 0.05$), RIPK2 ($p < 0.01$) and NF-κB P65 ($p < 0.05$) when the cells were treated with H_2O_2, but no change was observed in MAPK P38 ($p > 0.05$) (Fig. 4E, F, G, H, I). This means that yeast culture can inhibit NOD1/NF-κB P65 pathway to in response to H_2O_2 induced injury.

3.5. Correlation analysis of TJ proteins and NOD1-mediated signaling pathway related factors

To explore the relationship between TJ proteins and the NOD1-mediated signaling pathway, we analyzed the correlation between TJ proteins and NOD1-mediated signaling pathway related factors. As shown in Fig. 5, *Occludin* and *ZO-1* correlated significantly negatively with *NOD1* ($p < 0.01$), *RIPK2* ($p < 0.01$), *NF-κB* ($p < 0.01$) and *MAPK* ($p < 0.01$) at the mRNA level (Fig. 5A, B). At protein level, Occludin and ZO-1 correlated significantly negatively with NOD1 ($p < 0.01$), RIPK2 ($p < 0.01$) and NF-κB P65 ($p < 0.01$), but not with MAPK P38 ($p > 0.05$) (Fig. 5C, D).

4. Discussion

As a microecological preparation, yeast culture improves the intestinal barrier function in various ways. However, most reported studies have focused on modulating the intestinal microbiota to benefit the host. For example, Welty et al. (2019) showed that yeast culture positively influenced the bacterial sequence abundance in rumen fluid of cows. Liu et al. (2019a) showed that the ruminal genera were effectively influenced by yeast culture in sheep, and the effect was largely depended on the diet compositions. However, few studies have reported the effect of yeast culture on the intestinal TJ proteins. In the present

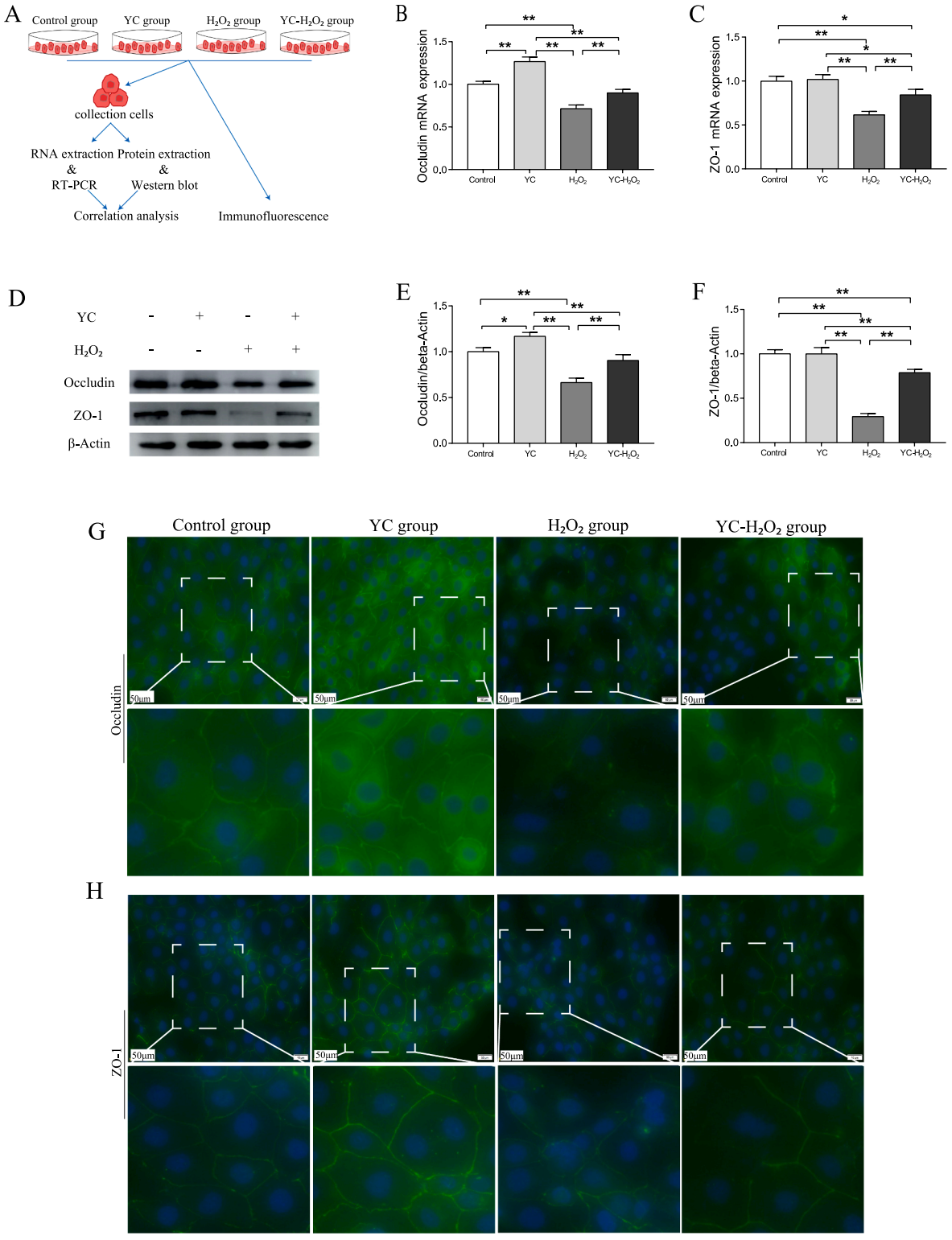

Fig. 3. Effect of yeast culture on the TJ proteins of IPEC-J2 cells. Schematic diagram illustrating the experimental design (A). Relative mRNA expression of *Occludin* (B) and *ZO-1* (C) in IPEC-J2 cells, (n = 6). Protein abundance of Occludin and ZO-1 in IPEC-J2 cells (D), (n = 6). Intensity analysis of Occludin (E) and ZO-1 (F). Effect of yeast culture on distribution of Occludin (G) and ZO-1 proteins (H) in IPEC-J2 cells. Distribution of TJ proteins was observed with fluorescence microscopy (scale bar = 50 μm). YC = yeast culture group. Bars with asterisk(s) (* or **) indicate statistically significant difference at $p < 0.05$ or $p < 0.01$, respectively.

study, we investigated the prebiotic effects of yeast culture and found that supplementation with yeast culture improved the growth performance, intestinal permeability, jejunum morphology, and the expression of intestinal TJ proteins in weaned piglets, which appears to be related to the reduction of TNF-α release via NOD1/NF-κB P65 pathway. In vitro study confirmed that yeast culture enhances the expression of TJ proteins and inhibit the NOD1/NF-κB P65 pathway triggered in response to H_2O_2 induced injury. Moreover, correlation analysis found that Occludin and ZO-1 correlated negatively with NOD1/NF-κB P65 pathway related factors in vitro, suggesting that

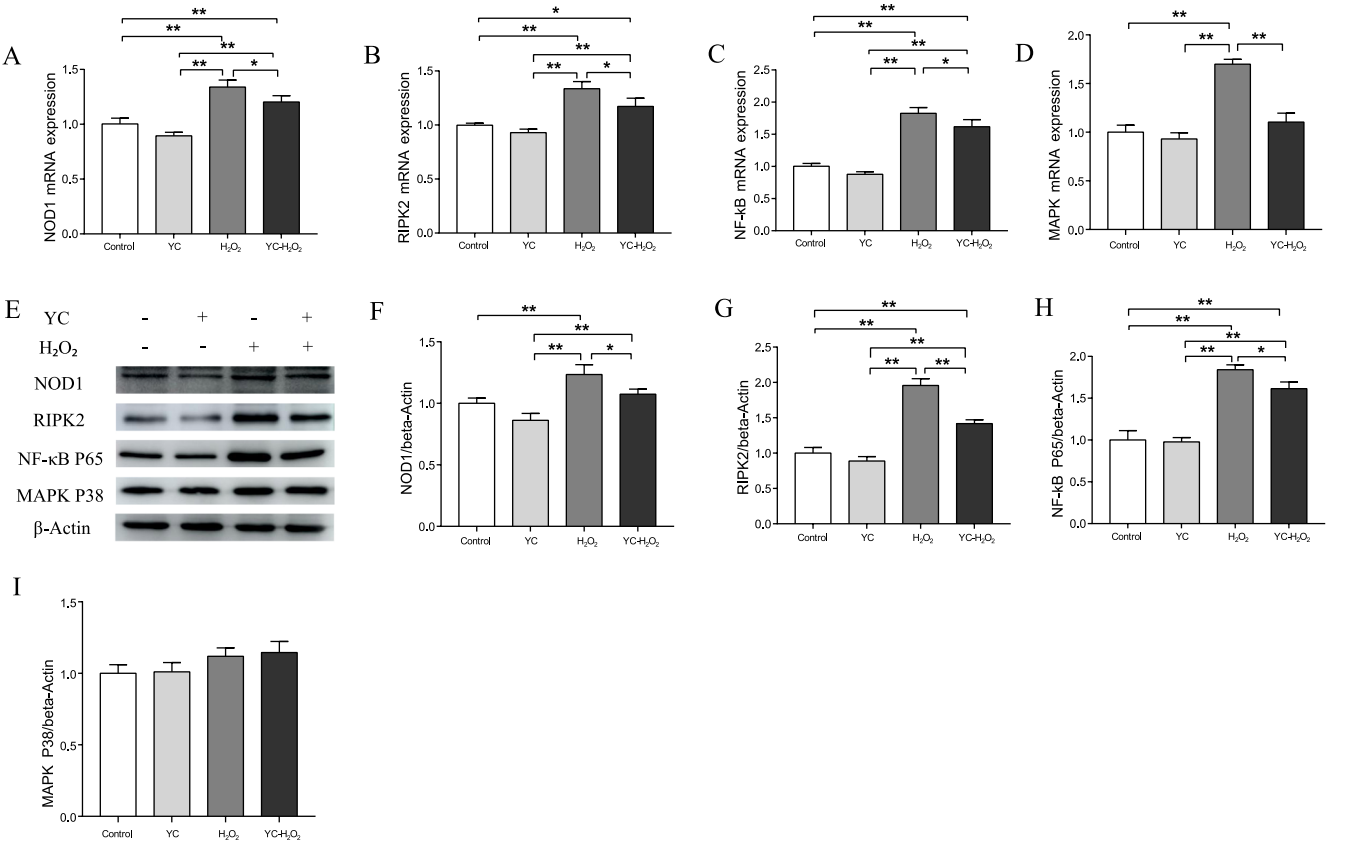

Fig. 4. Effect of yeast culture on the NOD1-mediated signaling pathway related factors in IPEC-J2 cells. Relative mRNA expression of *NOD1* (A), *RIPK2* (B), *NF-κB* (C), and *MAPK* (D) in IPEC-J2 cells, (n = 6). Protein abundance of NOD1, RIPK2, NF-κB P65 and MAPK P38 in IPEC-J2 cells (E), (n = 6). Intensity analysis of NOD1 (F), RIPK2 (G), NF-κB P65 (H) and MAPK P38 (I). YC = yeast culture group; RIPK2: receptor interacting serine/threonine kinase 2; NF-κB: nuclear transcription factor κB; MAPK: mitogen-activated protein kinases. Bars with asterisk(s) (* or **) indicate statistically significant difference at $p < 0.05$ or $p < 0.01$, respectively.

Occludin and ZO-1 play key roles in the activation of NOD1-mediated pathway. These results suggest that the NOD1/NF-κB P65 pathway is involved in a mechanism by which yeast culture improves the integrity of the intestinal TJ barrier.

Yeast culture has a relatively high protein content and is frequently used as a protein supplement for ruminants (Welty et al., 2019; Nasseri, Rasoul-Amini, Morowvat, & Ghasemi, 2011). In recent years, many researchers have reported that yeast culture promotes nutrient absorption and strengthens the host's immunity, which have been confirmed in a variety of animals. Sun, Kim, Zhang, and Kim (2018) reported that yeast culture linearly increased the bodyweight of male broilers and linearly reduced the feed conversion ratio during the whole experimental period. Liu et al. (2019b) showed that yeast culture improved the growth performance and carcass trait of lambs. This is similar to our results in weaned piglets. However, Yang, Li, Liang, and Kim (2018) demonstrated that yeast culture had no effect on ADG or feed efficiency in weanling pigs. Although most studies have reported that yeast culture improves animal growth, growth is also greatly influenced by the dietary composition, the feeding cycle, and other factors.

DAO and D-lactate are two important markers of intestinal mucosal injury (Luk, Bayless, & Baylin, 1980; Vella & Farrugia, 1998). We found that yeast culture reduced the DAO activity in the serum, indicating that it reduced intestinal permeability in weaned piglets. Intestinal permeability is mainly controlled by the TJ proteins between epithelial cells (Suzuki, 2013; Oshima & Miwa, 2016). Further studied suggested that yeast culture improved the intestinal morphology and promoted the expression of intestinal TJ proteins in vivo. Normal exfoliation of intestinal epithelial cell never causes a breach in the epithelial barrier because the redistribution of TJ proteins can facilitate the closure of the gap (Patterson & Watson, 2017). When TJ structure is destroyed, the host initiates a series of protective mechanisms to restore the normal state. Many factors, such as disease, stress and other strong oxidants, can destroy the integrity of intestinal TJ structure. Using the H_2O_2-treated IPEC-J2 cell model, we found that yeast culture strengthened the structural basis of the TJ strands by increasing the expression of Occludin, and reduced H_2O_2-induced TJ breakdown by enhancing the expression of Occludin and ZO1. We also found that yeast culture facilitated the assembly of Occludin. These evidences suggest that yeast culture is beneficial to TJ barrier in vitro. Wu et al. (2018) showed that yeast products (contain yeast culture) significant increased ileal villus height and V/C ratio in specific pathogen-free chickens, and also increased the expression of *Occludin* and *ZO-1* gene in the ileum, which are consistent with our results. However, Yang et al. (2016) showed that yeast products (contain yeast culture) adversely effected the intestinal morphology, intestinal permeability and intestinal mRNA expression of *Occludin* and *ZO-1* in weaned piglets. As Liu et al. (2019a) noted, the effects of yeast culture were largely depended upon the dietary composition. It has also been suggested that optimizing dietary protein levels is conducive to enhancing intestinal TJ barrier integrity (Beutheu et al., 2014; Tanabe, 2012).

The regulation of intestinal permeability depends on the TJ proteins between cells. ZO family is considered to play a key role in the regulation of epithelial permeability because it can trigger the reorganization of the cytoskeleton (Tornavaca et al., 2015). Occludin protein forms a TJ structure with scaffold proteins such as ZO-1 protein (Li, Fanning, Anderson, & Lavie, 2005), which can not only regulate the paracellular pathway, but also participate in the regulation of leak pathway (Raleigh et al., 2011). Knockout of Occludin in Caco-2 cells (Al-Sadi et al., 2011; Buschmann et al., 2013) will cause increased

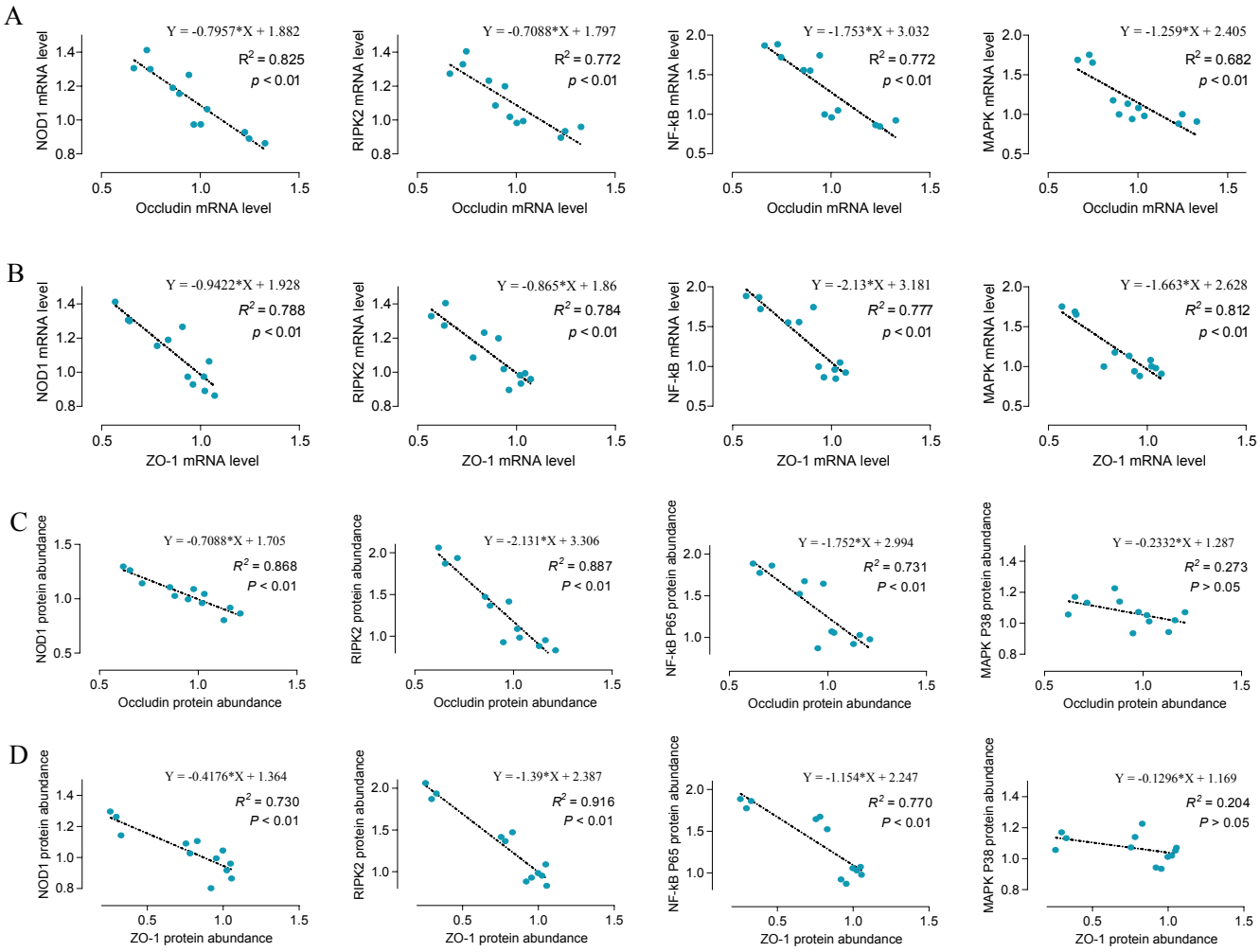

Fig. 5. Correlation analysis of TJ proteins and NOD1-mediated signaling pathway related factors. Correlation analysis of Occludin and NOD1-mediated signaling pathway related factors at the mRNA (A) and protein levels (C). Correlation analysis of ZO-1 and NOD1-mediated signaling pathway related factors at the mRNA (B) and protein levels (D). RIPK2: receptor interacting serine/threonine kinase 2; NF-κB: nuclear transcription factor κB; MAPK: mitogen-activated protein kinases.

permeability of monovalent cation and uncharged solute. Overexpression of Occludin limits the increased permeability of leak pathway induced by cytokines (Marchiando et al., 2010). Nevertheless, the role of Occludin in TJ remains controversial. Some researchers have suggested that the main role of Occludin may be to regulate immune signaling rather than epithelial permeability (Luo et al., 2017; Balda & Matter, 2016). As a regulator of TJ, Occludin may also have some functions that have not been untapped.

The integrity of the intestine is indispensable for the normal permeability function of the intestine. Strong evidences suggested that NOD1 plays an essential role in intestinal integrity (Caruso et al., 2014; Keestra-Gounder & Tsolis, 2017). NOD1 is expressed by various epithelial cells types, and is required for antimicrobial peptide production (Uehara, Fujimoto, Fukase, & Takada, 2007). Therefore, it can be considered a “mucosal gatekeepers”. On sensing its peptidoglycan ligands, NOD1 undergo auto-oligomerization, leading to the activation of RIPK2, which play crucial role in the NOD1 response (Magalhaes et al., 2011). We found that yeast culture reduced the expression of NOD1 and RIPK2 in the jejunum of weaned piglets. Yeast culture also downregulate NOD1 and RIPK2 expression in response to the injury induced by H_2O_2 in vitro. Similar results have not been reported, despite more than a decade’s work on the regulation of the NOD1-mediated signaling pathway in the host defense response and intestinal homeostasis. Intestinal homeostasis is a dynamic process regulated by many factors and can change at any time to achieve a balanced state. Our study provides a reference for yeast culture to participate in the regulation of the integrity of the intestinal barrier through NOD1-mediated signaling pathway in piglets.

The two major outcomes of signaling through the NOD1 pathway are the activation of NF-κB and MAPK, resulting in the secretion of proinflammatory cytokines, such as TNF-α, IL-1β and 1L-18 (Strober et al., 2006). Yeast culture and their glycan components improve mucosal damage and inflammation in intestinal diseases (Han, Fan, Yao, Yang, & Han, 2017). Yang et al. (2016) reported that yeast products (contain yeast culture) increased the concentration of anti-inflammatory factor IL-10 in the jejunum and ileum of weaned piglets. Bu et al. (2019) found that yeast culture downregulated the expression of proinflammatory cytokines via NF-κB related pathway in Ussuri catfish. We found that yeast culture transmitted signals from NOD1 to NF-κB P65 rather than MAPK P38 in the jejunum of weaned piglets and H_2O_2-exposed IPEC-J2 cells. We also found that Occludin and ZO-1 correlated negatively with NOD1/NF-κB P65 pathway related factors in vitro, suggesting that Occludin and ZO-1 play key roles in the activation of NOD1-mediated pathway.

The release of proinflammatory cytokines can destroy the integrity of the intestinal TJ structure, which exacerbates intestinal permeability (AL-Sadi, Boivin, & Ma, 2009). In vivo study suggested that yeast culture reduced TNF-α level in the serum and intestinal tissues, but has no similar effect on IL-18. TNF-α causes an increase in epithelial TJ permeability (Al-Sadi, Guo, Ye, Rawat, & Ma, 2016), which may be related

to the activation of myosin light chain kinase (MLCK) and the MLCK-triggered opening of the TJ barrier. TNF-α directly disrupts TJ structure via the MLCK-mediated phosphorylation of the MLC (Ye, Ma, & Ma, 2006; Zolotarevsky et al., 2002) and downregulation of ZO-1 and Occludin (Zolotarevsky et al., 2002; Mankertz et al., 2000). Moreover, a κB binding site (*cis*-κB site) located within the minimal MLCK promoter region is an essential element mediating the activation of *MLCK* gene by NF-κB (Ye & Ma, 2008). Although several studies have shown that IL-18 dysregulation can influence intestinal inflammation, the contribution of IL-18 to increase the permeability of TJ remain unclear (Lei-Leston, Murphy, & Maloy, 2017). Because of the importance of intestinal barrier function, the regulation of TJ proteins involves a complex network that is affected by many factors. Our findings may provide new insights into the mechanism by which yeast culture regulates TJ proteins.

5. Conclusions

In conclusion, this study has shown that yeast culture supplementation is beneficial to the intestinal health of weaned piglets, and improved the integrity of the intestinal TJ in weaned piglets and H_2O_2-challenged IPEC-J2 cells. This may be related to the inhibition of TNF-α release via the NOD1/NF-κB P65 pathway. Our findings may provide new insights into the mechanism by which yeast culture regulates TJ proteins in weaned piglets and H_2O_2-challenged IPEC-J2 cells.

Ethics statement

Animal experimental protocols and sample collections were approved by the Review Committee for the Use of Institutional Animal Care and Use Committee of Henan Agricultural University (Protocol no. 20181228).

Author's contributions

Shiqiong Wang: Conceptualization, Investigation, Writing - original draft. **Suiliang Zhu:** Investigation. **Jingjing Zhang:** Investigation. **Haiyan Li:** Software. **Dongji Yang:** Software. **Shucheng Huang:** Writing - review & editing. **Zhanyong Wei:** Methodology. **Xiuli Liang:** Methodology. **Zhixiang Wang:** Conceptualization, Data curation, Supervision, Writing - review & editing. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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