Saccharomyces boulardii attenuates inflammatory response induced by *Clostridium perfringens* via TLR4/TLR15-MyD8 pathway in HD11 avian macrophages

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ABSTRACT Macrophages are professional phagocytic cells that play a critical role in initiating immune responses by presenting antigen and phagocytic clearance. The macrophages can be targeted for immunomodulation by beneficial microbes, such as probiotics. The aim of this study is to investigate the protective effect of *Saccharomyces boulardii* against *Clostridium perfringens* infection in avian macrophage cell line HD11. In this study, HD11 macrophages were prestimulated with *S. boulardii* for 6 h and then infected with *C. perfringens* for 3 h. Results showed that *S. boulardii* enhanced phagocytosis and bactericidal capacity against *C. perfringens* by HD11 cells. The *S. boulardii* effectively promoted the mRNA expression of CD80, CD83, and CD197 cell-surface molecules in *C. perfringens*-infected HD11 cells. Moreover, we found that prestimulation with *S. boulardii* reduced the mRNA expression of CD40, toll-like receptor [**TLR**] 4, and TLR15 induced by *C. perfringens* and thereby downregulated the mRNA expression of myeloid differentiation primary response 88, TNF receptor associated factor 6, nuclear factor kappa-B p65 subunit, and c-Jun N-terminal kinase genes in HD11 cells. The upregulation of cytokines (interleukin [**IL**]-6, tumor necrosis factor alpha, and IL-10) and inducible nitric oxide synthase mRNA expression in *C. perfringens*-infected HD11 cells were noticeably inhibited by *S. boulardii* pretreatment. Conclusively, these results might provide a new insight into the role of *S. boulardii* in regulating avian immune defense against *C. perfringens* invasion and immune escape.

Key words: *Saccharomyces boulardii*, HD11 macrophage, immunomodulation, *Clostridium perfringens*

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INTRODUCTION

Clostridium perfringens (***C. perfringens***) is an anaerobic spore-forming pathogenic bacterium that causes a wide variety of diseases ranging from gas gangrene and food poisoning in humans to necrotic enteritis in animals (Kiu and Hall, 2018). It is commonly found in soil, dust, drinking water, feed, litter, feces, wastewater, and the gastrointestinal tract of humans and animals as a normal component of microbiota (Fasina et al., 2016; Prescott et al., 2016). Normally, *C. perfringens* does not cause intestinal pathophysiological changes and gut microbiota imbalance of broilers (Lacey et al., 2016; Schoster et al., 2019). However, predisposing factors, such as intestinal mucosal damage induced by *Eimeria spp.* and virus, high dietary levels of animal protein (e.g., fish meal) and fat, intestinal microbiota imbalance, or improper management (e.g., overcrowding), may favor proliferation of *C. perfringens* and secretion of its toxins that induce intestinal mucosal damage in chickens, and thereby results in subclinical or clinical necrotic enteritis disease (Wu et al., 2014; Moore, 2016). It is reported that the total global economic loss caused by necrotic enteritis is estimated to be over

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$6 billion per year (Wade et al., 2015). With the prohibitions on the antibiotic growth promoters, proper alternatives such as dietary interventions are being evaluated to improve animal gut health and prevent *C. perfringens* infection (M'Sadeq et al., 2015). Notable among the feed interventions are the use of direct-fed microbials as feed additives in poultry industry, as they are considered to be "Generally Recognized as Safe" additives and have been shown to be effective pathogen inhibitors and immune modulators in response to pathogenic bacterial infections (Buntyn et al., 2016).

As an antigen-presenting cell, the macrophages play an important role in host defense, tissue repair, and homeostasis (Mao et al., 2015). It is reported that macrophages are plastic, dynamic, and heterogeneous phagocytes (Mills, 2012). On one hand, macrophages phagocytize and kill pathogens by secreting proinflammatory cytokines (e.g., interleukin [**IL**]-6, IL-12, tumor necrosis factor alpha [**TNF-α**], and IL-1β), chemokines (e.g., CCL2, CCL5, and CXCL8), and chemical species (reactive oxygen species, reactive nitrogen species) to induce inflammation (Benoit et al., 2008; Murray, 2017). On the other hand, macrophages secrete anti-inflammatory cytokines (e.g., IL-4, IL-10, and TGF-β) and growth factors (e.g., PDGF, VEGF, and EGF) to dampen excessive or prolonged inflammation which can result in tissue injury and contribute to pathogenesis (Vannella and Wynn, 2017). Depending on the converging signals from inflammatory stimuli and the cellular environment, macrophages activation is broadly described as M1 and M2 phenotype (Vergadi et al., 2017). M1 phenotypic macrophages can be activated by bacteria-derived lipopolysaccharide (**LPS**) via toll-like receptors/myeloid differentiation primary response 88 (**TLR/MyD88**) signaling pathway, which has shown to be of vital importance in controlling intracellular pathogens (Da Silva et al., 2007; Mosser and Edwards, 2008).

Probiotics are live microorganisms, which exert a beneficial influence on animal health by reshaping gut microbiota, enhancing digestive capacity, improving gastrointestinal mucosal barrier, inhibiting intestinal pathogenic bacteria growth and colonization, or regulating mucosal immune system (Sánchez et al., 2017). Several selected probiotics, such as lactic acid bacteria, *Bacillus* species, *Bifidobacterium*, and yeast, have been applied to improve animal performance and health in food animal production (Wang et al., 2016). A number of studies in vivo have shown that *Saccharomyces boulardii*, as a nonpathogenic yeast, has been introduced to prevent or treat various gastrointestinal complications both

Table 1. Primer sequences used for qRT-PCR.

Gene name	Primer sequence (5′-3′)	Product size	Accession No.
CD40	F: GGCACCTTCTCCAATGTATCTTC R: GTTCGTCCCTTTCACCTTCAC	96	NM_204665
CD80	F: CAGCAAGCCGAACATAGAAAGA R: AGCAAACTGGTGGACCTGAGA	270	NM_001079739
CD83	F: GCTGACTTGCCTCGGGATT R: TCACTCCGCTATCCGTCTCA	272	XM_418929.5
CD197	F: GACGACTATGACGCCAACAC R: CCAGGTTCAGCAAGTAGATGTC	211	NM_001198752
IL-6	F: CTCCTCGCCAATCTGAAGTC R: CCTCACGGTCTTCTCCATAAAC	99	NM_204628
IL-1β	F: CGACATCAACCAGAAGTGCTT R: GTCCAGGCGGTAGAAGATGA	298	NM_204524
TNF-α	F: GGACAGCCTATGCCAACAAG R: GCGGTCATAGAACAGCACTAC	81	NM_204267
IL-10	F: ACCAGTCATCAGCAGAGCAT R: CCTCCTCATCAGCAGGTACTC	222	NM_001004414
iNOS	F: TACTCTTGGCGTCATTACTC R: GCATAGATCACAGTCACCTT	67	NM_204961
TLR1	F: CCGTTCAAGTGTTCGTGTGA R: CCGCTCAAGTCTTCTGGGTA	116	NM_001007488
TLR2	F: TGTTCCTGTTCATCCTCATCCT R: AGTTGGAGTCGTTCTCACTGT	168	NM_204278
TLR4	F: GAATGACACGGACACTCTT R: ACATAGGAACCTCTGACAAC	95	NM_001030693
TLR15	F: CTTGTCGTTCTGGTGCTAA R: ATCGTGCTCGCTGTATGA	156	NM_001037835
MyD88	F: GGATGGTGGTCGTCATTTCA R: GAGATTTTGCCAGTCTTGTCCA	225	NM_001030962
TRAF6	F: ATATCCAGTTACCAAGTGCTCAG R: CAAGGCAGATGGCTGTCATAT	91	XM_004941548
NF-κB p65	F: CTTCAATGTGCCAATGGAGGAG R: CTCAGCCCAGAAACGAACCT	271	NM_205129
JNK	F: GTAGTAGATCCAGACAAGAGAA R: TCATACCAGACCGTAATATAGG	71	NM_205095
β-actin	F: TATGTGCAAGGCCGGTTTC R: TGTCTTTCTGGCCCATACCAA	110	NM_205518

Abbreviations: CD, cluster of differentiation; F, forward; IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response 88; NF-κB p65, nuclear factor kappa-B p65 subunit; R, reverse; TLR, toll like receptor; TNF-α, tumor necrosis factor alpha; TRAF6, TNF receptor associated factor 6.

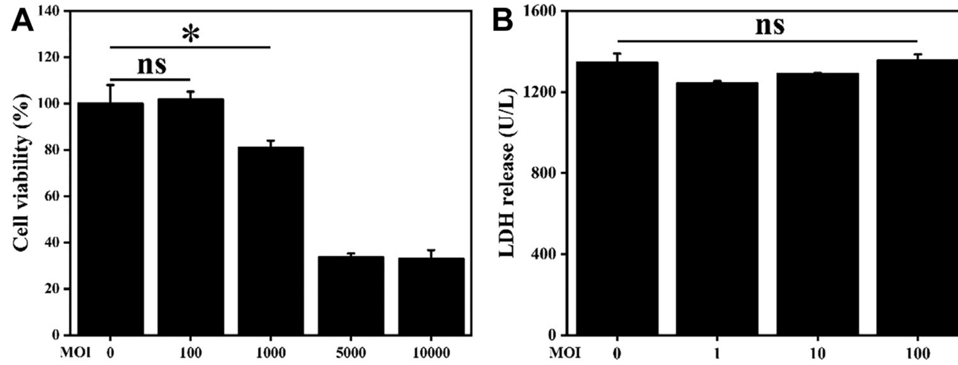

Figure 1. Cytotoxicity analysis of *S. boulardii* on chicken HD11 cells. HD11 cells were incubated with PBS or *S. boulardii* (MOI = 0, 100, 1,000, 5,000 or 10,000) for 6 h. (A) Cell viability was determined by the CCK-8 assay. (B) Cell damage was measured by testing the release of LDH. Results are presented as mean ± SD of 8 (CCK-8 assay) or 6 (LDH assay) samples. $^{*}P < 0.05$ (t test). ns indicates no significance ($P > 0.05$). Abbreviations: LDH, lactate dehydrogenase; MOI, multiplicity of infection.

in human and animals (Anand et al., 2018). Our previous in vivo study showed that *S. boulardii* supplementation modulates intestinal ultrastructure by upregulating the tight junction protein expression in broilers (Rajput et al., 2013). However, the role of *S. boulardii* on *C. perfringens*-induced inflammatory response in chicken macrophages is less studied, and the mechanism remains obscure. In this study, we investigated the protective effect of *S. boulardii* against *C. perfringens* infection in avian HD11 cell lines and its underlying immune mechanism.

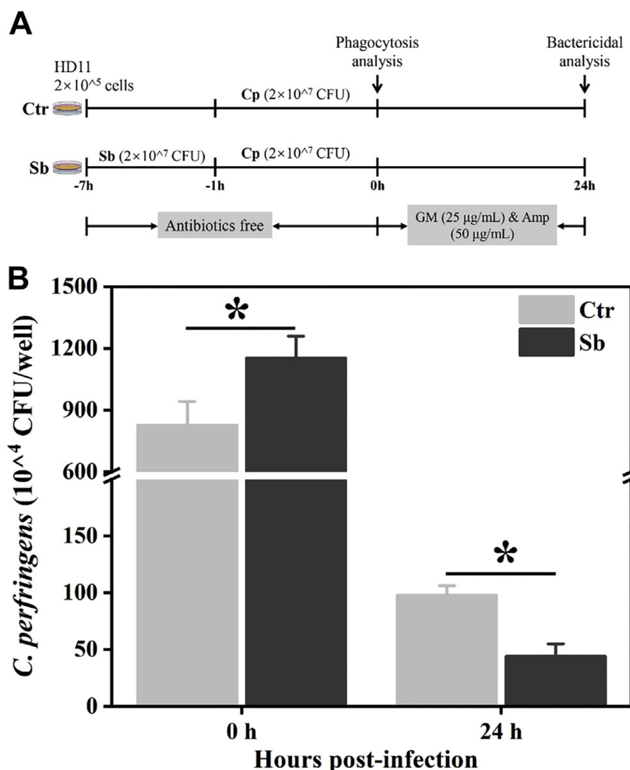

Figure 2. *S. boulardii* enhances phagocytosis and bactericidal capacity of HD11 cells. (A) Schematic illustration of *C. perfringens*-*killing* assay. HD11 cells were pretreated with *S. boulardii* (MOI = 100) for 6 h. After being infected with *C. perfringens* (MOI = 100) for 1 h, washed, and incubated in RPMI 1640 medium with gentamicin (25 μg/mL) and ampicillin (100 μg/mL) for 0 h or 24 h, these cells were lysed, diluted, and plated on reinforced clostridium medium (**RCM**) agar plates for colony enumeration. (B) *S. boulardii* enhances phagocytosis and bactericidal activity of macrophages. Results are mean ± SD for 3 independent experiments. $^{*}P < 0.05$ (t test). Abbreviations: Amp, ampicillin; Cp, *C. perfringens*; Ctr, control group; GM, gentamicin; MOI, multiplicity of infection; Sb, *S. boulardii* treated group.

MATERIALS AND METHODS

Strains and Culture Conditions

S. boulardii was isolated and identified by our lab and was cultured overnight at 30°C in yeast peptone dextrose broth (OXOID; UK) under aerobic conditions. *Clostridium perfringens* type A (ATCC 13124) was cultured in reinforced clostridium medium at 37°C for 20 h in anaerobic gas generating packs (Mitsubishi Gas Chemical Company Inc., Tokyo, Japan). *S. boulardii* and *C. perfringens* were harvested by centrifugation at 3,500 × g for 10 min, respectively. After 3 times washing with phosphate-buffered saline (**PBS**, pH = 7.2), the pellets of *S. boulardii* and *C. perfringens* were resuspended in RPMI 1640 medium (antibiotics free) for preincubation or infection. Optical density method (SpectraMax M5, Molecular Devices, San Jose, CA) was performed to adjust the final concentration of *S. boulardii* and *C. perfringens*.

Cell Culture

The avian macrophage cell line HD11 was kindly provided by Dr. Shou-Qun Jiang (GDAAS, Guangzhou, China) and was cultured in RPMI 1640 medium (Gibco, Carlsbad, CA) supplemented with 10% chicken serum (Gibco), 100 μg/mL streptomycin, and 100 U/mL penicillin (Sigma-Aldrich, St. Louis, MO), 1× nonessential amino acids (Invitrogen, Waltham, MA), sodium pyruvate (1 mmol/L), L-glutamine (2 mmol/L), and 2-mercaptoethanol (5×10^{-5} mol) at 41°C in a 5% humidified CO_2 incubator. If not mentioned, the antibiotics (100 μg/mL streptomycin and 100 U/mL penicillin) will not add into the RPMI 1640 medium in the further experiment.

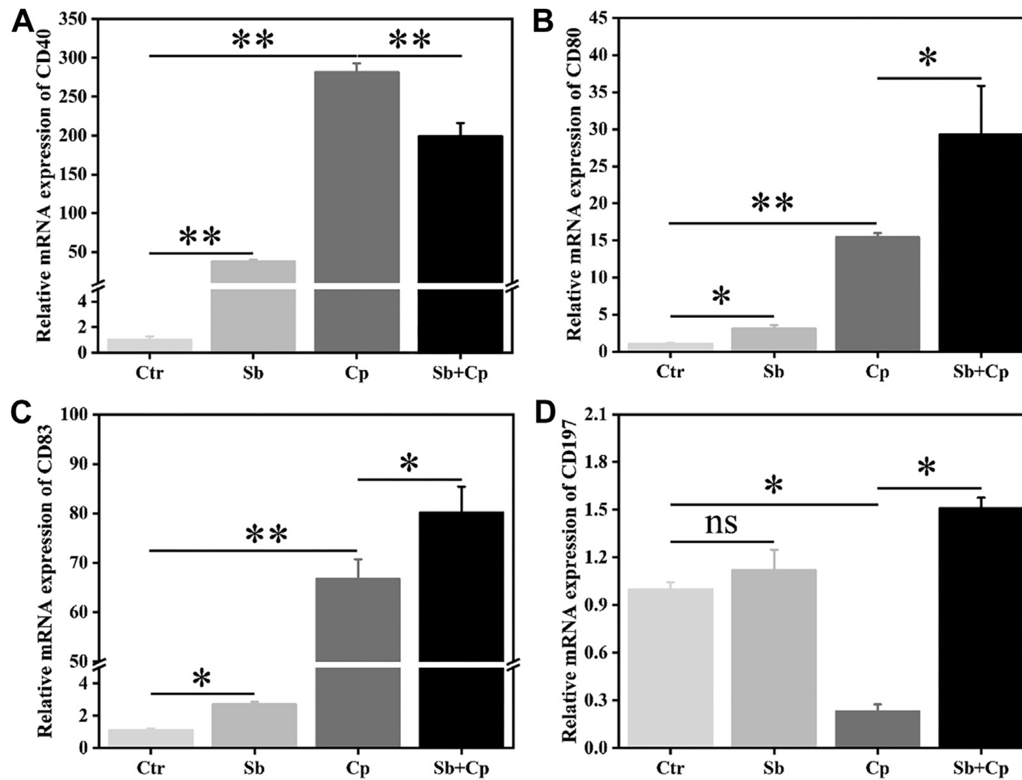

Figure 3. Effect of *S. boulardii* on cell surface molecule mRNA expression of HD11 macrophages. HD11 cells were preincubated with *S. boulardii* (MOI = 100) for 6 h and then infected with *C. perfringens* (MOI = 100) for 3 h. Total RNA was isolated, and the mRNA expression of cluster of differentiation 40 (CD40) (A), CD80 (B), CD83 (C), and CD197 (D) was analyzed by real-time PCR. Results are mean ± SD for 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (*t* test). ns indicates no significance ($P > 0.05$). Abbreviations: Cp, *C. perfringens* treated group; Ctr, control group; MOI, multiplicity of infection; Sb, *S. boulardii* treated group; Sb + Cp, *S. boulardii* pretreated and then Cp infected group.

Cytotoxicity Assay

Cell viability was determined by cell counting kit-8 (**CCK-8**, Beyotime, Nanjing, China) according to the manufacturer's instruction. Briefly, HD11 cells were seeded into 96-well microplate at 2.0×10^4 cells/mL (Corning Inc., Corning, NY) and incubated with *S. boulardii* (multiplicity of infection (**MOI**) = 0, 100, 1,000, 5,000, 10,000) for 6 h. After washed 3 times with sterile PBS, the cell wells were added with CCK-8 kit solution (10 μL/well) and further incubated for 1 h. Subsequently, the optical density value was measured by SpectraMax M5 (Molecular Devices) at OD450, and the relative cell viabilities was calculated as previously described (Mosmann, 1983). The release of lactate dehydrogenase (**LDH**) from the damaged cells was quantified using LDH kit (Beyotime) after HD11 cells incubated with *S. boulardii* (MOI = 0, 1, 10, 100) for 6 h (Wu et al., 2017).

Clostridium perfringens*-Killing Analysis*

The effect of *S. boulardii* on the *C. perfringens*-killing capacity of chicken macrophage HD11 cells was measured by a viable count method, as described previously (Wang et al., 2018). Briefly, HD11 cells seeded into 12-well plates (2×10^5 cells/mL) were preincubated with *S. boulardii* (MOI = 100, 2×10^7 CFU/well) for 6 h under aerobic conditions. For phagocytosis analysis, after washed 3 times with sterile PBS, HD11 cells were then infected with *C. perfringens* (MOI = 100, 2×10^7 CFU/well) in the RPMI 1640 medium (absence of chicken serum) at 41°C for 1 h under aerobic conditions. For bactericidal analysis, the infected HD11 cells were washed 3 times with sterile PBS and then incubated in RPMI 1640 containing gentamicin (25 μg/mL) and ampicillin (50 μg/mL) for another 24 h. At each of time points (0 h and 24 h), infected HD11 cells were washed 5 times with sterile PBS and lysed with 0.01% Triton X-100 diluted in PBS (1 mL/well). The serial 10-fold dilutions of cell lysates were immediately plated on reinforced clostridium medium agar plates in triplicate and then incubated at 37°C for 24 h under anaerobic conditions. The number of surviving intracellular bacteria was determined by bacterial colony counting in the next day.

RNA Isolation and Quantitative Real-Time PCR

HD11 cells seeded into 12-well plates (2×10^5 cells/mL) were pretreated with *S. boulardii* (MOI = 100) for 6 h, and then infected with *C. perfringens* (MOI = 100) for 3 h, and then washed by PBS 3 times to collect the cell pellets. Total RNA isolated from HD11 cells using RNAiso Plus (TaKaRa, Dalian, China) was reverse-transcribed by PrimeScript II first Strand cDNA Synthesis Kit (TaKaRa). Reverse transcription-

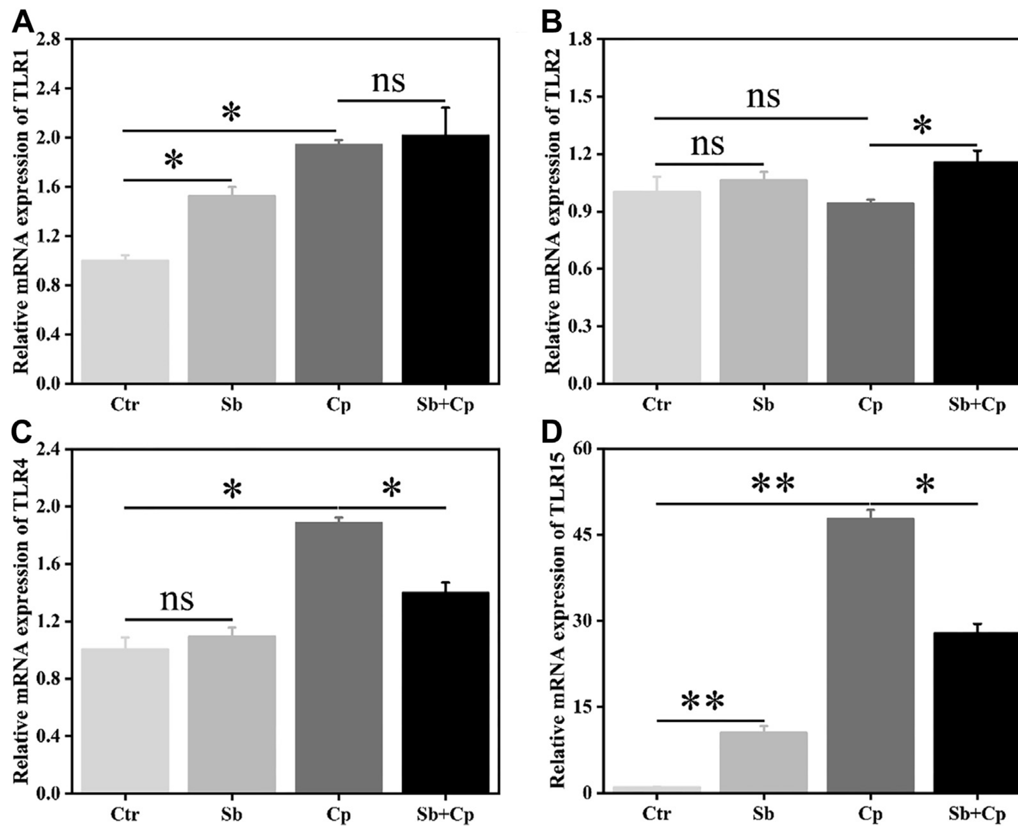

Figure 4. Effects of *S. boulardii* on TLRs mRNA expression of HD11 macrophages. HD11 cells were preincubated with *S. boulardii* (MOI = 100) for 6 h and then infected with *C. perfringens* (MOI = 100) for 3 h. Then, total RNA was isolated, and the mRNA expression of toll like receptor 1 (TLR1) (A), TLR2 (B), TLR4 (C), and TLR15 (D) was analyzed by real-time PCR. Results are mean ± SD for 3 independent experiments. $*P < 0.05$, $**P < 0.01$ (*t* test). ns indicates no significance ($P > 0.05$). Abbreviations: Cp, *C. perfringens* treated group; Ctr, control group; MOI, multiplicity of infection; Sb, *S. boulardii* treated group; Sb + Cp, *S. boulardii* pretreated and then Cp infected group.

qPCR was performed using SYBR PremixExTaq II (TaKaRa) and the StepOne Plus Real-Time PCR system (Applied Biosystems, Carlsbad, CA). All primer sequences for chicken macrophages genes are listed in Table 1. The housekeeping gene β-actin was selected as reference gene, and relative quantification was calculated using the $2^{-\Delta\Delta C_q}$ method (Bustin et al., 2009). ΔC_q is $C_{q,\ target} - C_{q,\ reference}$, and $\Delta\Delta C_q$ is $\Delta C_{q,\ treatment} - \Delta C_{q,\ control}$.

Statistical Analysis

Results between 2 groups were analyzed by 2-tailed Student *t* test using SPSS 22.0 (SPSS Inc., Chicago, IL), and graphs were represented as mean ± SD of at least 3 independent experiments and visualized by Origin 8.0 (OriginLab, Berkeley, CA). $*P < 0.05$, $**P < 0.01$.

RESULTS

Cytotoxicity Analysis of S. boulardii

The cytotoxicity of *S. boulardii* on chicken HD11 cells was measured by the CCK-8 kit. Significant ($P < 0.05$) cell cytotoxicity was observed when macrophages were incubated with *S. boulardii* at MOI of 1,000 but not at MOI of 100 ($P > 0.05$, Figure 1A). Moreover, the safety of *S. boulardii* at MOI of 100 was further confirmed by testing the release of the cytosolic marker LDH (Korzeniewski and Callewaert, 1983). There was no significant ($P > 0.05$) difference in LDH release among the groups (Figure 1B). Therefore, *S. boulardii* at MOI of 100 was selected for the further experiments.

S. boulardii Enhances Phagocytosis and Intracellular Bactericidal Capacity of Macrophages

Phagocytosis and intracellular bactericidal capacity play vital roles in macrophage-mediated host defense, which leads to internalization and death of pathogens (Mao et al., 2015). We further examined whether *S. boulardii* improved the phagocytosis and intracellular bactericidal capacity of the HD11 macrophages. The results showed that *S. boulardii* treatment significantly ($P < 0.05$) increased the uptake of *C. perfringens* in HD11 cells (Figure 2B, 0 h), suggesting that *S. boulardii* enhances the phagocytosis of HD11 cells. After 24 h incubation, the intracellular survival of *C. perfringens* in *S. boulardii*-pretreated HD11 cells significantly ($P < 0.05$) reduced compared with the unpretreated macrophages (Figure 2B, 24 h). The results demonstrated that *S. boulardii* could enhance phagocytosis

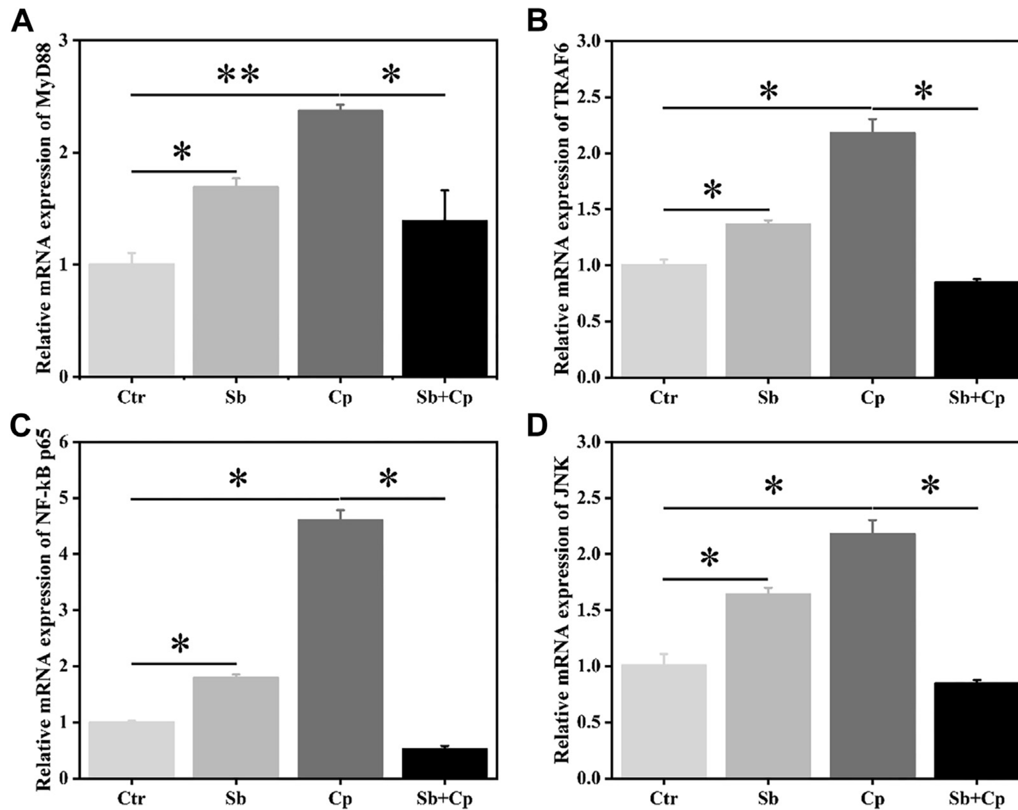

Figure 5. Effect of *S. boulardii* on the signaling pathways related mRNA expression of HD11 macrophages. HD11 cells were preincubated with *S. boulardii* (MOI = 100) for 6 h and then infected with *C. perfringens* (MOI = 100) for 3 h. Then total RNA was isolated and the mRNA expression of MyD88 (A), TRAF6 (B), NF-κB p65 (C), and JNK (D) was analyzed by real-time PCR. Results are mean ± SD for 3 independent experiments. $^{*}P < 0.05$, $^{**}P < 0.01$ (*t* test). Abbreviations: Cp, *C. perfringens* treated group; Ctr, control group; JNK, c-Jun N-terminal kinase; MOI, multiplicity of infection; MyD88, myeloid differentiation primary response 88; NF-κB p65, nuclear factor kappa-B p65 subunit; Sb, *S. boulardii* treated group; Sb + Cp, *S. boulardii* pretreated and then Cp infected group; TRAF6, TNF receptor associated factor 6.

and intracellular bactericidal capacity of avian macrophages.

Effect of S. boulardii *on Cell-Surface Molecules mRNA Expression of Macrophages*

S. boulardii-induced phagocytosis and bactericidal capacity were confirmed by the activation of macrophages. As shown in Figure 3, both *S. boulardii* and *C. perfringens* exposure markedly ($P < 0.05$ or $P < 0.01$) upregulated the mRNA expression of CD40, CD80, and CD83, whereas *C. perfringens* significantly ($P < 0.05$) inhibited the CD197 mRNA expression in HD11 cells. *S. boulardii* pretreatment significantly ($P < 0.05$) enhanced *C. perfringens*-induced mRNA expression of CD80, CD83, and CD197, whereas significantly ($P < 0.05$) decreased *C. perfringens*-induced mRNA expression of CD40 in HD11 cells (Figure 3).

Effect of S. boulardii *on mRNA Expression of TLR Signaling Molecules in Macrophages*

The TLR signaling pathways play an important role in the initiation and activation of macrophage inflammatory responses (Fitzgerald and Kagan, 2020). As shown in Figure 4, both *S. boulardii* and *C. perfringens* exposure significantly enhanced TLR1 and TLR15 mRNA expression ($P < 0.05$ and $P < 0.01$, respectively), and *C. perfringens* infection upregulated ($P < 0.05$) the TLR4 mRNA expression in HD11 cells. *S. boulardii* pretreatment markedly ($P < 0.05$) downregulated the mRNA expression of TLR4 and TLR15 induced by *C. perfringens*, whereas upregulated ($P < 0.05$) TLR2 mRNA expression in HD11 cells.

As shown in Figure 5, both *S. boulardii* and *C. perfringens* exposure significantly ($P < 0.05$ or $P < 0.01$) enhanced the mRNA expression of MyD88, TNF receptor associated factor 6 (**TRAF6**), nuclear factor kappa-B p65 subunit (**NF-κB p65**), and c-Jun N-terminal kinase (**JNK**), whereas *S. boulardii* pretreatment markedly ($P < 0.05$) downregulated *C. perfringens*-induced the mRNA expression of MyD88, TRAF6, NF-κB p65, and JNK in macrophages (Figure 5). To sum up, these data demonstrated that *S. boulardii* pretreatment inhibited *C. perfringens*-induced activation of TLR4/TLR15-MyD88 signaling pathways and thus inflammation.

Effect of S. boulardii *on mRNA Expression of Inflammatory Factors in Macrophages*

The downstream target mRNA expression of TLRs signaling pathways was further measured by qPCR (Figure 6). The results showed that both *S. boulardii*

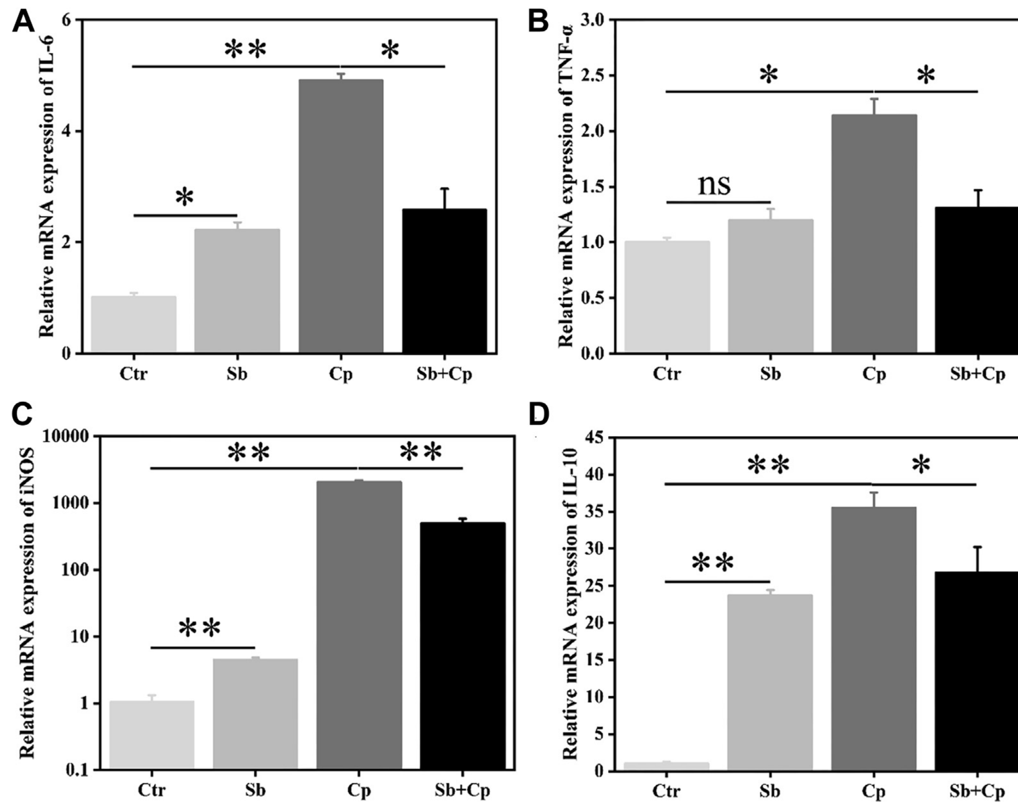

Figure 6. Effect of *S. boulardii* on inflammatory factors mRNA expression of HD11 macrophages. HD11 cells were preincubated with *S. boulardii* (MOI = 100) for 6 h and then infected with *C. perfringens* (MOI = 100) for 3 h. Then total RNA was isolated, and the mRNA expression of IL-6 (A), TNF-α (B), iNOS (C), and IL-10 (D) was analyzed by real-time PCR. Results are mean ± SD for 3 independent experiments. *$P < 0.05$, **$P < 0.01$ (*t* test). ns indicates no significance ($P > 0.05$). Abbreviations: Cp, *C. perfringens* treated group; Ctr, control group; IL, interleukin; iNOS, inducible nitric oxide synthase; MOI, multiplicity of infection; Sb, *S. boulardii* treated group; Sb + Cp, *S. boulardii* pretreated and then Cp infected group; TNF-α, tumor necrosis factor alpha.

and *C. perfringens* exposure significantly ($P < 0.05$ or $P < 0.01$) upregulated the mRNA expression of IL-6, inducible nitric oxide synthase (**iNOS**), and IL-10 in HD11 cells, whereas *C. perfringens* also significantly increased the mRNA expression of TNF-α ($P < 0.05$). Additionally, *S. boulardii* pretreatment significantly ($P < 0.05$) reduced *C. perfringens*-induced mRNA expression of IL-6, TNF-α, iNOS, and IL-10 in HD11 cells. These results indicated that *S. boulardii* alleviates *C. perfringens*-induced excessive proinflammatory response.

DISCUSSION

Macrophages play indispensable roles in initiating the innate immune response, shaping the adaptive immune response, and the defense against pathogens (Hoebe et al., 2004). Once stimulated by foreign antigens and inflammatory stimuli, immature macrophages undergo maturation, evolving from antigen-presenting cells to T cell-priming cells to promote T lymphocyte activation (Laskin, 2009). Previous studies in vivo have evidenced that probiotics, including *S. boulardii*, have a beneficial effect in improving growth performance and prevention of pathogenic infections in poultry (Li et al., 2018; de Oliveira et al., 2019; Nari and Ghasemi, 2020; Ramlucken et al., 2020). However, the protective mechanism of *S. boulardii* mediated by chicken macrophages remains unclear. Phagocytosis by macrophages is critical for the uptake and degradation of intracellular pathogens and for the initiation of the innate immune response (Aderem and Underhill, 1999). However, some pathogens, such as *Listeria*, *Salmonella typhimurium* and *Clostridium perfringens*, can survive and replicate in macrophages by escaping the phagosome and modifying the vacuolar maturation process of macrophages (Aderem and Underhill, 1999; O'Brien and Melville, 2004). Previous studies found that *Bacillus amyloliquefaciens* could enhance the phagocytosis and bactericidal activity of murine macrophages against *S. Typhimurium* or *E.coli* (Wu et al., 2017; Fu et al., 2019), which was consistent with our results. The present results showed that *S. boulardii* enhanced the HD11 macrophages' phagocytosis and its ability to clear intracellular *C. perfringens* (Figure 2), which was further confirmed by the activation and maturation of the macrophages (Figure 3). Activated macrophages can be characterized by the high expression of costimulatory molecules (CD40, CD80, and CD83) or surface marker (CD197), which are necessary for the activation of T and B lymphocytes in the host (Zotos and Tarlinton, 2012; Wang et al., 2018). The present results showed that *S. boulardii* pretreatment promoted the *C. perfringens*-induced activation and maturation of HD11 macrophages, as evidenced by the upregulation of macrophage surface

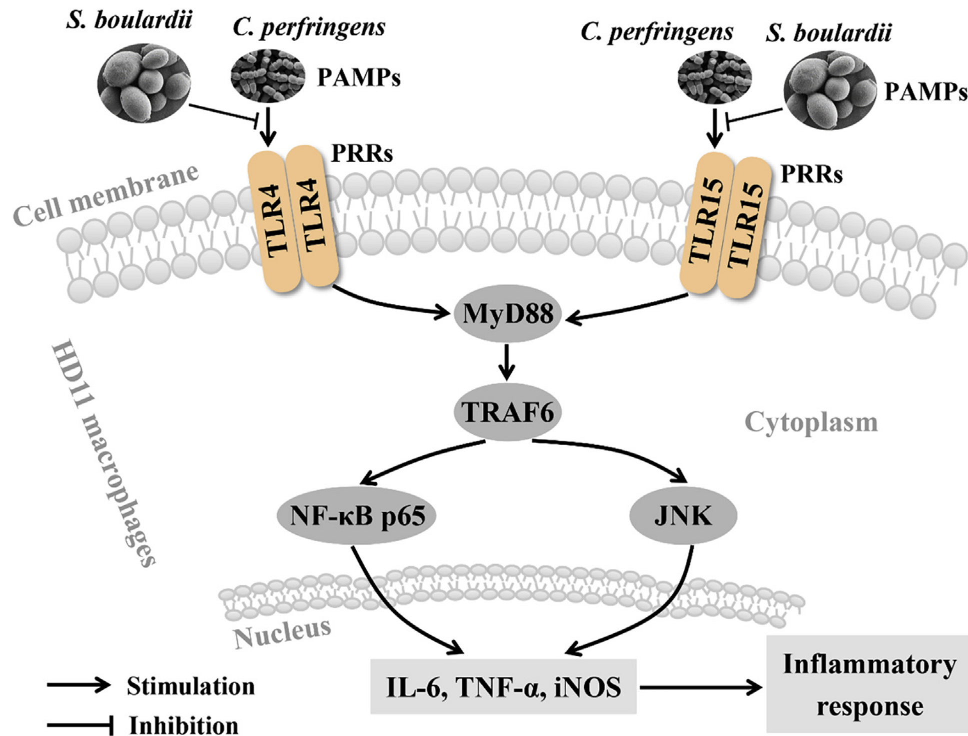

Figure 7. Graphical summary of the anti-inflammatory role of *Saccharomyces boulardii* against *Clostridium perfringens* infection in chicken HD11 macrophages. *S. boulardii* induces HD11 macrophages activation to clear *C. perfringens* and attenuates *C. perfringens*-induced proinflammatory responses mediated by TLR4/TLR15-MyD88 signaling pathway. Abbreviations: IL, interleukin; iNOS, inducible nitric oxide synthase; JNK, c-Jun N-terminal kinase; MyD88, myeloid differentiation primary response 88; NF-κB p65, nuclear factor kappa-B p65 subunit; PRRs, pattern recognition receptors; TLR, toll like receptor; TNF-α, tumor necrosis factor alpha; TRAF6, TNF receptor associated factor 6.

marker (CD197) and costimulatory receptors (CD80 and CD83) mRNA expression, consistent with previous observations (Wang et al., 2018). These results indicate that *S. boulardii* activates an innate immune response in HD11 macrophages to eliminate intracellular *C. perfringens*. CD40 (also known as tumor necrosis factor receptor superfamily 5) is a member of the tumor necrosis factor receptor superfamily that is expressed on macrophages, dendritic cells, and B cells (Croft et al., 2013). Not only is CD40 essential for activation and proliferation of B cells by T cell-dependent antigens, but also it is an important regulator to induce the production of inflammatory cytokines in macrophages and dendritic cells (Quezada et al., 2004; Croft et al., 2013; Croft and Siegel, 2017). In the present study, *S. boulardii* pretreatment downregulated *C. perfringens*-induced CD40 mRNA expression (Figure 3A), indicating that *S. boulardii* might attenuate *C. perfringens*-induced inflammatory response by blocking CD40.

Inflammatory response is regulated by a complex and cross-linked endogenous cellular signaling pathways and their modulators (Takeuchi and Akira, 2010). The immune responses of the host are initiated immediately after pattern recognition receptors recognize pathogen-associated molecular patterns, which is essential for effective clearance of infectious microbes (Palm and Medzhitov, 2009). Evidence has shown that the host–microbe interactions depend on the pattern recognition receptors, such as the TLRs and the pathogen-associated molecular patterns (Fitzgerald and Kagan, 2020). TLR2 can recognize many different microbial and synthetic components (Beutler et al., 2006), whereas TLR1/2 heterodimers recognize di- or tri-acylated lipoproteins or lipopeptides (Jin et al., 2007). Although TLR4 specifically recognizes bacteria-derived LPS (Lee and Kim, 2007), many studied have recorded that TLR4 is also involved in the host immune responses induced by *C. perfringens*. Shi et al. (2019) reported that TLR4/MyD88/NF-κB signaling pathway was involved in the inflammatory responses in piglet diarrhea induced by *C. perfringens*. *C. perfringens* α-toxin disturbs host defense by modulating TLR4-mediated inflammatory response (Takehara et al., 2019). Avian-specific TLR15 recognizes nonsecreted, heat-stabile components of both gram-positive and gram-negative pathogens and plays a constitutive role in the immune defense of chickens (Higgs et al., 2006; Nerren et al., 2010). In the current study, we found that *S. boulardii* treatment specifically upregulated TLR1 and TLR15 mRNA expression (Figure 4). The constant stimulation of TLR is necessary for maintaining immune homeostasis in the intestine (Rajput et al., 2014). We found that *C. perfringens* infection significantly increased the mRNA expression of TLR1, TLR4 and TLR15, whereas *S. boulardii* pretreatment markedly decreased *C. perfringens*-induced mRNA expression of TLR4 and TLR15 (Figure 4). After recognition, TLR trigger the activation of the downstream signaling pathways (such as mitogen-activated protein kinase and transcription factors nuclear factor **[NF]**-κB) by MyD88-dependent

and TRIF-dependent (MyD88-independent) ways, which leads to the production of inflammatory cytokines and chemokines (Lee and Kim, 2007). Our data demonstrated that *S. boulardii* treatment significantly increased the expression of MyD88, TRAF6, NF-κB p65, and JNK (Figure 5), indicating that *S. boulardii* activated HD11 macrophages via TLR1/TLR15-MyD88-dependent manner. In addition, we also found that *S. boulardii* pretreatment downregulated *C. perfringens*-induced overexpression of MyD88, TRAF6, NF-κB p65, and JNK (Figure 5), indicating that *S. boulardii* attenuated *C. perfringens*-induced TLR4/TLR15-MyD88 signaling.

The main role of the TLR-MyD88-dependent pathway is to induce the expression of inflammatory cytokines (such as IL-6, IL-12, and TNF-α), which is necessary for pathogen clearance (Lee and Kim, 2007). However, exaggerated immune responses can be detrimental to the host (Kawasaki and Kawai, 2014). Therefore, the excessive immune responses are tightly controlled by associated negative feedback loops and anti-inflammatory cytokines (Martinez et al., 2009; Ip et al., 2017). Evidences have shown that IL-10 and TGF-β are the 2 main potent anti-inflammatory cytokines (Opal and DePalo, 2000). In our study, we found that *S. boulardii* treatment significantly increased the mRNA expression of both pro- and anti-inflammatory cytokines (IL-6, iNOS, and IL-10) (Figure 6), suggesting that *S. boulardii* might serve as a regulator to maintain the anti- and pro-inflammatory response in macrophages. Furthermore, we also found that *C. perfringens* infection significantly upregulated the mRNA expression of inflammatory cytokines (IL-6, TNF-α, iNOS, and IL-10), whereas *S. boulardii* pretreatment decreased *C. perfringens*-induced inflammatory cytokines mRNA expression (Figure 6). Similar to our results, Thomas et al. (2009) also found that *S. boulardii* cultured supernatant reduced secretion of proinflammatory cytokines (IL-6 and TNF-α) in LPS-stimulated dendritic cells. These results indicate that *S. boulardii* alleviates *C. perfringens*-induced excessive proinflammatory responses mediated by TLR4/TLR15-MyD88 signaling pathway.

CONCLUSION

Overall, this study demonstrates that *S. boulardii* induces HD11 macrophages activation to clear *C. perfringens* and attenuates *C. perfringens*-induced inflammatory responses mediated by TLR4/TLR15-MyD88 signaling pathway (Figure 7). These findings might provide a new perspective into the function of *S. boulardii* in regulating avian immune defense against *C. perfringens* invasion and immune escape. Given that avian necrotic enteritis is mainly caused by *C. perfringens*, these findings also provide a potential preventive approach against avian necrotic enteritis caused by *C. perfringens*. However, further investigations about the protective effect of *S. boulardii* against *C. perfringens* infection in broilers are needed.

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