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# *Saccharomyces boulardii* and *Bacillus subtilis* B10 Modulate TLRs Mediated Signaling to Induce Immunity by Chicken BMDCs

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## ABSTRACT

Dendritic cells (DCs) are professional antigen-presenting cells (APCs) that play a critical role to activate immune response. They may be targeted for immunomodulation by microbes, including probiotics. In this study, chicken bone marrow dendrite cells (chi-BMDCs) were stimulated with lipopolysachride (LPS), *Saccharomyces boulardii* (Sb), *Bacillus subtilis* B10 (Bs), co-culture of Sb + Bs and phosphate buffer saline (PBS) as a control group (Ctr) at 3, 6, and 12 h intervals. Results revealed that treatment groups modulated the phenotype and biological functions of chi-BMDCs. Scan electron microscopy showed attachment of probiotics on the surface of chi-BMDCs. Additionally transmission electron microscopy (TEM) revealed efficiently engulfing and degradation of probiotics. Gene expression levels of MHC-II, CD40, CD80 and CD86 up-regulated in stimulated groups. Furthermore, toll-like receptors TLR1, TLR2, TLR4, and chicken specific TLR15 expressions were improved and downstream associated factors MyD88, TRAF6, TAB1, and NFκ-B mRNA levels increased in all treatment groups as compared to control. Surprisingly, NFκ-B response was noted significant higher in LPS treatment among all groups. Moreover, IL-1β, IL-17, IL-4, TGF-β, and IL-10 production levels were found higher, and lower concentration of INF-γ and IL-8 were observed in Sb, Bs, and Sb + Bs treatment groups. In contrast, LPS groups showed prominent increase in IL-12, INF-γ, and IL-8 concentration levels as compared to control group. Altogether, these results emphasize a potentially important role of *Saccharomyces boulardii* and *Bacillus subtilis* B10 in modulating immunological functions of chi-BMDCs by targeting specific toll like receptors (TLRs) and associated factors. The role of probiotics on chi-BMDCs functionality in a non-mammalian species have been presented for the first time. J. Cell. Biochem. 115: 189–198, 2014. 

**KEY WORDS:** PROBIOTICS; DCs; TLRs; SIGNALING; IMMUNITY

Dendritic cells (DCs) are potent APCs that stimulate the immune system to respond against the entire damage and/or foreign invaders. However, recognition of antigens and inflammatory stimulants in response DCs maturation, phonotypical changes and functional modulation emerge. The organisms induce DCs maturation can be characterized by up regulation of co-stimulatory molecules; cytokine production and activation of T cells by antigen presentation [Banchereau and Steinman, 1998]. Conversely, The immature state of DCs plays their role on the deletion of effector T cells or generation of T cells [Steinman et al., 2003; Al-Bader et al., 2004]. DCs intellect with probiotics or their components through pattern recognition receptors (PRRs) and escort the subsequent innate and adaptive immune responses [Keestra et al., 2007]. Most of the probiotics have also been used to develop the innate immunity and prevent some clinical conditions [Schultz et al., 2002; Isolauri, 2003]. Previously findings of Rakoff et al. [2004] revealed the critical importance of toll-like receptors (TLRs) and associated factors in the control of immune homeostasis. These receptors are one of PRRs that can recognize a wide variety of microbial compounds Rajput and Li [2012] and elicit immune activation [Keestra et al., 2007]. Simultaneously, probiotics

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modulate the TLRs expression and induce cytokine production [Li et al., 2012], and a constant TLRs stimulation may be necessary for maintaining immune homeostasis [Rakoff et al., 2004]. Although some degree of low level "surveillance" NF-κB activation might be a normal physiological state [Gewirtz et al., 2004]. However, innate receptors play an important role to balance the induction and reduction of inflammation in the host [Wen et al., 2011]. Conditionally, Neish et al. [2000] suggested that commensal bacteria might have an anti inflammatory effect through an inhibition of nuclear transcription factor (NFκ-B) by inhibition of IκB ubiquitination. Oral administration of probiotics may modulate DCs response, as a result up-regulation of DCs surface expression and cytokine production [Christensen et al., 2002]. Rajput et al. [2013a,b] recently found that *Saccharomyces boulardii* and *Bacillus subtilis* B10 induce cytokines production to enhance immunity in broiler chickens. However, it remained an attractive notion that the maturation of DCs by probiotics in vitro, the outcome of an immune response through TLRs signaling can be modulated. The addressed question is focusing in the present work on *S. boulardii and B. subtilis* B10 modulate toll-like receptor-mediated signaling to induce immunity by chi-BMDCs.

## MATERIAL AND METHODS

### ISOLATION AND CULTURING OF CHICKEN BONE MARROW DENDRITE CELLS (CHI-BMDCs)

Chinese cross breed chicken (Sanghuang) was kept under standard hygienic conditions at Animal Center at Institute of Medical Science, Zhejiang University, Hangzhou, P.R. China. The procedure was followed according to method of [Rajput et al., 2013a]. In briefly, femur was removed and surrounding muscle tissues detached aseptically. Bones were flushed and transfer into sterilized tubes for centrifugation at (1,400*g* for 5 min). Red blood cells (RBCs) lysis was added and centrifuged at 1,300*g* for 25 min. Latter, they were washed with PBS and complete medium was added. Culturing of chicken bone marrow DCs were maintained by pre-warmed RPMI-1640 (Invitrogen, USA). Complete medium containing 10% chicken serum (Kingfisherbiotech, USA), 1% non-essential amino acids, 1% L-glutamine, 1% streptomycin was added for 6 days at 41°, 5% $CO_2$. Recombinant chicken GM-CSF was obtained according to [Rajput et al., 2013b] and IL-4 (Kingfisher Biotech) to add into the culture medium throughout the growth period of 7 days. Complete medium devoid of antibiotic was used at 7th day of culturing before stimulation with treatments.

### PROBIOTICS AND CULTURE CONDITIONS

*S. boulardii* was cultured in yeast peptone dextrose (YPD) broth (Oxoide; England) in aerobic conditions at 30°C for 24 h and *B. subtilis* in Luria Bertani (LB) broth (Oxoide; England) for 12 h. Probiotics were harvested by centrifugation at 6,000*g* for 10 min and after twice washing with phosphate-buffered saline (PBS, pH 7.2), the pellets were resuspended in DCs culture medium. Optical density method (Spectrophotometer, LAMBDA 850, USA) was used to adjust the final concentration of the probiotics, and the exact numbers ($1 \times 10^6$ cfu/ml) were determined.

### EXPERIMENTAL DESIGN

Cultured cells ($1 \times 10^7$ cells/ml) were divided into five groups and each group was stimulated. Phosphate buffer saline (PBS) was added into control group (Ctr), and as treatment lipopolysachride (LPS), 1 μg/ml (Sigma–Aldrich), *S. boulardii* (Sb), *B. subtilis* B10 (Bs) and co-culture of *S. boulardii* and *B. subtilis* B10 (Sb + Bs) with concentration of ($1 \times 10^6$ cfu/ml) were used to stimulate the dendritic cells for 3, 6, and 12 h, respectively.

### MORPHOLOGICAL OBSERVATION

Effects of treatment on cells segregation were recorded by observing cells morphology and maturation. In addition, MHC-II antibody was used to confirm the maturation according to the method of (Kaufman et al., 1990). Cultured cells were photographed at 6th and 7th day of culturing by confocal microscope (Nikon, Japan).

### SCAN ELECTRON MICROSCOPY (SEM)

Chicken bone marrow derived DCs ($1 \times 10^7$ cells/ml) were cultured in 6-well plates and Ctr and treatment groups were collected at 3, 6, and 12 h time intervals. Media was removed and cells were collected and fixed in 2.5% glutaraldehyde. The preparations were post-fixed in osmium tetraoxide, dehydrated in ethanol and critical point of drying determined using carbon dioxide, and finally coated by gold sputtering. The DCs were examined with a (JMC 500, Nikon) benchtop scanning electron microscope.

### TRANSMISSION ELECTRON MICROSCOPY

Cells were collected from 6-well plates (n = 6), placed into vial containing sufficient fixative solution (2.5% glutaraldehyde buffer 0.1 M cacodylate, pH 7.4) for 24 h at room temperature. Moreover, cells were rinsed and subsequently processed for TEM in routine processing operation by Institute of Biotechnology, Zhejiang University, P.R China.

### EXTRACTION OF RNA AND cDNA SYNTHESIS

Total RNA was extracted from DCs ($1 \times 10^7$ cells/ml) using TRIzol (TaKaRa) and purified using RNeasy® MinElute™ (Qiagen, Hilden, Germany) following the manufacturer's instructions. The amount of total RNA was quantified by Nano Drop (Thermo Scientific, Shanghai, China). RNA was reverse transcribed using StrataScript first-strand synthesis system (TaKaRa) according to the provided protocol. In brief, RNA (1 μg) was combined with 10× first strand buffer, 1.0 μl of oligo (dT) primer and RNase-free water upto 10 μl, mixed well and incubated at 65°C for10 min. 5× M-MLV buffer (2 μl), 1 μ1 of dNTP mix (10 mM), RNase inhibitor 0.5 μl (40 U/μ1), TRase M-MLV 2 μ1 (200 U/μ1) and RNase-free water 4.5 μl was added. The mixture was incubated at 42°C for 60 min, and the reaction stopped by heating at 70°C for 10 min, and placed on ice for 5 min.

### QUANTITATION OF mRNA qRT-PCR

Oligonucleotide primers for chicken DCs surface receptors, associated factors and β-actin were designed based upon sequences available from public databases (Table I). Real-time qRT-PCR was performed using real-time PCR (iQ5, Bio-Rads). A total of 20 μl volume was prepared for amplification, containing 10 ml of 2× SYBR Green I real-time PCR Master Mix (Takara), diluted cDNA 1 μl, and 0.8 μl forward

TABLE I. Real-time Quantitative Reverse Transcriptase Polymerase Chain Reaction (qRT-PCR) Primers Sequences Were Used For Gene Expression

| Gene name | Sequence (5′-3′) F: forward R: reverse | Accession numbers | Base pair |
|---|---|---|---|
| β-Actin | F″GAGAAATTGTGCGTGACATCA<br>R″CCTGAACCTCTCATTGCCA | JN639846.1 | 107 |
| MHC-II | F-GGGGTTTACGACAGCGTCTATT<br>R-TTCCGGGTCCCACATCCT | NM001001762.1 | 130 |
| CD40 | F-AACGCAACGCACAACACTG<br>R-GTCCCTTTCACCTTCACCACA | EF554723.1 | 161 |
| CD80 | F-CAGCAAGCCGAACATAGAAAGA<br>R-AGCAAACTGGTGGACCTGAGAA | XM418929.3 | 270 |
| CD86 | F-GCCTACACTCTACTCTTCACCCTG<br>R-TATTCTGTCGCCAACTCC | EF554724.1 | 272 |
| TLR1 | F:5′GGCAGTGGACGCAGACAAA3′<br>R:5′GTAGGAAATGAAGGCGTGGAA3′ | AB109401.1 | 89 |
| TLR2 | F:5′CTGAAGCCACAGACATTCCTAAC3′<br>R:5′CTTGTACCCAACGACCACCA3′ | NM_001161650.1 | 209 |
| TLR4 | F:5′GGCAAAAAATGGAATCACGA3′<br>R:5′CTGGAGGAAGGCAATCATCA3′ | NM_001030693.1 | 201 |
| TLR15 | F:5′ATCCTTGTCGTTCTGGTGCTAA3′<br>R:5′TCAGTAGATGCTCCTTCGTCCA3′ | JN112029.1 | 187 |
| MyD88 | F:5′GGATGGTGGTCGTCATTTCA3′<br>R:5′GAGATTTTGCCAGTCTTGTCCA3′ | NM_001030962.1 | 226 |
| TRAF6 | F:5′CACAGAGGAGACGCAGGGATA3′<br>R:5′AACAGATCGGGCACTCGTATTT3′ | XM_001235884.1 | 74 |
| TAB2 | F:5′GAGTTTGCCAAGCAGACATCG3′<br>R:5′GCACAGAGACTGGGTAGACACG3′ | NM_001006240.1 | 226 |
| NF-kB | F:5′ACCCCTTCAATGTGCCAATG3′<br>R:5′TCAGCCCAGAAACGAACCTC3′ | D13721 | 274 |

and 0.8 μl reverse primer. Afterward qRT-PCR followed, denaturing at 95°C for 1 min, stepped by 40 cycles of 95°C for 10 s and 60°C for 55 s. The baseline was automatically adjusted by the software and data were analyzed with software (iQ5, Bio-Rads). Livak and Schmittgen [2001], comparative CT value method was applied to determine the fold-changes in gene expression, calculated as $2^{\Delta\Delta C_t}$.

### CYTOKINES PRODUCTION BY ELISA ESSAY

The ELISA tests of IL-1β, IL-17, IL-12, IL-4, TGF-β, IL-10, INF-γ, and IL-8, were performed as manufacturer's instructions, (Komabiotech, Ltd., Seoul, South Korea). Briefly, polyclonal goat anti-chicken IL-1β, IL-17, IL-12, IL-4, TGF-β, IL-10, INF-γ, and IL-8, antibodies were applied as capturing antibodies, biotinylated polyclonal goat anti-chicken IL-1β, IL-17, IL-12, IL-4, TGF-β, IL-10, INF-γ, and IL-8, antibodies as detecting antibodies. Streptavidin-RP and TMBS were used as color indicator and subsequently color reaction was stopped with acid. Well plates were read at (450 nm) wavelength, right after incubation at (37°C for 10 min).

### STATISTICAL ANALYSIS

Data were analyzed using SPSS 16.0 for Windows (SPSS, Inc., Chicago) and values are presented as means standard deviation (mean ± SD). Analysis of variance (ANOVA) was used to calculate the differences among the groups and ($P < 0.05$) was considered to be significant.

## RESULTS

### MORPHOLOGICAL CHANGES IN CHICKEN BONE MARROW DERIVED DENDRITE CELLS (CHI-BMDCs)

Chicken bone marrow dendritic cells were cultured at $1 \times 10^7$ cells/ml to evaluate the stimulating effects of probiotics. At 6th day, cultured cells were observed under an inverted light microscope (Fig. 1A and B), and chi-BMDCs aggregation were appeared. Higher magnification revealed (Fig. 1C and D) that many individual cells at 7th day displayed a veiled and/or dendrites as a sign of maturation after stimulation with LPS, Sb, Bs, and Sb + Bs groups. However, staining with anti-chicken MHC class II, cells were aggregated (Fig. 1E–H), and in all the treatment groups found positive to MHC-II, however LPS group showed higher response among all treatment groups.

### SCAN ELECTRON MICROSCOPY

Scan electron microscopy was performed after stimulation with LPS, Sb, Bs, and Sb + Bs at different time (3, 6, and 12 h) intervals (Fig. 2). LPS treatment showed revealing of dendrites in initial and middle time intervals (3 and 6 h), whereas at 12 h veiled appearance decreased. Moreover, Sb, Bs, and Sb + Bs groups also manifested the dendrites and confirmed the attachment of probiotics with chi-BMDCs and engulfing stages at different time intervals. At 3 h fewer attachment of probiotic groups were appeared, but at 6 and 12 h stimulation time showed several attachments of probiotics with chi-BMDCs and various stages of engulfing were observed as compared with LPS stimulated group.

### TRANSMISSION ELECTRON MICROSCOPY (TEM)

We examined the chi-BMDCs by transmission electron microscopy (TEM) to understand the mechanism that how probiotics (bacteria or yeast) were internalized by chi-BMDCs. While, after 3 h stimulation at 41°C only small proportion of the probiotics (Sb, Bs, and Sb + Bs) were found inside the chi-BMDCs. Conversely, at 6 and 12 h incubation with probiotics (Sb, Bs, and Sb + Bs), most of the probiotics were internalized by the chi-BMDCs and showed various stages of degradation. All of the probiotic groups were internalized

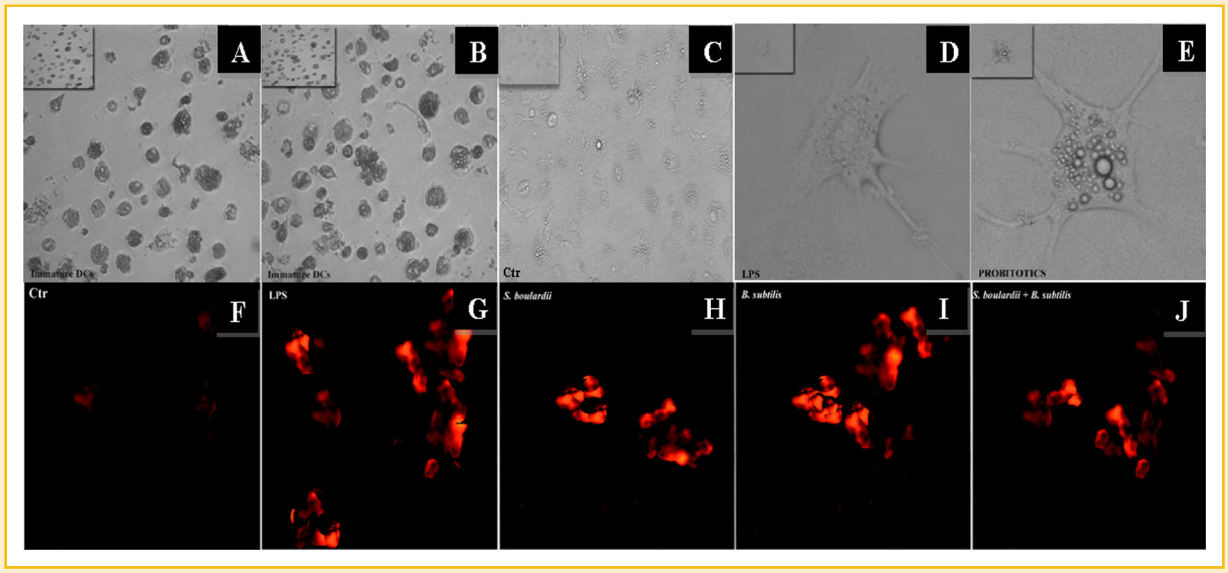


**Fig. 1.** Morphological structure of chicken bone marrow dendrite cells cultured for 7 days. Cell aggregates (A and B) at day 7 without stimulation (200×). Control group (C) stimulated with phosphate buffer saline (PBS). Single cell (D and E), after stimulation with lipopolysachride (LPS) and probiotics at day 7 (400×), respectively. Major histocompatibility complex class II (MHC II) staining after stimulation with PBS, LPS, *S. boulardii*, *B. subtilis* B10 and co culturing of *S. boulardii* and *B. subtilis* B10 (F–I) at magnification of (200×), respectively. Data is representative of six independent experiments in which bone marrow dendritic cells (chi-BMDCs) were cultured.

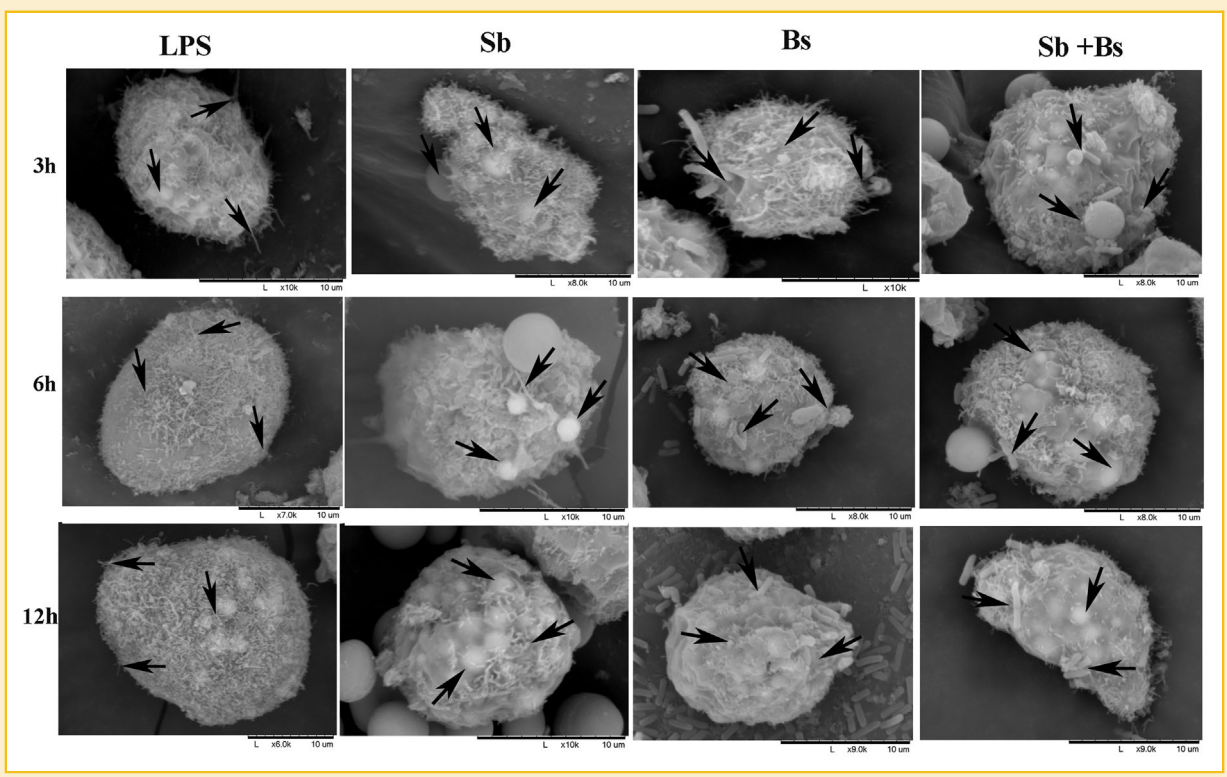


**Fig. 2.** Capturing of probiotics by chicken BMDCs. Chi-BMDCs were stimulated for 3, 6, and 12 h with lipopolysachride (LPS), probiotic strains *S. boulardii* (Sb), *B. subtilis* (Bs) B10 and co culturing of *S. boulardii* and *B. subtilis* B10 (Sb + Bs),and processed for scan electron microscopy. Bacteria are visible on the cells surface and are bounded with dendrites at (Bar, 10 $\mu$m) and various stages of probiotics capturing shown by (arrows).

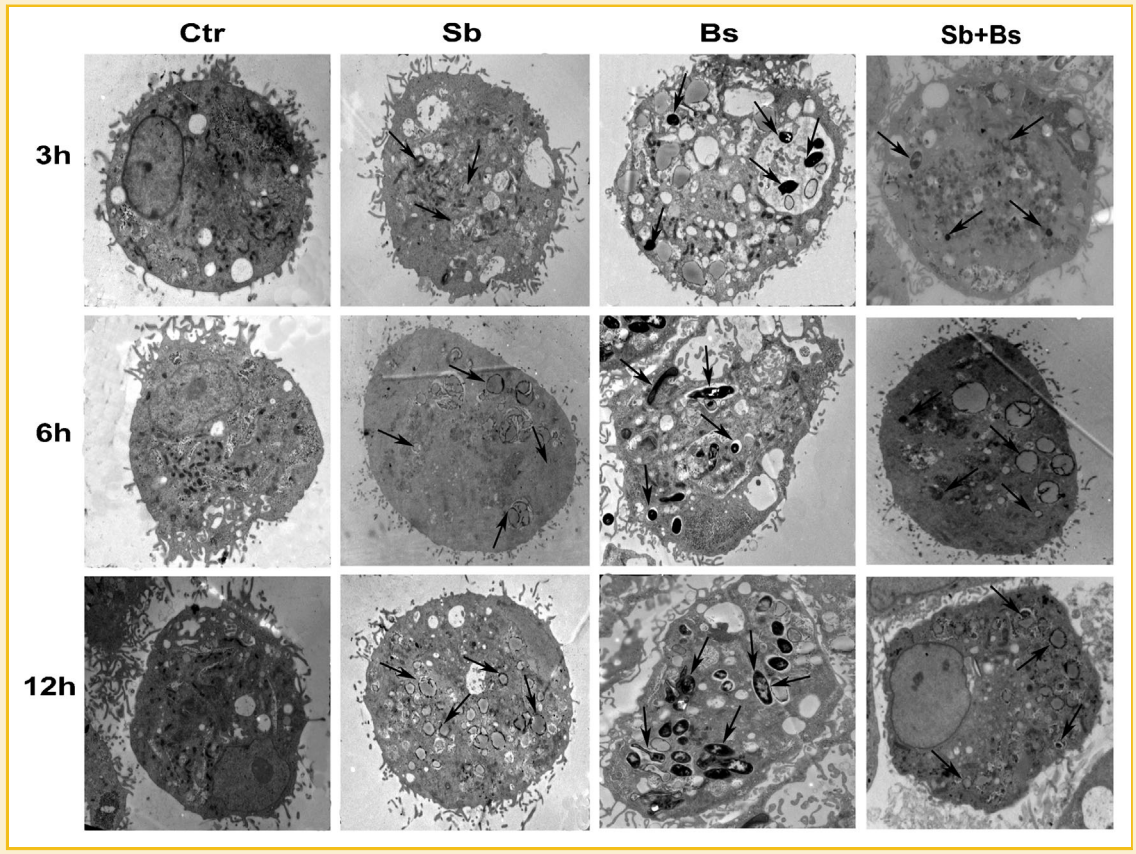


**Fig. 3.** Internalization of probiotics by chicken BMDCs. Chi-BMDCs were incubated for 3, 6, and 12 h. Different groups, phosphate buffer saline (Ctr), probiotic strains *S. boulardii* (Sb), *B. subtilis* B10 (Bs) and co culturing of *S. boulardii* and *B. subtilis* B10 (Sb + Bs). Latter processed for transmission electron microscopy (TEM). Bacteria are visible in the cytosol and are within membrane bounded phagosomes and vacuoles at various stages of degradation (arrows). Bar 2 $\mu$m.

and appeared in membrane bound intracellular vacuoles and/or phagosomes. Internalized probiotics were not diplayed in cytosol (Fig. 3), suggesting that *S. boulardii, B. subtilis* B10 and co-culture were taken up by conventional phagocytosis, as previously observed [Foligne et al., 2007], respectively.

## GENE EXPRESSION OF SURFACE MARKERS

Specific surface markers MHC-II, CD40, CD80, and CD86 gene expressions were determined to observe the maturation of chi-BMDCs (Fig. 4). The results compared to control (Ctr), all of the treatment groups showed significant up regulation of surface marker mRNA levels at different time intervals. Moreover, among the groups MHC-II gene response was significant higher in LPS group at 12 h in comparison of Sb, Bs, and Sb + Bs groups. Gene expression of CD40 up regulated in Bs group as compared to all of the treatment groups at 12 h. LPS showed prominent increase in mRNA expression levels of CD80 and CD86 at 12 h interval as compared to Sb, Bs, and Sb + Bs groups.

## TLRs AND ASSOCIATED FACTORS EXPRESSION RESPONSE

We determined the mRNA expression levels of chicken bone marrow derived denderitic cells stimulated with LPS, Sb, Bs, and Sb + Bs groups (Fig. 5). The expression of TLR1 tended to increase during different time (3, 6, and 12 h) intervals. Sb and Bs group showed significant up regulation of TLR1. However, Sb + Bs showed prominent expression level at 6 and 12 h. TLR2 gene expression increased in all the treatment groups, but Sb + Bs improved the response consistently during stimulation times. LPS and Sb group significantly increased TLR4 response than other treatments. Moreover, chicken specific receptor TLR15 gene expression improved by all the treatment at different times, while response to LPS was significantly higher among the groups. Intrinsic associated factors, MyD88 showed higher mRNA expression levels in all treatments, and LPS influence was found significantly higher as compared to probiotic groups. Gene expression level of TRAF6 and TAB1 were up regulated in all the treatment groups and Sb group prominently higher among the groups. The response of NFκ-B was noted higher in all the treatments. However, probiotic groups Sb, Bs, and Sb + Bs showed significant lower expressions of NFκ-B as compared to LPS group.

## CYTOKINES AND CHEMOKINE DETERMINATION

In chicken BMDCs cytokines and chemokine were determined after stimulation with LPS, Sb, Bs, and Sb + Bs using ELISA method (Fig. 6). IL-1β concentration showed significantly increase in Bs group and prominently decrease in LPS group. However, IL-12

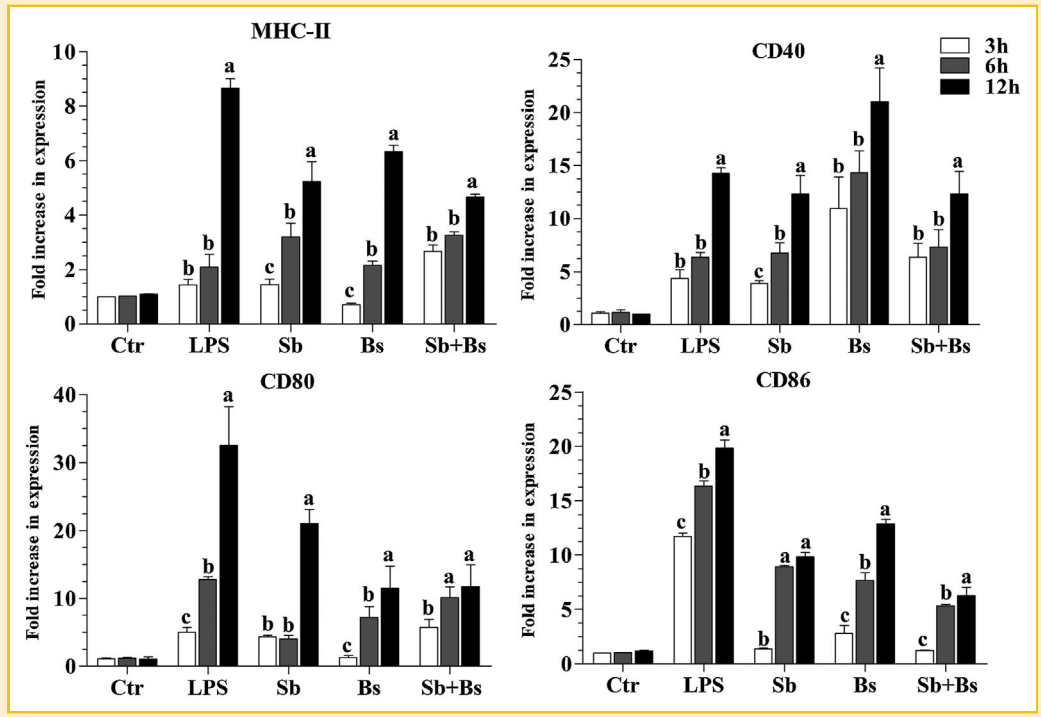


Fig. 4. Quantitative real time PCR analysis of mRNA expression of surface marker genes in chicken BMDCs after stimulation with lipopolysachride (LPS), probiotic strains *S. boulardii* (Sb), *B. subtilis* (Bs) B10 and co culturing of *S. boulardii* and *B. subtilis* B10 (Sb + Bs), Phosphate buffer saline (PBS) was added in control (Ctr) group and harvested at 12 h. The values are expressed as fold increased ± SD of mRNA levels (n = 6). Means with different alphabets (a, b, and c) are defined as significantly different ($P < 0.05$).

production decreased in Bs and Sb + Bs, and LPS group improved, while Sb group remained unchanged. Furthermore, IL-4 was found higher in Bs and Sb + Bs groups and showed low levels in LPS and Sb treatment groups. The production level of IL-17 decreased in Sb and Bs groups, and numerical drop appeared in Sb + Bs group. Moreover, higher production of TGF-β observed in Sb and Bs groups, and decreased level noted in LPS stimulated group. Also, IL-10 significantly increased in all treatment groups but LPS group showed lowest concentration and Sb group highest level of IL-10 among the treatment groups. Conversely, INF-γ level up regulated in LPS treatment group and significantly down regulated in probiotic treatment groups. Meanwhile, IL-8 production increased in LPS group and lower in Sb + Bs group, but Sb and Bs groups remained unchanged as compared to Ctr group.

## DISCUSSION

Immune system is regulated by professional APC, and antigen presentation, which is executed either in spleen or local diffuse structured of birds [Li et al., 2011], because most of the avian species are lacking of lymph nodes. One example of APC is DC and they have unique functions such as defence by capturing, degradation and antigens presentation to activate primary immune responses [Banchereau and Steinman, 1998; Steinman, 1991]. In this study, we for the first time, have addressed the role of chicken bone marrow dendrite cells in the protective effects meditated by direct interaction with probiotics to enhance immunity through toll like receptor (TLR) and associated factor mediated signaling in broiler chickens.

Presently we found that after stimulation at 7th day majority chi-BMDCs displayed a veiled or dendrites as a sign of maturation. In addition MHC-II reaction showed positive results after stimulation with LPS, Sb, Bs, and Sb + Bs groups. When immature DCs were induced into mature in vitro with different stimuli, it was revealed that LPS is a strong inducer of DCs maturation in mammals [Wurtzen et al., 2001]. Similarly, Zhiguang et al. [2010] reported that LPS and other stimulants can induce maturation of chicken immature DCs and phenotypically mature DCs displayed dendrites and be reacttive to MHC-II.

Scan electron microscopy revealed that dendrites of chi-BMDCs at different time intervals capture probiotics. The 12 h interval showed higher numbers of probiotics attachment on the surface of chi-BMDCs. Dendritic cells have many receptors to sense the microbes and their products from both pathogens and probiotics. These receptors are also known as sense endogenous molecules that may be presented in any inappropriate time, place or form to capture the organisms by their specific structures [Paul et al., 2011].

TEM showed internalization of probiotics at different time intervals and different stages of degradation of probiotics (yeast and bacterial) displayed. These phagocytosis activities were not able to proliferate within chi-BMDCs although probiotics eventually degraded. Similarly, Jen et al. [2008] observed that phagocytosis and bacteria were internalized by the macrophage and organisms were degraded. Other studies illustrated that degradation of probiotics probably depends on the cells and their stages in the cell cycle as well as other nutritional factors [Bielecki et al., 1991; Portnoy et al., 1992].

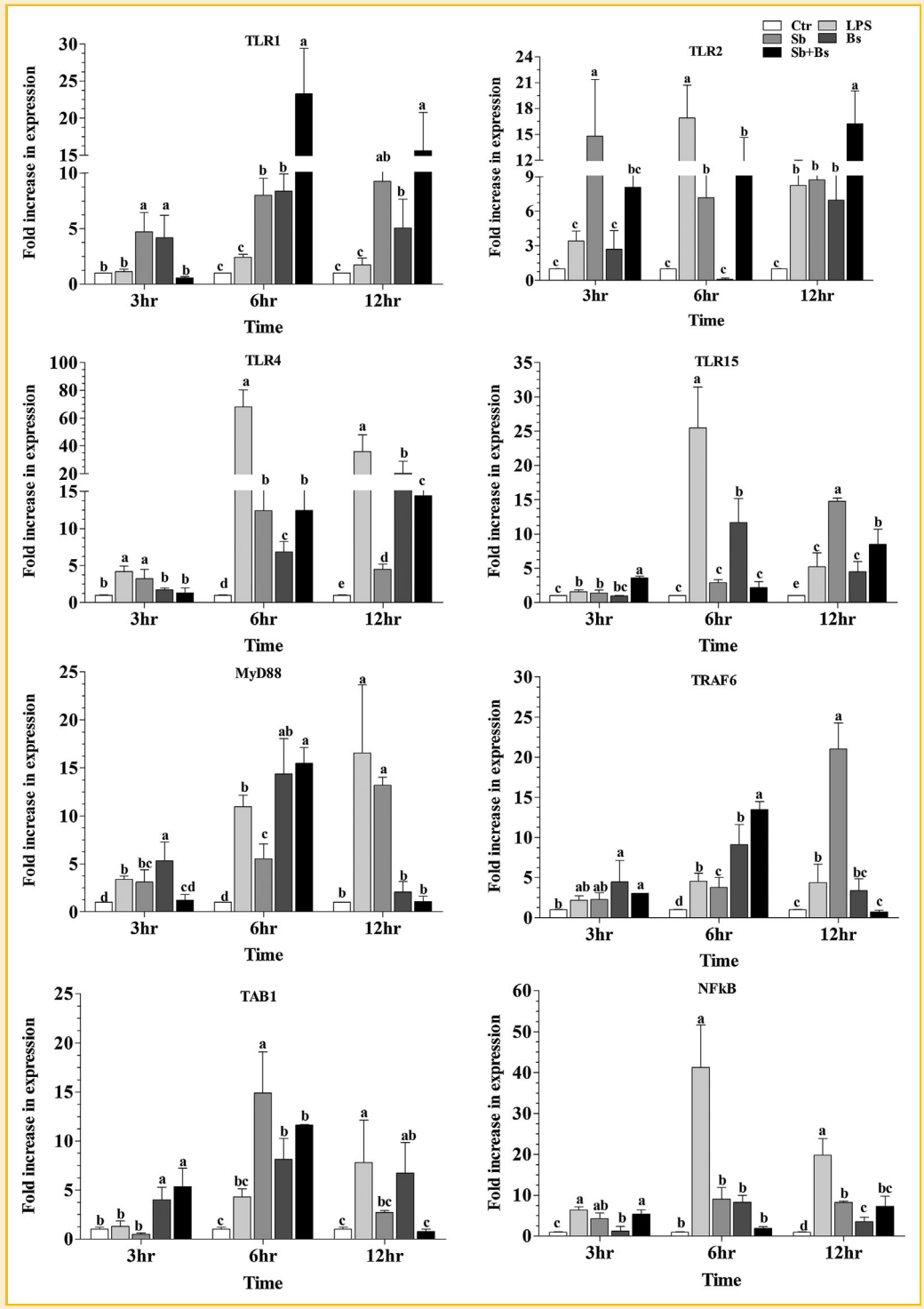


Fig. 5. Quantitative real time PCR analysis of mRNA expression of Toll like receptors (TLRs) and associated mediators in chicken BMDCs harvested at 3, 6, and 12 h after stimulation with lipopolysachride (LPS), probiotic strains *S. boulardii* (Sb), *B. subtilis* (Bs) B10 and co culturing of *S. boulardii* and *B. subtilis* B10 (Sb + Bs). Phosphate buffer saline (PBS) was added in control (Ctr) group and harvested at same times of treatment. The values are expressed as fold increased ± SD of mRNA levels (n = 6). Means with different alphabets (a, b, c, and d) are defined as significantly different ($P < 0.05$).

It is entirely possible that probiotics might exist in the phagosomes and vacuoles [Faille et al., 2002], but survival and germination require de novo protein synthesis, such as L-alanine and L-asparagine [Moir and Smith, 1990], and the unavailability of these proteins within vacuoles and phagosomes could be a reason of probiotics degradation after certain time within DCs. Thus, the true outcome in vivo, where probiotics come indirectly into contact with dendritic cells may be diverse.

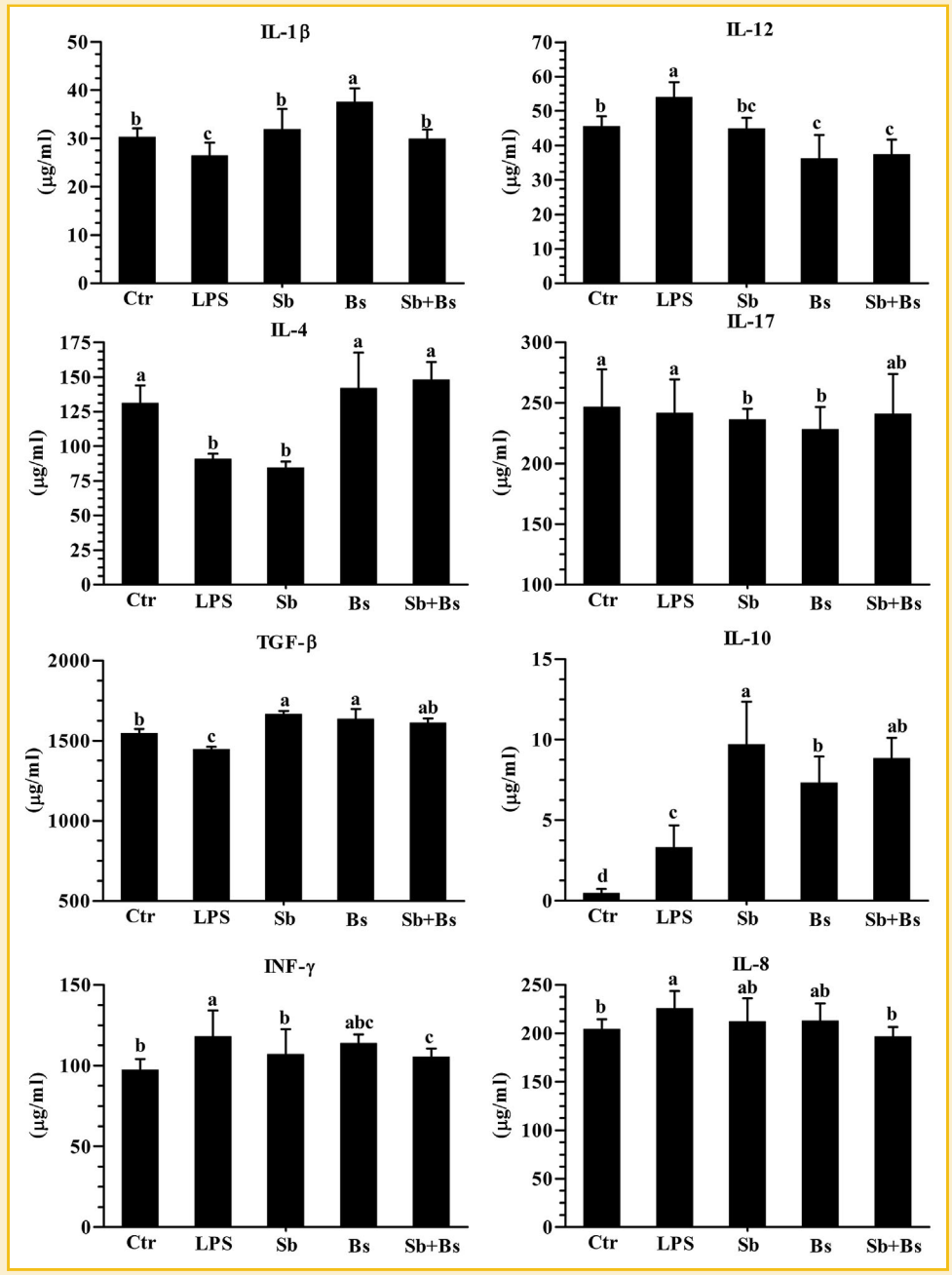


**Fig. 6.** Cytokines and chemokine response of chicken BMDCs ($1 \times 10^8$/ml) derived from Sanhuang broiler chicken (Chinese cross breed) to stimulate with lipopolysachride (LPS), probiotic strains *S. boulardii* (Sb), *B. subtilis* (Bs) B10 and co culturing of *S. boulardii* and *B. subtilis* B10 (Sb + Bs), phosphate buffer saline (PBS) was added in control (Ctr) group and supernatant was collected after 12 h stimulation. Results represent the mean ± SD, and different alphabets show significant differences among groups ($P < 0.05$), n = 6.

The cell surface markers, MHC class II, CD40, CD80, and CD86 expression is considered as a sign of DCs maturation in mammalian species [Banchereau and Steinman, 1998]. We stimulated chi-BMDCs with LPS and probiotic groups to observe the level of surface markers MHC-II, CD40, CD80, and CD86 and found higher gene expression as compared with non-stimulated chi-BMDCs. These results coincide with the findings of Lutz et al. [1999], who reported that immature bone marrow-derived DCs of mammalians showed only moderate mRNA expressions of MHC class II molecules. Conversely, mature BMDCs expressed high expression levels of MHC class II, CD40, CD80, and CD86 [Lutz et al., 2000]. Findings of Zhiguang et al. [2010] have similarity to the present study who reported that LPS and many stimuli increased the gene expression level of MHC-II, CD40, CD83, and CD86. Previously, Depaz et al. [2003] illustrated that stimulation of DCs express high expression levels of MHC-II, CD40, CD80, and CD86 surface markers that have significant role in maturation and functional activities. The results of earlier studies concluded that specific CD markers high expressions

show DCs maturation and functional response. In similar case, our results revealed that after stimulation with LPS and probiotics specific CD markers gene expression up-regulated significantly.

The role of PRRs signaling and associated factors in chi-BMDCs through LPS and probiotics (*S. boulardii and B. subtilis* B10) stimulation was investigated. Our findings showed that cell surface receptors TLR1, 2, 4, and 15 showed significant up-regulation at mRNA levels. In addition, associated factors MyD88, TRAF6, TAB1, and NF-κB were found responding to all treatment groups. NF-κB showed significant increase in LPS amongst the group. Previously, noted that administration of *C. albicans* and *S. boulardii* up-regulated TLR2 and TLR4 gene expressions, and chicken TLR2 (chiTLR2) gene expression was found higher after stimulation with probiotics [Samir and Daniel, 2007]. Moreover, intestinal isolated microbiota up regulated the TLR2, TLR3, TLR4, and TLR5 mRNA levels [Cario and Podolsky, 2000], and chicken specific surface receptor TLR15 showed up regulation in embryonic chicken fibroblasts [Higgs et al., 2006]. Another study showed that TLR2 and TLR4 expression level increased and finally NF-κB activation observed [Keestra et al., 2007]. TLRs are initial signaling tool to activate immune responses, and follow multiple pathways to maintaining immune homeostasis. They also play an important role in identification, degradation and antigens presentation to T-cells, which activate inflammatory response for the prevention of injury [Rakoff et al., 2004]. Additionally, effective role of MyD88 as an adapter protein in downstream signaling pathway to activate the innate immune response via TRAF 6 and TAB1 mediated proteins to further trigger the activation of NF-κB [Kiyoshi and Shizuo, 2004]. Current literature revealed that probiotics can trigger the MyD88-dependent signaling pathway to activate the immune system by stimulating TLRs and subsequently TRAF6 expression up regulate [Takeuchi et al., 2000]. TRAF6 is an important associated protein consisting of TRAF-N terminal and TRAF-C terminal, play a crucial role to mediate the cytokine signals [Arch et al., 1998]. It dissociates from the receptor and associates with TAB1 and TAB2, where the complex of TRAF6, TAB1, and TAB2 moves into the cytoplasm and make large complex with other proteins [Deng et al., 2000]. Thereafter, a lys 63-linked polyubiquitin of TRAF6 is synthesized; then it induces TRAF6 mediated stimulation of TAB1/2 and finally activate NF-κB [Deng et al., 2001]. The mechanism of response was similarly revealed in the present study.

The probiotic displayed different effects on cytokines and chemokine production by DCs [Hart et al., 2004], and their secretion levels depend on probiotic species and cellular response [Atarashi et al., 2011]. In this study, chi-BMDCs were cultured and stimulated with LPS and probiotics. The results showed that probiotics improved the production of IL-1β, IL-17, TGF-β, and IL-10 where as IL-8 and INF-γ were down-regulated. On the other hands, LPS group increased IL-8 and INF-γ production. Cytokine production response may vary from species to species, case in point, *B. bifidum*, *B. breve*, *and B. infantis* stimulation increased the production of IL-10 and TNFα yet less effective on IL-12 secretion level [He et al., 2002]. Furthermore, Jeon et al. [2012] also found that application of *B. breve* improved IL-10 concentration in SCID mice, whereas IL-8 and IFN-γ concentration were down-regulated [Marianna et al., 2006]. Inflammation is characterized by the initial release of inflammatory cytokines (IL-1 and INF-γ) and chemokine [Eckmann and Kagnoff, 2001], afterword IFN-γ, IL-12, and IL-17 are involved in the host defense against infection. The regulation of inflammatory cytokines is controlled by the release of IL-10, therefore ups and downs in $Th_1$ inflammatory response is possible [Groux and Powrie, 1999]. Because IL-4 and TGF-β initially up-regulate and down-regulate afterward during infection [Coburn et al., 2007]. In the present work, our findings clearly demonstrate that probiotic strains (*S. boulardii* and *B. subtilis* B10) promote the chicken bone marrow dendritic cells cytokine production in response of TLRs mediated signaling to develop immunity.

Conclusively, *S. boulardii* and *B. subtilis* B10 have prominent effects on TLRs mediated signaling to induce immunity in chi-BMDCs. Moreover, it might be a gateway to evaluate the effect of probiotics in vitro as a feed additive and their role in immunity development.

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