Modulatory Effects of *Bacillus subtilis* BS02 on Viability and Immune Responses of RAW 264.7 Murine Macrophages

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Abstract: The aim of this study is to investigate the effects of *Bacillus subtilis* BS02 on viability and immune responses of RAW 264.7 cells. RAW 264.7 cells were stimulated with and without *B. subtilis* spores for 12 h in the treatment group and the control group, respectively. Cytotoxic effect of *B. subtilis* BS02 on macrophages was measured by cell viability and LDH cytotoxicity assay. Immune responses of macrophage elicited by *B. subtilis* BS02 were analyzed by measuring the activities of Acid Phosphatase (ACP) and Lactate Dehydrogenase (LDH), the production of Nitric Oxide (NO) and inducible Nitric Oxide Synthase (iNOS) and the secretion of inflammatory cytokines. The results showed that after 12 h incubation, *B. subtilis* BS02 spores had no influence on viability of RAW 264.7 cells; ACP and LDH activities, the production of NO and iNOS, the levels of inflammatory cytokines [Tumor Necrosis Factor-alpha (TNF-α), Interferon-gamma (IFN-γ), Interleukin-1 beta (IL-1β), IL-6, IL-8, IL-10 and IL-12] were significantly higher than that in the control group (p<0.01). These results indicate that *B. subtilis* BS02 is not only safe for RAW 264.7 cells but also can activate macrophage immune function.

Key words: *Bacillus subtilis* spores, RAW 264.7 cells, cytotoxic effect, immune function, inflammatory cytokine

INTRODUCTION

Numerous reports support the use of spore-forming *Bacillus* species as probiotics for the prevention and the treatment of a broad variety of gastrointestinal disorders (Hong *et al.*, 2005; D'Arienzo *et al.*, 2006; Fujiya *et al.*, 2007; Williams, 2007; Cutting, 2011; El-Naggar, 2004; Deeseenthum *et al.*, 2007; Chantharasophon *et al.*, 2011). A number of studies have revealed that *Bacillus* species should be considered as inhabitants of the Gastrointestinal Tracts (GIT) of insects, animals and humans and *Bacillus* species are able to survive and persist within the GIT (Duc *et al.*, 2003, 2004).

B. subtilis spores can germinate, proliferate and sporulate in the murine GIT (Duc *et al.*, 2004). *B. subtilis* spores have been demonstrated to disseminate to Peyer's Patches (PPs) and Mesenteric Lymph Nodes (MLN) following oral inoculation (Duc *et al.*, 2003). When ingested spores were delivered to GIT, the interaction of *Bacillus* species with the GALT is an efficient mechanism to stimulate the immune system. Mounting evidence indicates that *Bacillus* species plays a key role in the development of the GIT including the Gut-Associated Lymphoid Tissue (GALT) and thus plays an important

role in defense against enteropathogenic bacteria (Rhee *et al.*, 2004; Huang *et al.*, 2008). In the GIT, *B. subtilis* spores are taken up by M cells and then carried to the PPs (Rhee *et al.*, 2004) where they could interact with Antigen-Presenting Cells (APCs; macrophages and dendritic cells), B and T cells to trigger humoral and cellular immune responses (Duc *et al.*, 2003, 2004; Huang *et al.*, 2008) before transportation to the efferent lymph nodes and the interaction between *B. subtilis* spores and macrophages plays an important role in both instructing the host innate and adaptive immune response.

Macrophages constitute an important part of the first line of defense against microbial invaders and malignancies by the nature of their phagocytic, cytotoxic and intracellular killing capacities. Once being activated after recognizing microorganisms and other antigens, macrophages will acquire the above capacities and immune functions in a multi-step process. The activities of ACP and LDH are closely related to the phagocytic activity of macrophages for eliminating foreign particles. Thus, increased LDH and ACP activities are seen as important symbols of the activated phagocytic function of macrophages (Huang *et al.*, 2001). Macrophage

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activation can also induce the production of oxygen-derived intermediates, NO, iNOS, inflammatory cytokines, antimicrobial peptides and other defense molecules (Ganz, 2003; Wu *et al.*, 2009).

In this study, co-culture of *B. subtilis* BS02 and RAW 264.7 cells was used to mimicking the interaction between macrophages and spores in the GIT. Firstly, cytotoxic effects of *B. subtilis* BS02 on macrophages were determined by measuring cell viability and extracellular LDH level. Then, the effects of *B. subtilis* BS02 on macrophage immune function were evaluated by measuring the activities of ACP and LDH, the production of NO and iNOS and the levels of inflammatory cytokines.

MATERIALS AND METHODS

Preparation of *B. subtilis* BS02 spores: Spores of *B. subtilis* BS02 were prepared by the exhaustion method as described elsewhere (Duc *et al.*, 2003) using difco sporulation medium. Spore suspensions were lysozyme-treated and then heat-treated (68°C, 1 h) to remove residual vegetative cells and stored as aliquots at -20°C prior to use.

Co-cultivation of *B. subtilis* BS02 spores and RAW 264.7 cells: Murine macrophage-like cell line RAW 264.7 (obtained from Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences) were cultured in RPMI-1640 medium (GIBCO) supplemented with 10% (v/v) Fecal Bovine Serum (FBS), 100 U mL^{-1} penicillin and 100 ug mL^{-1} streptomycin in an atmosphere containing 5% CO_2 at 90% humidity and 37°C.

About 1 mL of cell suspensions (1×10^6 cells mL^{-1}) was seeded onto six well cell culture plates and incubated for 48 h at 37°C in 5% CO_2. The 2 days old macrophages were then washed twice with Phosphate Buffered Saline (PBS) and treated with 1 mL RPMI-1640 medium with 10% (v/v) FBS but without antibiotics. In the *B. subtilis*-treated group, RAW 264.7 cells were treated with 1 mL suspensions of *B. subtilis* spores (1×10^8 mL^{-1} PBS) and cocultrued for 12 h at 37°C in 5% CO_2. The 1 mL of PBS was used instead of *B. subtilis* spores suspension in the control group.

Sampling: After 12 h co-culture, culture supernatants were collected, centrifuged and frozen immediately at -80°C until needed. Monolayers were washed thrice with sterile PBS and treated with two methods:

- Monolayers were exposed to trypsin-EDTA long enough to detach cells. The cells were neutralized with serum and harvested for the Trypan blue dye exclusion test

- Monolayers were lysed *in situ* by 1 mL 0.1% Triton X-100 for 5 min at 37°C. Cell lysates were collected and frozen immediately at -80°C before use

Cytotoxic analysis of *B. subtilis* B02 on RAW 264.7 cells: Cell viability was assessed by the Trypan blue dye exclusion test (Sarir *et al.*, 2010) and the level of LDH in the culture supernatants. The concentration of LDH was determined using a commercial kit (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's protocol.

Analysis of RAW 264.7 cell immune responses elicited by *B. subtilis* BS02: The activities of LDH, ACP and iNOS in cell lysates as well as the levels of NO in culture supernatants from six cell samples for each group were analyzed by commercial kits (Nanjing Jiancheng Bioengineering Institute, China) according to the manufacturer's instructions. The levels of inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10 and IL-12) in culture supernatants were measured by the commercially available ELISA kits (purchased from R and D Systems) according to the manufacturer's instructions.

Statistical analysis: All values were expressed as means±Standard Deviation (SD). The statistical significance of differences between the *B. subtilis*-treated group and the control group was examined by independent-samples t-test using SPSS 16.0 for Windows. The level of significance was set at p<0.05.

RESULTS AND DISCUSSION

Cytotoxic effect of *B. subtilis* B02 on RAW 264.7 cells: In this study, viabilities of macrophages treated and untreated with *B. subtilis* BS02 spores were >95% after 12 h incubation and no significant difference were observed in both groups (p>0.05, Table 1). Furthermore, there was no significant difference in the level of extracellular LDH between the *B. subtilis*-treated group and the control group (p>0.05, Table 1).

Effect of *B. subtilis* B02 on ACP and LDH activities of RAW 264.7 cells: Figure 1 shows ACP and LDH activities of RAW 264.7 cells. As shown in Fig. 1a, ACP activity of cell lysates in the *B. subtilis*-treated group was

Table 1: Effect of *B. subtilis* BS02 on cell viability and extracellular LDH activity of macrophages

Groups	Cell viability (%)	Extracellular LDH (U mL^{-1})
Control	97.08±1.24	41.02±4.54
B. subtilis BS02	96.67±0.88	45.42±3.59

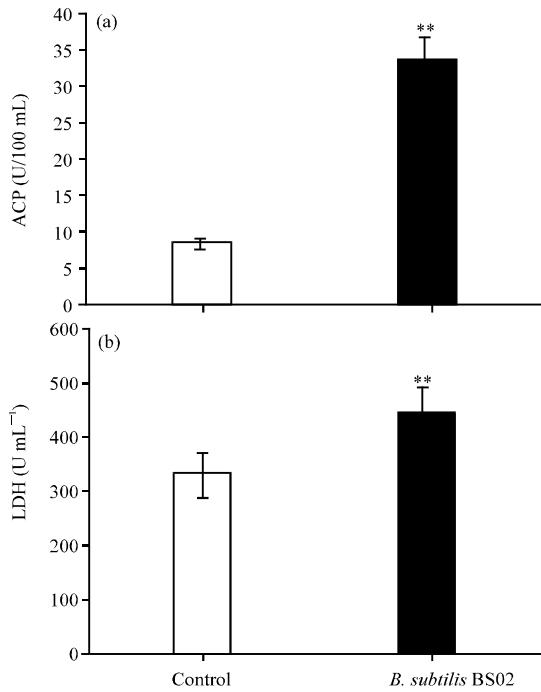Values are means±SD (n = 6); Values were compared in both groups

Fig. 1: Effect of *B. subtilis* BS02 on intracellular activities of ACP and LDH in macrophages: a) intracellular ACP activity; b) intracellular LDH activity. Each bar represents the mean±SD (n = 6). **Significantly different from the control group at p<0.01
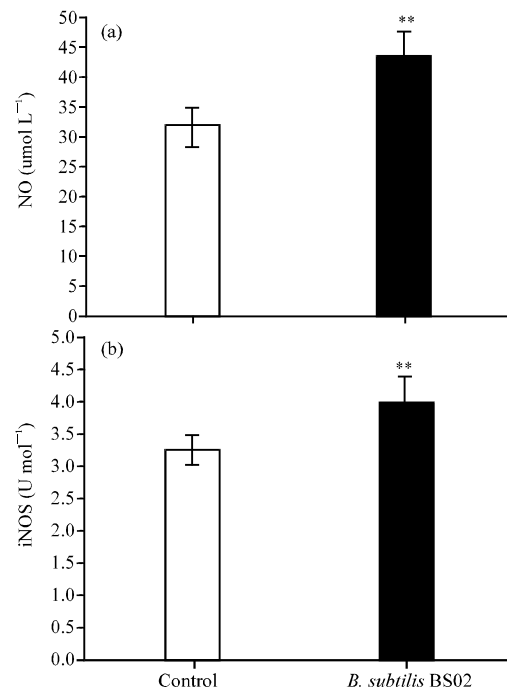

Fig. 2: Effect of *B. subtilis* BS02 on the extracellular NO production and intracellular iNOS activity of macrophages: a) extracellular NO production; b) intracellular iNOS activity. Each bar represents the mean±SD (n = 6); **Statistically significant differences (p<0.01) compared with the control group

significantly higher than that in the control group (p<0.01). Furthermore, LDH activity of cell lysates was also significantly higher (p<0.01, Fig. 1b) in the *B. subtilis*-fed group compared to the control group.

Effect of *B. subtilis* B02 on NO and iNOS production of RAW 264.7 cells: Figure 2 shows NO and iNOS production of RAW 264.7 cells. As compared with the control group, the level of NO in the *B. subtilis*-treated group was significantly higher (p<0.01, Fig. 2a) and a significant increase in the production of iNOS was also observed in the *B. subtilis*-treated group (p<0.01, Fig. 2b).

Effect of *B. subtilis* B02 on inflammatory cytokine secretion of RAW 264.7 cells: The expression of inflammatory cytokines in RAW 264.7 cells treated and untreated with *B. subtilis* spores is shown in Table 2. The levels of inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10 and IL-12) were significantly higher (p<0.01, Table 2) in the *B. subtilis*-fed group compared to the control group.

The safety of probiotics is of prime importance and should be taken into consideration in the selection process of probiotics (Saarela *et al.*, 2000). Therefore, as

Table 2: Effect of *B. subtilis* BS02 on the levels of cytokines in macrophages

Items (pg mL⁻¹)	Control	*B. subtilis* BS02
TNF-α	19.29±2.56	197.82±9.47**
IFN-γ	14.98±1.74	45.06±6.03**
IL-1β	ND	7.26±2.16**
IL-6	22.17±1.65	58.48±6.53**
IL-8	28.09±2.99	112.75±8.36**
IL-10	37.07±10.41	275.84±24.87**
IL-12	ND	59.53±6.83**

Data were presented as mean±SD (n = 6); **p<0.01 significantly different compared to the control group; TNF: Tumor Necrosis Factor; IFN: Interferon, IL: Interleukin; ND: Not Detected

a first footprint of this study, cytotoxic effect of *B. subtilis* BS02 spores on RAW 264.7 cells was investigated. The results showed that *B. subtilis* BS02 spores had no influence on viability of RAW 264.7 cells. Cell membrane damage and cell death result in a significant increase of extracellular LDH (Drent *et al.*, 1996). But no such increase was observed for extracellular LDH of RAW 264.7 cells exposed to *B. subtilis* spores which suggested that RAW 264.7 cells didn't suffer damage from co-culturing with *B. subtilis* spores. The above findings suggest that *B. subtilis* BS02 spores didn't exert any cytotoxic effect

on RAW 264.7 cells. The results agrees with previous reports that four *B. subtilis* strains were examined and none of them were found to have any pathogenic indications (Hong *et al.*, 2008; Sorokulova *et al.*, 2008; Tompkins *et al.*, 2008). The combined use of trypan blue exclusion test and LDH cytotoxicity assay can be an effective way to evaluate the safety of probiotics.

In the process of phagocytosis, macrophages phagocytoze foreign particles and then eliminate the foreign particles by lysosomal enzymes including ACP. The activities of ACP and LDH parallel the phagocytic function of macrophages in eliminating foreign particles. Thus, increased ACP and LDH activities are seen as important symbols of macrophage activation (Huang *et al.*, 2001). The results suggest that *B. subtilis* BS02 remarkably enhances the phagocytic function of macrophages through up-regulation of LDH and ACP activities, leading to the activition of RAW 264.7 cells.

NO, derived from L-arginine is mainly produced by iNOS in monocytes or macrophages. Mice whose genes for iNOS have been knocked out are more susceptible to infections with several intracellular pathogens (Han *et al.*, 2002). NO is widely recognized as an important messenger in numerous physiological and pathophysiological phenomena (Soszynski and Chelminiak, 2007). NO production in macrophages is essential for the defense mechanisms against microorganisms and tumor cells and the suppression of T cell proliferation to downregulate local inflammatory reactions (Friedl *et al.*, 2001). The study found that there is a significant increase in the level of NO with a concomitant increase in iNOS production from RAW 264.7 cells stimulated with *B. subtilis* BS02, suggesting that *B. subtilis* BS02 stimulated RAW 264.7 cells to produce iNOS and then leads to enhanced synthesis of NO. As NO plays an important role in immune function, *B. subtilis* could modulate several aspects of host defense mechanisms through the induction of large amounts of NO.

One component of the immune system that molds the host response to foreign microorganisms is cytokines which are secreted or membrane-bound proteins that regulate the growth, differentiation and activation of immune cells (Budhu and Wang, 2006). Inflammation is essentially the effects of rapidly produced cytokines in response to the activation of the innate immune system. This evolutionarily ancient innate immune response helps control infections at an early stage by activating pro-inflammatory signaling cascades and also by playing an important role in triggering and shaping the adaptive immune response through up-regulation of cytokines and costimulatory molecules (Lakshmanan and Porter, 2007). Cytokines are generally grouped into two categories, pro-inflammatory (TNF-α, IFN-γ, IL-1, IL-2, IL-6, IL-8 and IL-12) and anti-inflammatory (IL-4, IL-10 and IL-13) (Stoycheva and Murdjeva, 2005). Both TNF-α and IL-1β are early regulators of inflammatory response and cause the secondary release of other cytokines (IFN-γ, IL-6, IL-8 and IL-12). IL-10 is an anti-inflammatory cytokine that reduces the synthesis of proinflammatory mediators (e.g., IL-1, IL-6 and TNF-α).

IFN-γ activates macrophages and protects cells against viral infections. IL-6 stimulates proliferation and differentiation of B cells. IL-8 is a powerful chemotactic and paracrine mediator for neutrophils and infiltration of activated neutrophils plays an important role in inflammation and oxidative injury (Sarir *et al.*, 2010). IL-12 regulates lymphocyte growth, activation and differentiation. Researchers found that *B. subtilis* BS02 significantly increased the levels of inflammatory cytokines (TNF-α, IFN-γ, IL-1β, IL-6, IL-8, IL-10 and IL-12) in macrophages which suggested that *B. subtilis* BS02 could regulate host immunity through the induction of inflammatory cytokines in macrophages. The results are in agreement with previous reports that Bacillus strains can induce the mRNA expression levels of pro-inflammatory cytokines (TNF-α, IL-6 and IL-1α) in RAW 264.7 cells using RT-PCR (Duc *et al.*, 2004; Huang *et al.*, 2008).

CONCLUSION

This study shows that *B. subtilis* BS02 has no cytotoxicity for RAW 264.7 cells and also enhance the immune function of macrophages through stimulating the production of NO, iNOS and inflammatory cytokines. It is suggested that *B. subtilis* BS02 may have immunomodulatory function and exert part of its probiotic activities through activated macrophage functions. Thus, *B. subtilis* BS02 can be considered as a potential probiotic. This study provides valuable evidence for explaining the mechanism of immune modulation by *B. subtilis* spore-based probiotics.

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