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*Full Length Research Paper*

# Immunomodulatory effects of novel bifidobacterium and lactobacillus strains on murine macrophage cells

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**The principle purpose of this study was to investigate the immunomodulatory ability of three microbial strains isolated from Bama County centenarians in activating macrophages, and to characterize the effective component of these strains. A murine macrophage cell line, RAW264.7, was cultured in presence of intact bacterial cells, bacterial cell wall (CW) or cell free extract (CFE) . NO, IL-6, TNF- production and phagocytic activity of co-cultured macrophages were tested. *Bifidobacterium adolescentis* BBMN23, *Bifidobacterium longum* BBMN68 and *Lactobacillus saliva* Ren were demonstrated to enhance the activities of macrophages by increasing the phagocytic activity and NO, IL-6, TNF- production. The immunomodulatory activities of these microbial strains are mainly due to the CW fraction, although their CFE were also effective. The results of this study indicated that specific substrains of bifidobacterium and lactobacillus from guts of healthy centenarians (Bama County, China) may be of immunomodulatory value, and thus may have the potential to be used as probiotics.**

**Key words:** *Bifidobacterium*, lactobacillus; RAW 264.7 cells, immunomodulatory activities, bacterial fractions.

## INTRODUCTION

Probiotics, mainly lactobacilli and bifidobacteria, are reported to be able to stimulate as well as regulate several aspects of innate and acquired immune responses (Arunachalam et al., 2000; Gill et al., 2001; Parra et al., 2004; Olivares et al., 2006). Additionally, intake of probiotics is effective in the prevention and/or treatment of diseases related to abnormal immune functions, including allergic diseases (Shida et al., 2002) and intestinal inflammatory disorders such as Crohn's disease (Fujimori et al., 2007) . Probiotics thus could be used to improve the immune functions of whom these functions are diminished, or to prevent or treat diseases mediated by immune functions.

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**Abbreviations: CW,** cell wall; **CFE,** cell free extract; **PBS,** phosphate buffer saline; **CGMCC,** China general microbiological culture collection.

The beneficial effects of probiotics in regulating immune responses have stimulated interests of researchers all over the world in finding new strains. Bama County (Guangxi, China) is one of the world's five longevity districts identified by International Natural Medicine Society. The ratio of centenarians in the local population was 31.1 per 100, 000 reported by census record in year 2000, exceeding the international standard of 25 per 100, 000 for "hometowns of longevity". In our previous study, three strains, *Lactobacillus saliva* Ren, *Bifidobacterium adolescentis* BBMN23 and *Bifidobacterium longum* BBMN68, were isolated from the feces of centenarians in Bama County, and ingestion of *B. adolescentis* BBMN23 and *B. longum* BBMN68 were demonstrated to significantly boost the innate and acquired immune responses in mice (Yang et al., 2009). One hypothetical pathway by which probiotics modulate immune function in the intestine is that M (microfold) cells take up probiotics directly by transcytosis and macrophages who present immediately below M cells engulf probiotics and trigger immune responses (Shida and Nanno, 2008). Therefore, we hypothesize that the stimulation of immune function

by centenarians originated bacteria may be mediated by macrophages. In the present study, we focused on the immunomodulatory ability of these centenarians originated bacteria in activating macrophage cells.

Macrophages are key players in the innate immune response and function as antigen-presenting cells in initiating adaptive immune responses (Laskin, 2009). A variety of studies have demonstrated that lactobacilli and bifidobacteria are capable of enhancing the phagocytic activity, or stimulating the production of hydrogen peroxide, NO or cytokines in macrophage cells, and therefore activate a variety of immune responses (Park et al., 1999; Cross et al., 2004; Kim, 2006; Kim et al., 2007). These immune responses are reported to be stimulated not only by intact bacterial cells, but also their components, including peptidoglycan, lipoteichoic acids, intra- and extracellular polysaccharide products, cell wall (CW) and cell free extract (CFE) fractions (Hatcher and Lambrecht, 1993; Matsuguchi et al., 2003; Amrouche et al., 2006a; Shida et al., 2009). However, different probiotic strains vary in their ability to modulate the immune cells (Park et al., 1999; He et al., 2002). Therefore, efficacy of each specific strain needs to be demonstrated through a strictly designed study.

The aim of this study was to use the RAW 264.7 murine macrophage model to further explore the immunomodulatory potential of bifidobacterium and lactobacillus strains isolated from healthy centenarians in China, and to characterize the effective component of these strains. The immune-active abilities of different microbial strains and bacterial fractions were evaluated. Centenarians originated bacteria were compared with two commercial strains, Bifidobacterium animalis Bb12 and lactobacillus casei strain Shirota, so as to lay a basis for wider use as probiotics.

## MATERIALS AND METHODS

### Microbial strains and culture conditions

*B. adolescentis* BBMN23 (CGMCC 2264), *B. longum* BBMN68 (CGMCC 2265) and *L. saliva* Ren LSR (CGMCC 3606) were isolated from fecal samples of healthy centenarians living in Bama County located in the Guangxi Zhuang Autonomous Region of southwest China and were stored in China General Microbiological Culture Collection (CGMCC; Beijing, China). Two commercial strains, Bifidobacterium animalis subsp. Lactis Bb12 (ATCC 27536) and Lactobacillus casei strain Shirota LCS (ATCC 53103), were provided by Chr Hansen company (Moersolm, Denmark) and Yakult Central Institute (Kunitachi, Japan), respectively.

All strains were cultured in MRS (De Man, Rogosa and Sharpe) broth (LuQiao, China) at 37°C until late log phase. Bacterial cells were collected by centrifugation at 2,600 x g for 10 min and washed twice in phosphate buffer saline (PBS; pH 7.2). After re-suspended in distilled water, the bacterial cells were heat-inactivated at 95°C for 30 min, then lyophilized and stored at -20°C.

### Preparation of cellular fractions of bacteria

Harvested bacterial cells were fractionated by the method according to Orlando et al. (2009). After cultivation, all strains were harvested, washed twice with PBS (pH 7.2) and suspended in distilled water. Then the bacterial cells were sonicated (Sonics and Materials, Danbury, CT, USA) on ice at 50 W for 1 min at 30 s intervals until the bacterial cells were disrupted. The whole cells were removed from suspension by centrifugation (1,000 x g for 30 min at 4°C) . CWs were then separated from the CFE by centrifugation (35,000 x g for 20 min at 4°C). The supernatant and the precipitate were used as CFE and CW, respectively. Each fraction was freeze dried and suspended with DMEM (Invitrogen, Carlsbad, USA) to the desired bacterial concentration on a dry weight basis. Suspended bacterial cells were stored at -20°C until use.

### Murine macrophage cells culture

Mouse monocyte/macrophage cell line RAW 264.7 (ATCC TIB-71) was grown in DMEM media, supplemented with 10% foetal bovin serum, streptomycin (100 mg/ml) and penicillin (100 U/ml) at 37°C with 5% $CO_2$. Cells were transferred to 24-well culture clusters at a density of 5 x 105 cells per ml, and allowed to adhere for 4 h prior to bacterial activation. Bacteria or their fractions were incubated with cells for 24 h. Cells incubated without bacteria were used as negative control, and cells cultured with LPS from *Escherichia coli* serotype O127:B8 (Sigma-Aldrich, St. Louis, MO) at the concentration of 1 g/ml (Okada et al., 2009) were used as positive control. The culture supernatant was collected and stored at -80°C until analyzed for NO, IL-6 and TNF- .

### NO determination

The NO concentration was determined by measuring the amount of released nitrite with Griess reagent (Sigma-Aldrich, St. Louis, MO) according to the Griess reaction (Green et al., 1982). Briefly, 50 l of cell culture supernatant was added to a new 96-well plate and mixed with 50 l of modified Griess reagent. After incubation at room temperature for 15 min, absorbance was measured in a plate reader at 540 nm. Nitrite concentrations were calculated on the basis of $NaNO_2$ standard curve.

### Cytokine measurement

Concentrations of IL-6 and TNF- in culture supernatant were measured using the IL-6 or TNF- ELISA kits (Mouse IL-6 ELISA Kit and Mouse TNF- ELISA Kit; Rapidbio, Califonia, USA), according to the procedure described by the manufacturer.

### Phagocytosis analysis

The phagocytic ability of macrophage was measured by neutral red uptake as described (Yu et al., 2009) . RAW 264.7 cells were seeded in triplicates at the density of 1 x 105 cells per ml in 96-wells plates with complete DMEM media, and allowed to adhere for 4 h. Cells incubated without bacteria were used as negative control, and cells cultured with LPS (1 g/ml) as positive control. Bacteria or their fractions were incubated with cells for 24 h. After that, the cells were washed three times with PBS (pH 7.2) and 0.075% (w/v) neutral red was added. The plates were incubated for 30 min and cells were then washed with PBS (pH 7.2) three times to remove excess dye. Then cell lysate (ethanol and 0.01% (w/v) acetic acid at the volume ratio of 1:1) was added and cells were incubated at room temperature overnight for sufficient schizolysis. The optical density was determined at 540 nm and the relative phagocytosis was calculated by dividing experimental data by control value.

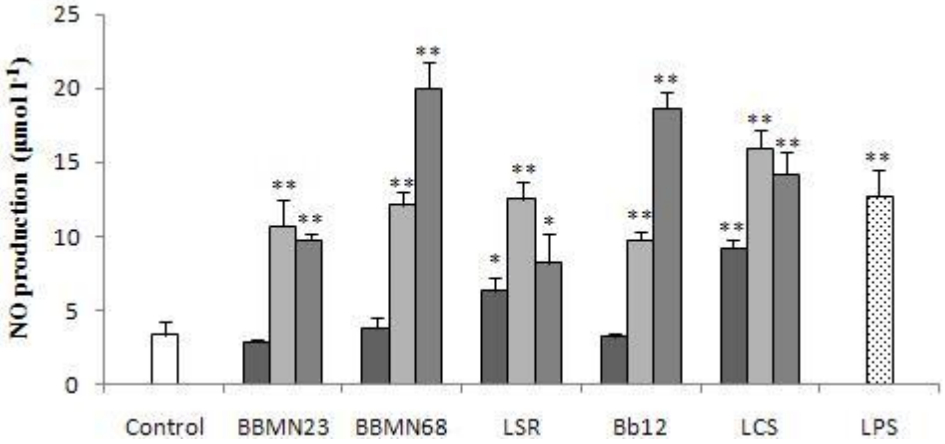


**Figure 1.** NO production by RAW 264.7 macrophage cells treated with various concentrations of probiotic strains or LPS. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean. *Statistically significant difference in comparison with the control group, P < 0.05, ** P < 0.01. (■) 10 g ml$^{-1}$; (□) 50 g ml$^{-1}$; (■) 250 g ml$^{-1}$; (□) Control; (⊠) LPS 1 g ml$^{-1}$.

**Statistic analysis**

Results are expressed as means ± standard deviations. Data were analyzed using Independent Samples T-test or one-way ANOVA (SPSS FOR WINDOWS version 13.0) at a significance level of P < 0.05.

## RESULTS

NO production in macrophages treated with bifidobacterium and lactobacillus strains. All strains increased the NO production significantly (P < 0.05) at the concentrations of 50 or 250 g/ml, which equal to 1 x 107 or 5 x 107 cfu/ml (Figure 1). For *B. longum* BBMN68 and *B. animalis* Bb12, NO production increased with concentration, whereas for other strains it tended to decline at the concentration of 250 g bacteria per ml. Macrophage cells cultured with LPS (1 g/ml), a macrophage activator, generated 12.8 ± 1.7 mol/l of NO in the culture supernatant. NO productions stimulated by *B. longum* BBMN68 and *B. animalis* Bb12 treatment at the concentration of 250 g/ml were significantly higher (P < 0.05) than those stimulated by LPS and the other strains. Cytokines induced in macrophages treated with bifidobacterium and lactobacillus strains.

All five strains affected both IL-6 and TNF- production of macrophage cells in a dose- dependent manner (Tables 1a and b). For IL-6 induction, two centenarians originated bifidobacterium strains were as potential as the commercial strain, *B. animalis* Bb12, at low and mid concentrations. *L. saliva* Ren induced the highest level of IL-6 production (1272.5 ± 116.5 pg/ml) when compared with other strains. IL-6 levels in the RAW 264.7 cells cultured with LPS (1 g/ml) were elevated at 1671.9 ± 29.2 pg/ml, and all the IL-6 productions induced by bacterial strains were lower than that of LPS stimulation (P < 0.05). As regard to TNF- induction, *B. adolescentis* BBMN23 appeared to be the most potent at high concentration. TNF - was elevated under LPS treatment (39.6 ± 2.0 ng/ml), which was lower than the production induced by three centenarians originated strains and higher than that by *L. casei* Shirota treatment. Lactobacillus showed significantly (P < 0.01) higher inducing ability of IL-6 than bifidobacterium. However, strains showing higher abilities of IL-6 induction did not show the same ability of TNF- induction. Enhanced phagocytosis of macrophages induced by bifidobacterium and lactobacillus strains.

The phagocytic activity of macrophages was monitored by measuring the amount of neutral red internalized in macrophages. Figure 2 shows that phagocytosis of RAW 264.7 cells increased with bacterial concentration, and the phagocytic activity was significantly (P < 0.05) enhanced at the concentration of 250 g/ml. *B. adolescentis* BBMN23 appeared to be the most potent. BBMN23 induced significantly (P < 0.05) higher levels of phagocytic activity (155.2 ± 11.7%) when compared with the other strains and the LPS treatment (133.7 ± 8.5%).

NO, cytokines and phagocytic activity induced in macrophages treated with CW and CFE fractions from microbial strains. In order to evaluate the effects of different fractions from bifidobacterium and lactobacillus strains on the induction of NO, IL-6, TNF- and the increasing of phagocytic activity in RAW 264.7 macrophages, cells were incubated with CW or CFE.

As shown in Figure 3, both fractions (CW and CFE) of five strains significantly (P < 0.05) increased the phagocytic activity of macrophage cells and the production of NO, IL-6 and TNF- . CW of *B. longum* BBMN68, *L. saliva* Ren and *L. casei* Shirota showed a

**Table 1(a).** Levels of IL-6 and TNF- production by RAW264.7 cells treated with heat-killed probiotic bacteria.

| Strains | IL-6 production (pg ml$^{-1}$)* | | |
|---|---|---|---|
| | 10 g ml$^{-1}$ | 50 g ml$^{-1}$ | 250 g ml$^{-1}$ |
| BBMN23 | 8.7 ± 1.3 | 85.4 ± 5.2** | 599.6 ± 34.1** |
| BBMN68 | 9.6 ± 1.1 | 99.7 ± 12.5** | 342.1 ± 49.9** |
| LSR | 130.8± 7.4** | 363.8 ± 29.9** | 909.0. ± 121.8** |
| Bb12 | 9.5 ± 1.1 | 109.7 ± 5.1** | 480.1 ± 25.8** |
| LCS | 152.2± 6.8** | 376.1 ± 16.3** | 1272.5± 116.5** |
| LPS | 1671.9± 29.2** | | |
| Control | 7.9 ± 2.8 | | |

**Table 1(b).** Levels of IL-6 and TNF- production by RAW264.7 cells treated with heat-killed probiotic bacteria.

| Strains | TNF- production (ng ml$^{-1}$)* | | |
|---|---|---|---|
| | 10 g ml$^{-1}$ | 50 g ml$^{-1}$ | 250 g ml$^{-1}$ |
| BBMN23 | 6.6 ± 1.6** | 26.4 ± 2.1** | 64.6 ± 2,1** |
| BBMN68 | 13.3 ± 1.1** | 39.1 ± 1.8** | 51.8 ± 2.4** |
| LSR | 17.0 ± 1.6** | 17.0 ± 2.1** | 51.0 ± 3.1** |
| Bb12 | 12.5 ± 1.7** | 21.2 ± 1.5** | 42.9 ± 1.6** |
| LCS | 13.6 ± 2.4** | 14.5 ± 2.7** | 22.7 ± 1.0** |
| LPS | 39.6 ± 2.0** | | |
| Control | 1.0 ± 0.1 | | |

*Data are presented as mean ± standard deviation (n = 3). **Statistically significant difference in comparison with the control group, P < 0.01.

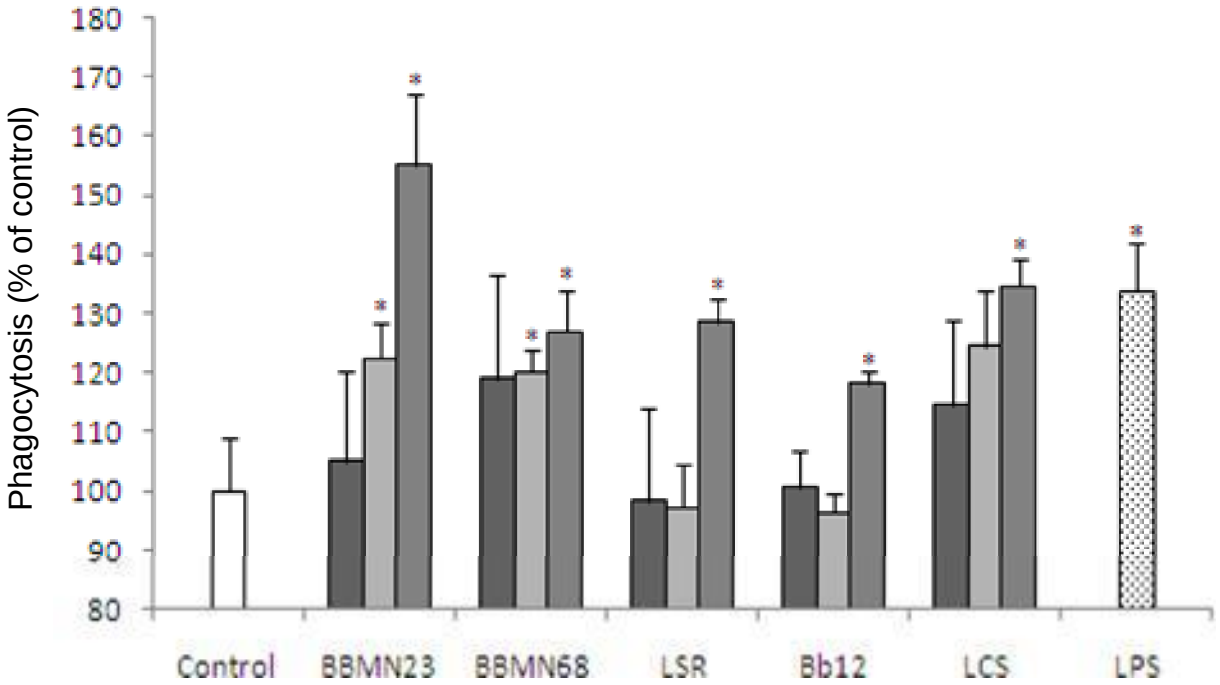


**Figure 2.** Phagocytic activity of RAW 264.7 macrophage cells treated with various concentrations of probiotic strains or LPS. Results were calculated by dividing experimental data by control values. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean. *Statistically significant difference in comparison with the control group, P < 0.05. (■) 10 g ml$^{-1}$; (■) 50 g ml$^{-1}$; (■) 250 g ml$^{-1}$; (□) Control; (▨) LPS 1 g ml$^{-1}$.

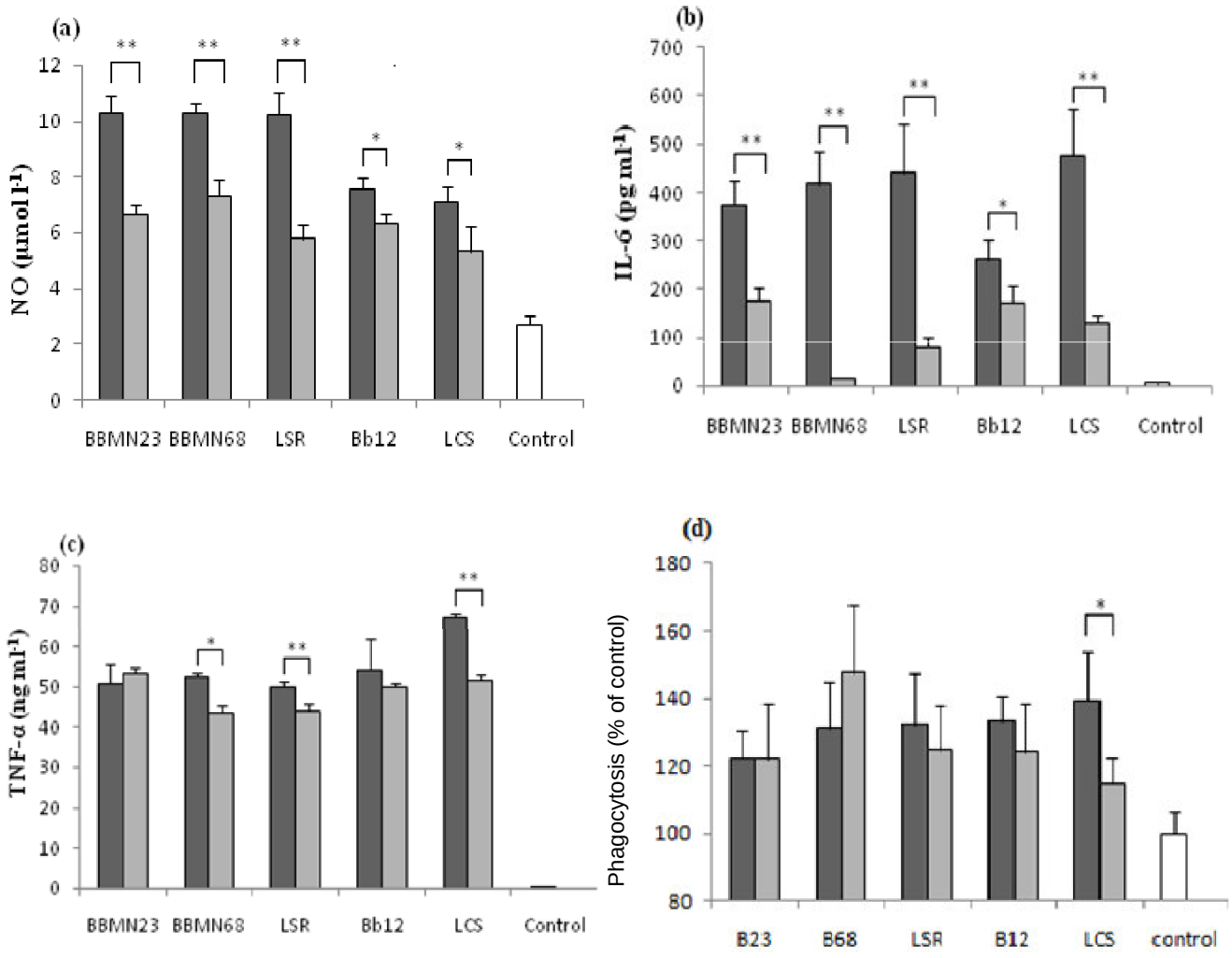


**Figure 3.** Effects of various bacterial components on NO and cytokine secretion by RAW 264.7 cells. Macrophage cells were cultured with cell wall (CW) or cell free extract (CFE) of probiotic bacteria at concentration of 50 g $ml^{-1}$ for 24 h. The production of NO (a), IL-6 (b), TNF- (c) and the phagocytic activity (d) was measured. Experiments were performed in triplicate, and error bars represent the standard deviations of the mean. *Statistically significant difference in comparison with the corresponding CFE, $P < 0.05$; ** $P < 0.01$. (■) CW; (▩) CFE; (□) Control.

significantly ($P < 0.05$) stronger capacity of NO, IL-6 and TNF- induction than CFE. For *B. adolescentis* BBMN23 and *B. animalis* Bb12, NO and IL- 6 productions induced by CW were significantly higher ($P < 0.05$) than the productions induced by CFE, while the TNF- induction by CW was equal to that of CFE stimulation. For phagocytic activity, only CW of *L. casei* Shirota showed a significantly ($P < 0.05$) stronger inducing capacity than CFE, while the CW and CFE fractions from other four strains did not showed a significant difference in increasing the phagocytic activity. The NO and cytokine values induced by CW or CFE fractions varied among strains. The ratio of IL-6 production induced by CW to CFE of *B. animalis* Bb12 was significantly ($P < 0.05$) lower than that of the other strains, while the ratio of *B. longum* BBMN68 was the highest, reaching approximately 26 fold.

## DISCUSSION

It is reported that age could have a major effect on human intestinal microflora. Compared with younger subjects, elders usually have increased numbers of facultative anaerobes and decreased numbers in beneficial organisms, such as the anaerobic lactobacilli and bifidobacteria (Woodmansey, 2007). However, the situation is different in healthy centenarians. Zhang et al. (1994) reported that the proportions of bifidobacteria in feces anaerobe (53 - 87%) from the healthy centenarians of Bama County were higher than the average (about 40%) from the younger subjects. Zhao et al. (2010)

observed a high proportion of bifidobacteria (up to 9.59%) in total fecal bacteria from some centenarians, and the proportion in younger adults is usually 3% (Trebichavsky et al., 2009). Therefore, bacteria from the centenarians of Bama County might be good resources for finding novel probiotic strains. This study focused on the immunomodulatory abilities of three centenarians originated strains, *B. adolescentis* BBMN23, *B. longum* BBMN68 and *L. saliva* Ren, which were newly isolated and not reported to exist in other subjects.

In present study, we used RAW 264.7 macrophages, a transformed peritoneal macrophage cell line from by Abelson murine leukemia virus, as reporter cells. They were demonstrated to have the properties of normal macrophages, and especially sensitive to macrophage activating agents, such as LPS (Raschke et al., 1978). Consistent with our previous *in vivo* results (Yang et al., 2009), phagocytic activity of RAW 264.7 macrophages was enhanced after inoculation with *B. adolescentis* BBMN23 or *B. longum* BBMN68, and BBMN23 was a more effective stimulator than BBMN68. *L. saliva* Ren and two commercial strains also showed an activating ability in phagocytosis. Phagocytosis is an early and crucial event in host defense against pathogens, and it may subsequently initiate the adaptive immune response. Several studies suggest that by stimulating phaocytic activity and other mediators in macrophage, probiotic bacteria consumption can enhance the intestinal mucosa immunity, and thus inhibit pathogen invasion of the host ((Lin et al., 2007; Medici et al., 2005).

In the present study, exposure of RAW264.7 cell line to *B. adolescentis* BBMN23, *B. longum* BBMN68 or *L. saliva* Ren resulted in marked increases of NO, IL-6 and TNF- production. NO is a major efficient molecule involved in the destruction of tumor cells and pathogenic microorganism (Duerksen-Hughes et al., 1992). NO can also regulate the functional activities of natural killer cells, neutrophil cells and mast cells, and affect the production of several cytokines (Armstrong, 2001; Cifone et al., 2001; Forsythe et al., 2001; Klimp et al., 2002). IL-6 and TNF- are pro-inflammatory cytokines usually secreted by activated macrophages. They may activate neutrophils and monocytes to initiate bacterial or tumor cell killing and stimulate T- or B-lymphocyte proliferation (Calder, 2007). In this sense, the tested five strains may be able to promote immune responses against tumors and intracellular pathogenic infections.

The ability of the tested bacteria to activate macrophage cells was different among strains. In the present study, *B. adolescentis* BBMN23 was demonstrated to be a stronger stimulator for TNF- production and the phagocytic activation than the two commercial strains. Whereas commercial strain, *L. casei* Shirota induced the highest IL-6 production among tested strains. Earlier research reports that bifidobacteria isolates can stimulate $H_2O_2$, NO, TNF- , and IL-6 production in macrophage cells. $H_2O_2$ tends to decline slightly at high concentration while NO increased with concentration (Park et al., 1999). Our results showed that NO production tended to decline when co-cultured with some strains at high concentration. This effect was demonstrated to be strain-dependent, and strains that induced a decreased production of NO at high concentration might be more susceptible to saturation or feedback mechanisms. Lee (2005) reports that lactobacilli strains show much lower activity of IL-6 induction in macrophage cells than bifidobacteria. However, we found that lactobacilli showed stronger ability than bifidobacteria in IL-6 induction, which suggests that this stimulatory capacity might be related to the specific strain rather than the genus.

The various stimulatory capacities of different probiotic strains may arise from the different proportion and various modifications of immune- active components in CW and CFE fractions of specific strains. Studies show that probiotics, mostly belong to gram-positive bacteria, contain lipoteichoic acids and peptidoglycan as CW components, which can active macrophages to secret cytokines or important mediators (Korhonen et al., 2001; Wang et al., 2001; Matsuguchi et al., 2003), whereas the stimulation of macrophage activity by CFE fraction may be due to the DNA containing unmethylated CpG motifs and bioactive peptides or proteins (Li et al. 2005; Takahashi et al., 2006; Amrouche et al., 2006a). Our research demonstrated that both CW and CFE fractions from bifidobacteria and lactobacilli strains enhanced the capacity of NO, IL-6 and TNF- production and the phagocytic activity of macrophages, but CW extract was a more efficient stimulator of macrophage activity than the CFE fraction. However, this effect was strain-dependent. The CW fraction from *B. longum* BBMN68 was found to be much more effective than CFE in macrophage activation, while the CFE fraction from *B. lactis* Bb12 was no less effective than CW. Amrouche et al. (2006b) report that peptides and proteins from *B. lactis* Bb12 cytoplasm may significantly stimulate cell proliferation and cytokine secretion by mouse splenocytes. The inducing ability of CFE fraction from *B. lactis* Bb12 might be due to the immunomodulatory function of peptides and proteins in the cytoplasm. However, the mechanisms by which component the tested bacteria stimulate cellular responses are still unclear and require further study.

The study overall demonstrated that intact cells and cell components from centenarians originated bifidobacteria and lactobacilli promote *in vitro* immune responses in mouse macrophages. Intact bacterial cells, CFE fraction and mainly CW extract were found to stimulate phagocytic activity and increase NO, IL-6 and TNF- production. This report suggests that specific strains of bifidobacteria and lactobacilli from guts of healthy centenarians in China and their cellular components may be of immunomodulatory value and have the potential to be used as probiotics. They may used as nutritional

supplements to improve the immune function or as immunomodulators for the prevention or treatment of diseases mediated by immune responses.

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