Saudi Journal of Biological Sciences 28 (2021) 5115–5118

Contents lists available at ScienceDirect

Saudi Journal of Biological Sciences

journal homepage: www.sciencedirect.com

Original article

Lysed and disrupted *Bifidobacterium bifidum* BGN4 cells promote anti-inflammatory activities in lipopolysaccharide-stimulated RAW 264.7 cells

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ARTICLE INFO

Article history:
Received 17 June 2020
Revised 11 May 2021
Accepted 12 May 2021
Available online 20 May 2021

Keywords:
Paraprobiotic
Bifidobacterium bifidum BGN4
Anti-inflammation
Immunoregulatory

ABSTRACT

Bifidobacterium bifidum BGN4 has been shown to improve the immune system by regulating interleukin (IL)-6 in RAW 264.7 macrophage cells. In this study, the dead cells of *B. bifidum* BGN4 were produced by enzymatic and physical processing to enhance the inhibition properties of pro-inflammatory cytokines using lipopolysaccharide (LPS)-stimulated RAW 264.7 cells. Notably, the secretion levels of cytokines such as interleukin (IL)-1β, IL-6, IL-8, and tumor necrosis factor (TNF)-α were decreased by the cell-wall disrupted extracts compared to heat-killed cells. The result suggests that the exposed interior-surface of *B. bifidum* BGN4 has a potential ability to regulate the immune-responses in the gastrointestinal tract due to major substances in inside-cell wall such as peptidoglycan and teichoic acids. In conclusion, the lysed and disrupted cells from the inside out of *B. bifidum* BGN4 have anti-inflammatory properties as paraprobiotic agents to control chronic inflammatory related-diseases.
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1. Introduction

Probiotics such as *Bifidobacterium* spp. and *Lactobacillus* spp. have been used as health functional materials to enhance immunomodulatory activities (Hirose et al., 2006; Lee and Ji, 2005; Matsuguchi et al., 2003). Recent studies showed that heat-killed cells of lactic acid bacteria (LAB) also have a beneficial effect on the immune regulation similar to live bacteria (Jeong et al., 2019; Moon et al., 2019) because of the teichoic acid which is one of components in the cell surface of peptidoglycan layer of gram-positive bacteria (Taverniti and Guglielmetti, 2011). Furthermore, it was validated that the released cytoplasmic contents from dead LAB can modulate the pro-inflammatory cytokine production by gastric epithelial cells (Tejada-Simon and Pestka, 1999). Thus, the use of inactivated cells or cell extracts of probiotics have a potential to beneficial effects on the immune system, and suggested to be called as 'paraprobiotic' (Taverniti and Guglielmetti, 2011).

Bifidobacterium bifidum is the second most species identified in breast-fed infants, and *B. bifidum* BGN4 is obtained from a breast-fed infant's fecal sample (Ku et al., 2016). *B. bifidum* BGN4 has bio-functional characteristics such as anti-tumor effects, production of biogenic metabolites, as well as the regulation of immune system by reducing levels of pro-inflammatory cytokines [e.g., Interleukin (IL)-6 and tumor necrosis factor (TNF)-α] (Kim and Ji, 2006; Kim et al., 2007). Moreover, heat-treated dead cells of *B. bifidum* BGN4 also had a physiological property to decrease the secretion of IL-6 with the combination of cell wall and cell-free extracts by synergistic effects on immune system (Lee and Ji, 2005). Here, we hypothesized that the dead cell extracts of *B. bifidum* BGN4, ceased by the enzymatic lysis with physical treatment, have an enhanced immune functionality as the interior surface of cell walls includes more essential components such as teichoic and lipoteichoic acids (Piqué et al., 2019; Tejada-Simon and Pestka, 1999). The results can be applied to produce the dead-cell of other LAB as well as *B. bifidum* BGN4 with enhanced immune functionalities.

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Peer review under responsibility of King Saud University.

Production and hosting by Elsevier

https://doi.org/10.1016/j.sjbs.2021.05.028
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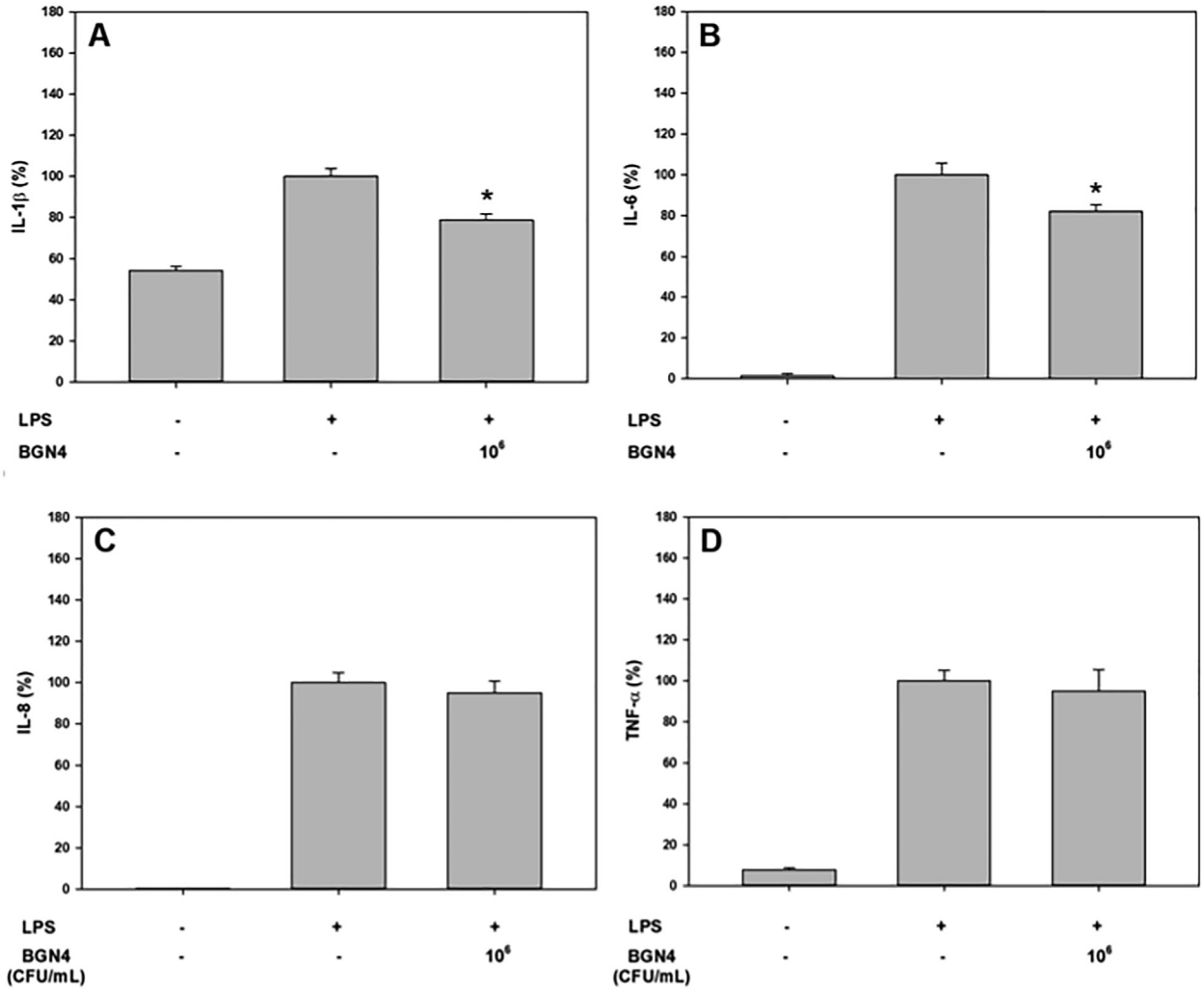

Fig. 1. Regulation of different types of cytokine secretion [A: Interleukin (IL)-6, B: Interleukin (IL)-6, C: Interleukin (IL)-8, and D: Tumor necrosis factor (TNF)-α] in RAW 264.7 macrophage cells by heat-killed *B. bifidum* BGN4. (LPS; lipopolysaccharides, BGN4; *B. bifidum* BGN4). "+" and "-" indicate 'treated with' and 'not treated with', respectively. Values are the means ± standard deviations (n = 3). $p < 0.05$(*) were used to indicate significance compared with the LPS-stimulated value.

2. Materials and methods

2.1. Preparation of live and dead bacteria fractions

B. bifidum BGN4 was gifted from BIFIDO Co. Ltd. (Hongcheon, South Korea), and cells were cultured anaerobically in de Man, Rogosa and Sharpe broth (MRS, Oxoid, Basingstoke, UK) containing L-cysteine hydrochloride monohydrate (0.1%, w/v) at 37 °C until growing up to 9 log CFU/mL (You et al., 2004). After centrifugation at 8000*g* for 20 min, the precipitated-culture was mixed with phosphate-buffered saline (PBS), and the suspension was collected to produce the dead-cell with various treatments. The suspension was autoclaved at 121 °C for 15 min to prepare the heat-killed samples (Peng and Hsu, 2005), and food-grade lysozyme (Neova Technologies Inc., Abbotsford, Canada; 6%,w/v) was reacted with the suspension for 1 h at 37 °C to assist the cell disruption as a cell-lysis treatment (Ibrahim et al., 1994; Tejada-Simon and Pestka, 1999). The cell surface of lysozyme-treated suspension was further broken down by sonication (turn on for 5 sec following 2 sec for turn off; VCX-400, Sonics and Materials, Newtown, CT) for 90 min at 30% amplitude (Duckhouse et al., 2004). All fractions were stored at −80 °C before used.

2.2. Preparation of RAW 264.7 cell culture

The mouse macrophage cell line RAW 264.7 was obtained from Korean Cell Line Bank (KCLB) and cultured in Dulbecco's modified Eagle's complete medium (DMEM, Welgene, Daegu, Korea) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin in a humidified incubator with 5% CO_2 at 37 °C (He et al., 2010).

2.3. Determination of cytokine production by RAW 264.7 macrophage cells

RAW 264.7 cells were incubated in a 48-well plate cell (1×10^5 cells/mL per well) for 18 h (Wong et al., 1998), and lipopolysaccharide (LPS, 1 μg/mL) was added into each cell culture with dead bacteria fractions of *B. bifidum* BGN4 at concentrations of 10^6 CFU/mL. The concentrations of TNF-α, IL-6, and IL-1β in cultured-supernatant after 24 h were determined by ELISA kits from BD Biosciences (San Diego, CA) and IL-8 was measured by kits from R&D Systems (Minneapolis, MD) according to the manufacturer's instructions. The amounts of cytokines (pg/mL) were calculated to relative amount (%) of the control. For the statistical analysis, analysis of variance (ANOVA) was used to analyze the

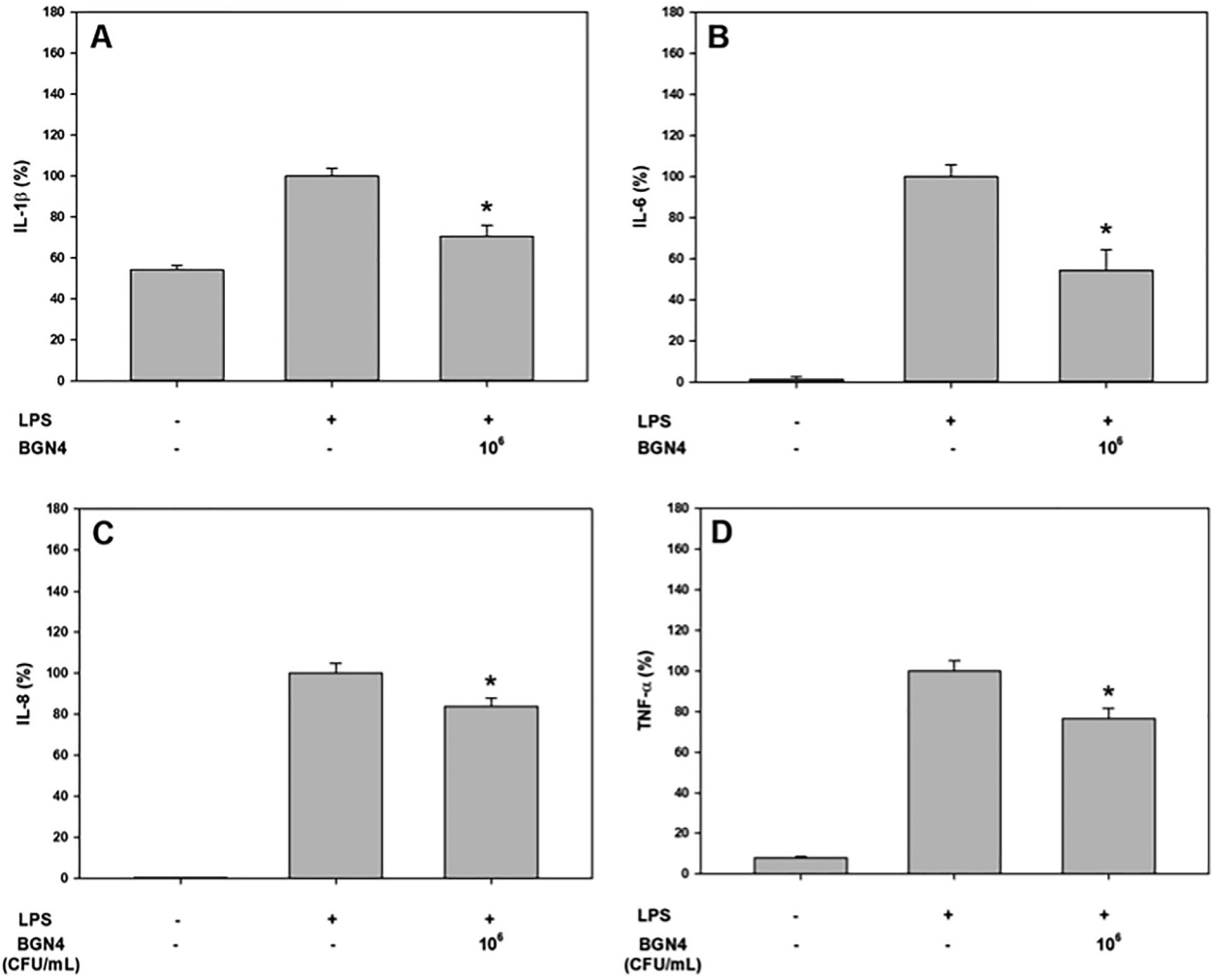

Fig. 2. Regulation of different types of cytokine secretion [A: Interleukin (IL)-6, B: Interleukin (IL)-6, C: Interleukin (IL)-8, and D: Tumor necrosis factor (TNF)-α] in RAW 264.7 macrophage cells by enzymatically-lysed and physically-disrupted *B. bifidum* BGN4. (LPS; lipopolysaccharides, BGN4; *B. bifidum* BGN4). "+" and "-" indicate 'treated with' and 'not treated with', respectively. Values are the means ± standard deviations (n = 3). $p < 0.05$(*) were used to indicate significance compared with the LPS-stimulated value.

experimental results from triplicate trials followed by Tukey's multiple comparison tests. All statistical analyses were performed with SAS software (version 9.4, SAS institute, Cary, NC); the statistical significances were considered at $p < 0.05$ and $p < 0.01$.

3. Results and discussion

3.1. Effects of heat-killed B. bifidum BGN4 on cytokine secretion in RAW 264.7 cells

The production of inflammatory cytokines was analyzed in the cell culture treated by heat-killed bacteria fraction of *B. bifidum* BGN4 (Fig. 1). Heat-killed cells significantly inhibited the IL-1β secretion in LPS-treated RAW 264.7 cells at a concentration of 10^6 CFU/mL (Fig. 1A), and also decreased the release of IL-6 (Fig. 1B). However, heat-killed *B. bifidum* BGN4 showed slightly inhibitory effects on IL-8 and TNF-α secretions compared to LPS-treated control (Fig. 1C and D). The inhibition of IL-6 production by the heat-killed *B. bifidum* BGN4 is corresponding to previous study (Lee and Ji, 2005), and the current study further confirmed that the heat-treated dead-cells also had possible influences to other pro-inflammatory cytokines such as IL-1β, IL-8, and TNF-α which are considered as important roles in the immune responses against external stress.

3.2. Regulation of the cytokine secretion in RAW 264.7 macrophage cells by enzymatically-lysed and physically-disrupted B. bifidum BGN4

Effects the production of inflammatory cytokines such as IL-1β, IL-6, IL-8, and TNF-α in RAW 264.7 macrophage cells by enzymatically-lysed and physically-disrupted *B. bifidum* BGN4 were analyzed. Interestingly, the secretions of all cytokines were significantly decreased on LPS-stimulated macrophage cells (Fig. 2). The secretion level of IL-1β was remarkably reduced by the enzymatically- and physically-treated *B. bifidum* BGN4 cell walls at 10^6 CFU/mL compared to heat-killed cells (Fig. 2A). The secretion of IL-6 was also significantly different in 10^6 concentrations of cell walls compared to only LPS-treated cells as a control (Fig. 2B). These results suggest the disrupted cell wall components have higher inhibitory effects compared to heat-killed cells. Previous study showed that live or heat-killed *B. bifidum* BGN4s inhibited the IL-6 expression as an immunomodifier by peptidoglycan layers (Lee and Ji, 2005). Notably, it is worth to note that cell wall extracts by enzymatic and physical treatments further suppressed IL-8 formation compared to heat-killed BGN4 cells as well as the expression of TNF-α (Fig. 2C and 2D). The inhibitory properties of *B. bifidum* BGN4 may be explained that the inside of disrupted-dead cell walls have higher amounts of lipoteichoic acids or teichoic acids which have anti-inflammatory effects in peptidoglycan

layer and the cytoplasmic membrane (Matsuguchi et al., 2003; Taverniti and Guglielmetti, 2011).

In current study, it was clearly showed that the dead cells of *B. bifidum* BGN4 by bio-catalytical and physical treatments inhibit various pro-inflammatory cytokines in LPS-activated RAW 264.7 cells because of anti-inflammatory agents indwelled in inlayer of cell walls. Thus, the cell wall fragments from inside out of *B. bifidum* BGN4 suggest potential recovery effects for inflammatory diseases in the gastrointestinal tract with suppressing the pro-inflammatory overproduction, and it can be used in various probiotic research and industrial applications.

We wish to confirm that there are no known conflicts of interest associated with this publication and there has been no significant financial support for this work that could have influenced its outcome.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Acknowledgment

This work was supported by the Technology Innovation Program (#20014744) funded by the Ministry of Trade, Industry & Energy (MOTIE, Republic of Korea).

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