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Research Article

A Strain-Defined IDCC 3201+IDCC 3501 Mixture Modulates Inflammatory and BDNF-Related Biomarker Responses in LPS-Stimulated BV-2 Microglial Cells

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Abstract

Neuroinflammation is closely associated with microglial activation, inflammatory mediator production, and alterations in neurotrophic and synapse-related biomarkers. This study investigated whether heat-killed *Lacticaseibacillus rhamnosus* IDCC 3201, heat-killed *Lactiplantibacillus plantarum* IDCC 3501, and the IDCC 3201+IDCC 3501 mixture modulate inflammatory and BDNF-related biomarker responses in lipopolysaccharide (LPS)-stimulated BV-2 microglial cells. BV-2 cells were pretreated with heat-killed bacterial samples at 10^5 - 10^7 cells/mL for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. Cell viability, inflammatory gene expression, inflammatory cytokine secretion, *Bdnf* and *Dlg4* mRNA expression, BDNF secretion, and intracellular BDNF protein expression were evaluated using qRT-PCR, ELISA, and western blotting. The heat-killed bacterial samples showed no cytotoxicity in BV-2 cells. LPS stimulation increased the expression of *Tnf*, *Il1b*, *Il6*, and *Ptgs2* mRNA and the secretion of TNF-α, IL-1β, and IL-6, whereas treatment with the heat-killed IDCC 3201+IDCC 3501 mixture broadly reduced these inflammatory markers. In addition, LPS decreased *Bdnf* and *Dlg4* mRNA expression, BDNF secretion, and intracellular BDNF protein expression, while the mixture increased these BDNF/*Dlg4*-related biomarkers. The mixture showed a broad and coordinated biomarker-modulating pattern across inflammatory and BDNF-related markers, with significant differences from the single-strain treatments observed for selected biomarkers at 10^7 cells/mL. These findings indicate that heat-killed IDCC 3201+ IDCC 3501 may represent a postbiotic-type candidate material that modulates inflammatory and BDNF-related biomarker responses in LPS-stimulated BV-2 cells. Further pathway-level and in vivo studies are required to determine whether these biomarker changes are linked to neuroprotective or cognitive outcomes.

Keywords

BV-2 microglia; heat-killed bacteria; postbiotic-type preparation; neuroinflammation; BDNF

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Introduction

Neuroinflammation is an important biological interface linking innate immune activation, neuronal stress, synaptic dysfunction, and vulnerability to cognitive impairment (Muzio *et al.*, 2021; Tastan and Heneka, 2024). Microglia are the resident immune cells of the central nervous system and continuously survey the brain microenvironment, contributing to tissue homeostasis, clearance of cellular debris, and synaptic remodeling (Colonna and Butovsky, 2017; Pallarés-Moratalla and Bergers, 2024). However, under persistent inflammatory stimulation, microglia can acquire a pro-inflammatory phenotype characterized by increased production of cytokines, inflammatory enzymes, and oxidative mediators (Lull and Block, 2010). If sustained, this response may contribute to neuronal injury and disruption of synaptic integrity (Wang *et al.*, 2015).

Lipopolysaccharide (LPS), a structural component of the outer membrane of Gram-negative bacteria, is widely used to induce inflammatory activation in microglial models (Dai *et al.*, 2015; Skrzypczak-Wiercioch and Sałat, 2022). LPS activates Toll-like receptor 4-dependent signaling and downstream inflammatory pathways, including nuclear factor-κB (NF-κB) and mitogen-activated protein kinase (MAPK) cascades. These pathways regulate the expression of inflammatory mediators such as TNF-α, IL-1β, IL-6, COX-2, and iNOS (Kang *et al.*, 2024; Kim *et al.*, 2023; Park *et al.*, 2020). Although BV-2 cells do not fully recapitulate the complexity of primary microglia or the *in vivo* brain microenvironment, they are widely used as a practical platform for preliminary screening of candidate materials that modulate anti-inflammatory and neurotrophic biomarker responses (Henn *et al.*, 2009; Timmerman *et al.*, 2018).

Microglial inflammatory activation is also associated with alterations in neurotrophic and synapse-related biomarkers (Prowse and Hayley, 2021). Brain-derived neurotrophic factor (BDNF) is a representative neurotrophin involved in neuronal survival, synaptic plasticity, and memory-associated processes (Cunha *et al.*, 2010; Kowiański *et al.*, 2018; Miranda *et al.*, 2019). Postsynaptic density protein 95 (PSD-95) is a major postsynaptic scaffold protein associated with excitatory synapse organization, receptor anchoring, and long-term potentiation (Ehrlich and Malinow, 2004; Xu, 2011). In the present study, the expression of *Dlg4* mRNA, which encodes PSD-95, was evaluated as a synapse-related biomarker together with BDNF-related markers. Therefore, a response characterized by suppression of LPS-induced inflammatory mediator production and increased BDNF-related biomarkers and *Dlg4* mRNA expression can be interpreted as a biomarker profile reflecting attenuation of inflammatory activation and improvement of neurotrophic/synapse-related marker responses in BV-2 cells.

With increasing interest in the gut–microbiota–brain axis, probiotics and postbiotic-type microbial preparations have been investigated as candidate materials capable of modulating neuroimmune responses and neurotrophic pathways (Abdel-Haq *et al.*, 2019; Baek *et al.*, 2024). In particular, lactic acid bacteria have attracted attention in gut–brain axis-related research because of their potential to modulate immune responses, improve the intestinal environment, and regulate host-cell responses (Cristofori *et al.*, 2021). However, when viable bacteria are directly applied to cell culture systems, bacterial viability, proliferation, metabolite production, and changes in the culture environment may influence cellular responses. In contrast, heat-killed bacterial cells can minimize the effects associated with bacterial proliferation while retaining bacterial surface structures, cell wall components, and heat-stable bacterial components. Therefore, they can be used as postbiotic-type preparations to evaluate strain-dependent host-cell responses (Geraldo *et al.*, 2020; Piqué *et al.*, 2019).

Strain-level identification is important in the development of microbial-based functional materials. Even within the same species, immunomodulatory activity, cellular response induction, and physiological activity may vary depending on the strain; therefore, functional properties cannot be generalized at the genus or species level

(McFarland *et al.*, 2018). IDCC 3501 is a kimchi-derived strain with reported safety and anti-inflammatory potential, and IDCC 3201 has also been reported to have safety and anti-inflammatory potential (Chae *et al.*, 2022; Yang *et al.*, 2021). However, it remains unclear how these strains, when applied as heat-killed bacterial cell preparations either individually or in combination, regulate microglial inflammatory responses, BDNF-related biomarkers, and *Dlg4* mRNA expression.

Therefore, this study evaluated whether heat-killed IDCC 3201, heat-killed IDCC 3501, and the IDCC 3201+ IDCC 3501 mixture modulate inflammatory mediators, BDNF-related biomarkers, and *Dlg4* mRNA expression in LPS-stimulated BV-2 microglial cells. To this end, inflammatory mRNA expression, inflammatory cytokine secretion, *Bdnf* and *Dlg4* mRNA expression, BDNF secretion, and intracellular BDNF protein expression were analyzed. In particular, this study aimed to compare the biomarker response patterns induced by each single strain and by the IDCC 3201+IDCC 3501 mixture in LPS-stimulated BV-2 cells.

Materials and Methods

Sample Preparation

The samples used in this study were heat-killed *Lacticaseibacillus rhamnosus* IDCC 3201 (IDCC 3201), heat-killed *Lactiplantibacillus plantarum* IDCC 3501 (IDCC 3501), and a 1:1 mixture of heat-killed IDCC 3201 and IDCC 3501 (IDCC 3201+IDCC 3501 mixture). IDCC 3201 and IDCC 3501 were cultured in de Man, Rogosa, and Sharpe medium (MRS; BD Difco, Franklin Lakes, NJ, USA) at 37°C for 24 h. The bacterial cultures were centrifuged at 8,000 ×g for 5 min, and the collected pellets were washed with phosphate-buffered saline (PBS; Thermo Fisher Scientific, Waltham, MA, USA) and resuspended in PBS. The bacterial suspensions were adjusted to 1 × 10^8 CFU/mL before heat treatment. Subsequently, the bacterial suspensions were heat-treated at 100°C for 30 min to prepare heat-killed bacterial cells. To confirm bacterial inactivation, the heat-treated suspensions were plated on MRS agar and incubated at 37°C for 48 h, and no colony formation was observed. The IDCC 3201+ IDCC 3501 mixture was prepared by mixing equal volumes of each heat-killed bacterial cell suspension. For the IDCC 3201+IDCC 3501 mixture, the indicated concentration represents the total heat-killed bacterial cell concentration, with each strain contributing equally.

Cell Culture

BV-2 mouse microglial cells were cultured in Dulbecco's modified Eagle's medium (DMEM; Thermo Fisher Scientific) supplemented with 10% fetal bovine serum (FBS; Thermo Fisher Scientific) and 1% penicillin/streptomycin (P/S; Thermo Fisher Scientific). Cells were maintained at 37°C in a humidified incubator with 5% CO_2. The culture medium was replaced every 2–3 days, and cells were used for experiments when they reached approximately 80% confluence.

Cell Viability Assay

The cytotoxicity of IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture was evaluated in BV-2 cells using the MTT assay. BV-2 cells were seeded in 96-well plates at a density of 1 × 10^5 cells/well and incubated at 37°C for 24 h. The cells were then treated with IDCC 3201, IDCC 3501, or the IDCC 3201+IDCC 3501 mixture at concentrations of 10^5–10^7 cells/mL for 24 h.

After treatment, MTT solution (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Duchefa, Haarlem, Netherlands) was added to each well at a final concentration of 0.5 mg/mL, followed by incubation at 37°C for 2 h. The MTT solution was then removed, and the resulting formazan crystals were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA). Absorbance was measured at 570 nm using a microplate reader. Cell viability was calculated using the following equation:

Cell viability (%) = Absorbance of treated group / Absorbance of untreated control × 100

LPS-Stimulated BV-2 Microglial Model

BV-2 cells were seeded in 6-well plates and cultured for 24 h until they reached approximately 80% confluence. The cells were pretreated with IDCC 3201, IDCC 3501, or the IDCC 3201+IDCC 3501 mixture at concentrations of 10^5, 10^6, or 10^7 cells/mL for 1 h. Subsequently, lipopolysaccharide (LPS; *Escherichia coli* O111:B4; Sigma-Aldrich) was added at a final concentration of 1 μg/mL, and the cells were incubated for 24 h.

The experimental groups consisted of the non-LPS control, LPS control, IDCC 3201 10^5–10^7 cells/mL + LPS, IDCC 3501 10^5–10^7 cells/mL + LPS, and IDCC 3201+ IDCC 3501 10^5–10^7 cells/mL + LPS. Cell pellets were used for qPCR and western blotting analyses, and culture supernatants were used for ELISA.

Quantitative Real-Time PCR

Total RNA was extracted from BV-2 cell pellets using the RNeasy Kit (QIAGEN, Hilden, Germany). RNA quantity and purity were evaluated by measuring absorbance at 260 and 280 nm, and samples with an OD_{260}/OD_{280} ratio of 1.8 or higher were used for cDNA synthesis. cDNA was synthesized using SuperiorScript III Reverse Transcriptase (Enzynomics, Daejeon, Republic of Korea) according to the manufacturer's instructions.

qPCR was performed using SYBR Green Real-time PCR Master Mix (Toyobo, Osaka, Japan) on a QuantStudio 3 Real-Time PCR System (Applied Biosystems, Thermo Fisher Scientific). Relative mRNA expression levels were calculated using the $2^{-\Delta\Delta Ct}$ method, and the primer sequences used in this study are listed in Table 1. The qPCR cycling conditions were as follows: 95°C for 10 min, followed by 40 cycles of 95°C for 15 s, annealing at 60°C for 20 s, and 72°C for 30 s.

ELISA

Culture supernatants were collected after 1 h of sample pretreatment and 24 h of LPS stimulation. The concentrations of TNF-α, IL-1β, IL-6, and BDNF were measured using corresponding ELISA kits (R&D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions.

Table 1. Primer sequences used for quantitative real-time PCR

Target gene	Primer	Sequence (5′–3′)
Il1b	Forward	ACCTGGGCTGTCCTGATGAGAG
	Reverse	TGTTGATGTGCTGCTGCGAGAT
Il6	Forward	GTTCTCTGGGAAATCGTGGA
	Reverse	GGAAATTGGGGTAGGAAGGA
Ptgs2	Forward	CTGGTGCCTGGTCTGATGATGTATG
	Reverse	TCTCCTATGAGTATGAGTCTGCTGGTT
Tnf	Forward	AACTAGTGGTGCCAGCCGAT
	Reverse	CTTCACAGAGCAATGACTCC
Dlg4	Forward	AGTTGCAGGTGAACGGAACA
	Reverse	ATGCTGTCGTTGACCCTGAG
Bdnf	Forward	TATTTCATACTTCGGTTGC
	Reverse	CCTTCTGGTCCTCATCC
Gapdh	Forward	ACTCCACTCACGGCAAATTC
	Reverse	GTCATGAGCCCTTCCACAAT

Western Blotting

BDNF protein expression was evaluated by western blotting. The analyzed groups were the non-LPS control, LPS control, IDCC 3201 10^7 cells/mL + LPS, IDCC 3501 10^7 cells/mL + LPS, and IDCC 3201+IDCC 3501 10^7 cells/mL + LPS. Cell pellets were lysed using ice-cold RIPA buffer (Thermo Fisher Scientific), and the cell lysates were centrifuged at 13,000 ×g for 30 min at 4°C to collect the supernatants. Protein concentrations were determined using a BCA protein assay kit (Thermo Fisher Scientific).

A total of 30 μg of protein from each sample was denatured at 95°C for 5 min and separated on a 10% SDS-PAGE gel. The separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-Rad, Hercules, CA, USA), and the membrane was blocked with 3% bovine serum albumin (BSA; Sigma-Aldrich). The membrane was then incubated overnight at 4°C with an anti-BDNF primary antibody (1:1,000; catalog no. ab108319; Abcam, Cambridge, UK). β-actin was used as an internal loading control and detected using an anti-β-actin antibody (1:5,000; catalog no. A5441; Sigma-Aldrich).

After primary antibody incubation, the membrane was washed and incubated with an HRP-conjugated goat anti-rabbit IgG secondary antibody (1:5,000; catalog no. ab6721; Abcam) and an HRP-conjugated goat anti-mouse IgG secondary antibody (1:3,000; catalog no. ab6789; Abcam) at room temperature for 1 h. Protein bands were visualized using enhanced chemiluminescence (ECL) reagent (Bio-Rad). BDNF band intensity was quantified using ImageJ software (National Institutes of Health, Bethesda, MD, USA) and normalized to β-actin band intensity.

Statistical Analysis

Data are presented as the mean ± SD from three independent experiments. Statistical analyses were performed using GraphPad Prism software (GraphPad Software, San Diego, CA, USA). One-way analysis of variance (ANOVA) followed by Dunnett's multiple comparisons test was used to compare treatment groups with the LPS control. For the cell viability assay, treatment groups were compared with the untreated control. To directly compare the IDCC3201+IDCC3501 mixture with each single-strain treatment at the highest tested concentration (10^7 cells/mL), one-way ANOVA followed by Šídák's multiple comparisons test was performed for selected pairwise comparisons. A value of $p < 0.05$ was considered statistically significant. In the figures, statistical significance from Dunnett's multiple comparisons test is indicated as $*p < 0.05$, $**p < 0.01$, and $***p < 0.001$ versus the LPS-treated control or untreated control, as appropriate. Statistical significance from Šídák's multiple comparisons test is indicated as $\#p < 0.05$, $\#\#p < 0.01$, and $\#\#\#p < 0.001$ between the indicated groups.

Results

IDCC 3201, IDCC 3501, and Their Mixture did not Induce Cytotoxicity in BV-2 Cells

The cytotoxicity of IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture was evaluated in BV-2 cells using the MTT assay (Fig. 1). BV-2 cells were treated

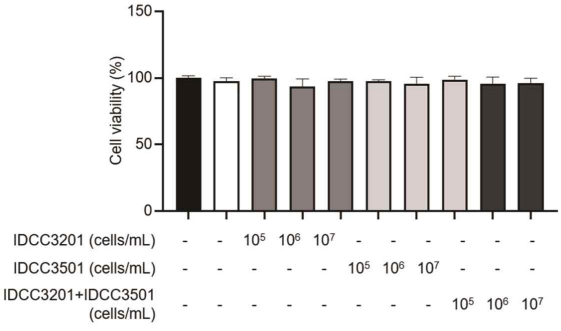

Fig. 1. Cytotoxicity of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture in BV-2 cells. BV-2 cells were treated with heat-killed IDCC 3201, IDCC 3501, or the IDCC 3201+IDCC 3501 mixture at 10^5–10^7 cells/mL for 24 h. Cell viability was measured using the MTT assay. Data are presented as the mean ± SD from three independent experiments. Statistical significance was determined by one-way ANOVA followed by Dunnett's multiple comparisons test versus the untreated control.

with IDCC 3201, IDCC 3501, or the IDCC 3201+IDCC 3501 mixture at concentrations of 10^5–10^7 cells/mL for 24 h. Cell viability was maintained at levels comparable to the untreated control in all treatment groups.

These results indicate that the test materials did not induce non-specific cytotoxicity in BV-2 cells under the present experimental conditions. Therefore, the concentration range of 10^5–10^7 cells/mL was used for subsequent experiments.

The IDCC 3201+IDCC 3501 Mixture suppressed LPS-Induced Inflammatory mRNA Expression

To evaluate the anti-inflammatory effects of IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture, the mRNA expression of inflammatory genes, including *Tnf*, *Il1b*, *Il6*, and *Ptgs2*, was measured in LPS-stimulated BV-2 cells (Fig. 2). LPS stimulation markedly increased the expression of these inflammatory genes, confirming that the inflammatory activation model was successfully established in BV-2 microglial cells.

Among the treatment groups, the IDCC 3201+IDCC 3501 mixture showed the most consistent suppressive pattern across the four inflammatory genes. At 10^7 cells/mL, the mixture significantly reduced the mRNA expression of *Tnf*, *Il1b*, *Il6*, and *Ptgs2* by 43.3% (p = 0.0002), 45.4% (p = 0.0001), 47.2% (p = 0.0004), and 35.0% (p = 0.0377), respectively, compared with the LPS control.

The single-strain treatments showed relatively selective

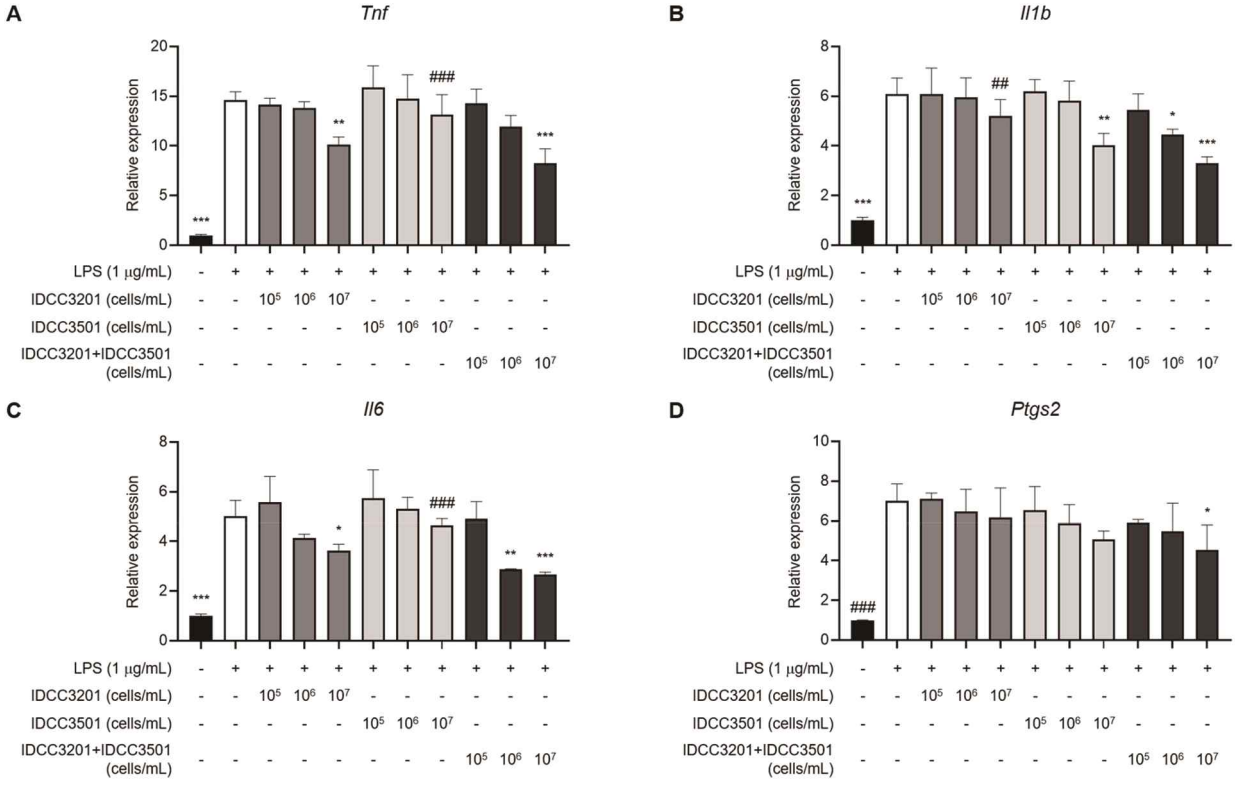

Fig. 2. Effects of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture on inflammatory gene expression in LPS-stimulated BV-2 cells. BV-2 cells were pretreated with heat-killed bacterial samples at 10^5–10^7 cells/mL for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. The mRNA expression levels of (A) *Tnf*, (B) *Il1b*, (C) *Il6*, and (D) *Ptgs2* were measured by qRT-PCR. Data are presented as the mean ± SD from three independent experiments. Statistical significance versus the LPS-treated control was determined by one-way ANOVA followed by Dunnett's multiple comparisons test. Direct comparisons between the IDCC 3201+IDCC 3501 mixture and each single-strain treatment at 10^7 cells/mL were performed using Šídák's multiple comparisons test for selected pairwise comparisons. *p < 0.05, **p < 0.01, ***p < 0.001 versus the LPS-treated control; ###p < 0.001 between the indicated groups.

responses. IDCC 3201 at 10^7 cells/mL significantly reduced *Tnf* and *Il6* mRNA expression, whereas IDCC 3501 at 10^7 cells/mL significantly reduced *Il1b* mRNA expression. In contrast, the IDCC 3201+IDCC 3501 mixture significantly modulated all four inflammatory transcripts. At 10^7 cells/mL, direct comparisons using Šídák's multiple comparisons test identified significant differences between the IDCC 3201+IDCC 3501 mixture and the single-strain treatments for selected inflammatory transcripts, as indicated in Fig. 2. These results indicate that the mixture modulated multiple inflammatory transcripts, while the direct differences from the single-strain treatments were marker-dependent.

The IDCC 3201+IDCC 3501 Mixture restored LPS-Suppressed *Bdnf* and *Dlg4* mRNA Expression

The mRNA expression of the neurotrophic and synapse-related markers *Bdnf* and *Dlg4* was evaluated in LPS-stimulated BV-2 cells (Fig. 3). LPS stimulation reduced the expression of these markers, indicating that inflammatory activation was accompanied by decreased neurotrophic and synapse-related biomarker expression in BV-2 cells. *Dlg4* encodes postsynaptic density protein 95 (PSD-95) and was interpreted as a synapse-related biomarker in this study.

LPS treatment decreased *Dlg4* mRNA expression from 1.00 ± 0.06 in the non-LPS control to 0.64 ± 0.03 in the LPS control. *Bdnf* mRNA expression was also decreased to 0.71 ± 0.13 in the LPS control. These results indicate that LPS induced an inflammatory response while reducing BDNF/PSD-95-related marker expression in BV-2 cells.

Bacterial treatment partially restored these LPS-induced reductions. IDCC 3201 at 10^7 cells/mL significantly increased *Dlg4* mRNA expression by 53.9% relative to the LPS control (p = 0.0211), whereas the increase in *Bdnf* mRNA expression was not statistically significant. IDCC 3501 at 10^7 cells/mL increased *Dlg4* and *Bdnf* mRNA expression by 82.0% (p = 0.0003) and 46.8% (p = 0.0152), respectively.

The IDCC 3201+IDCC 3501 mixture showed the most consistent restoration pattern across both markers. The mixture increased *Dlg4* mRNA expression by 94.1% (p < 0.0001) and *Bdnf* mRNA expression by 56.4% (p = 0.0029) relative to the LPS control. These findings indicate that the IDCC 3201+IDCC 3501 mixture partially restored LPS-suppressed *Bdnf* and *Dlg4* mRNA expression. Direct

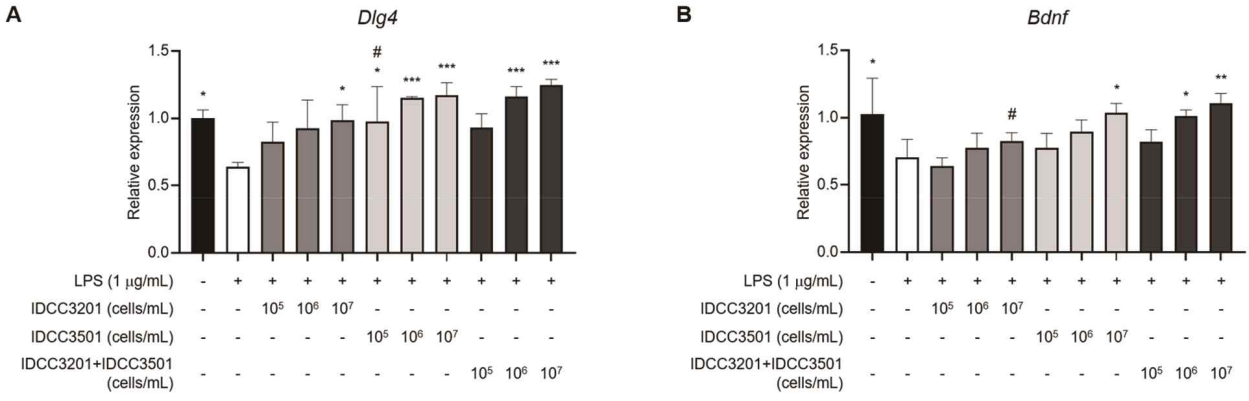

Fig. 3. Effects of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture on *Bdnf* and *Dlg4* mRNA expression in LPS-stimulated BV-2 cells. BV-2 cells were pretreated with heat-killed bacterial samples at 10^5–10^7 cells/mL for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. The mRNA expression levels of (A) *Dlg4* and (B) *Bdnf* were measured by qRT-PCR. Data are presented as the mean ± SD from three independent experiments. Statistical significance versus the LPS-treated control was determined by one-way ANOVA followed by Dunnett's multiple comparisons test. Direct comparisons between the IDCC 3201+IDCC 3501 mixture and each single-strain treatment at 10^7 cells/mL were performed using Šídák's multiple comparisons test for selected pairwise comparisons. $^*p$ < 0.05, $^{**}p$ < 0.01, $^{***}p$ < 0.001 versus the LPS-treated control; #p < 0.05 between the indicated groups.

comparisons at 10^7 cells/mL identified significant differences between the mixture and the single-strain treatments for selected markers, as indicated in Fig. 3.

The IDCC 3201+IDCC 3501 Mixture reduced LPS-induced Inflammatory Cytokine Secretion

To determine whether the suppression of inflammatory mRNA expression was reflected at the cytokine secretion level, the concentrations of TNF-α, IL-1β, and IL-6 in culture supernatants were measured by ELISA (Fig. 4). LPS stimulation markedly increased the secretion of all three inflammatory cytokines.

TNF-α secretion increased from 47.10 ± 40.96 pg/mL in the non-LPS control to 823.81 ± 13.65 pg/mL in the LPS control. IDCC 3201 and IDCC 3501 at 10^7 cells/mL reduced TNF-α secretion to 616.05 ± 57.88 pg/mL and 553.62 ± 25.58 pg/mL, respectively. The mixture reduced TNF-α secretion to 303.81 ± 32.60 pg/mL, corresponding to a 63.1% decrease relative to the LPS control ($p < 0.0001$). IL-1β secretion increased from 5.63 ± 1.20 pg/mL in the non-LPS control to 86.48 ± 1.62 pg/mL in the LPS control. IDCC 3201 and IDCC 3501 at 10^7 cells/mL reduced IL-1β secretion to 65.93 ± 2.10 pg/mL and 69.02 ± 3.28 pg/mL,

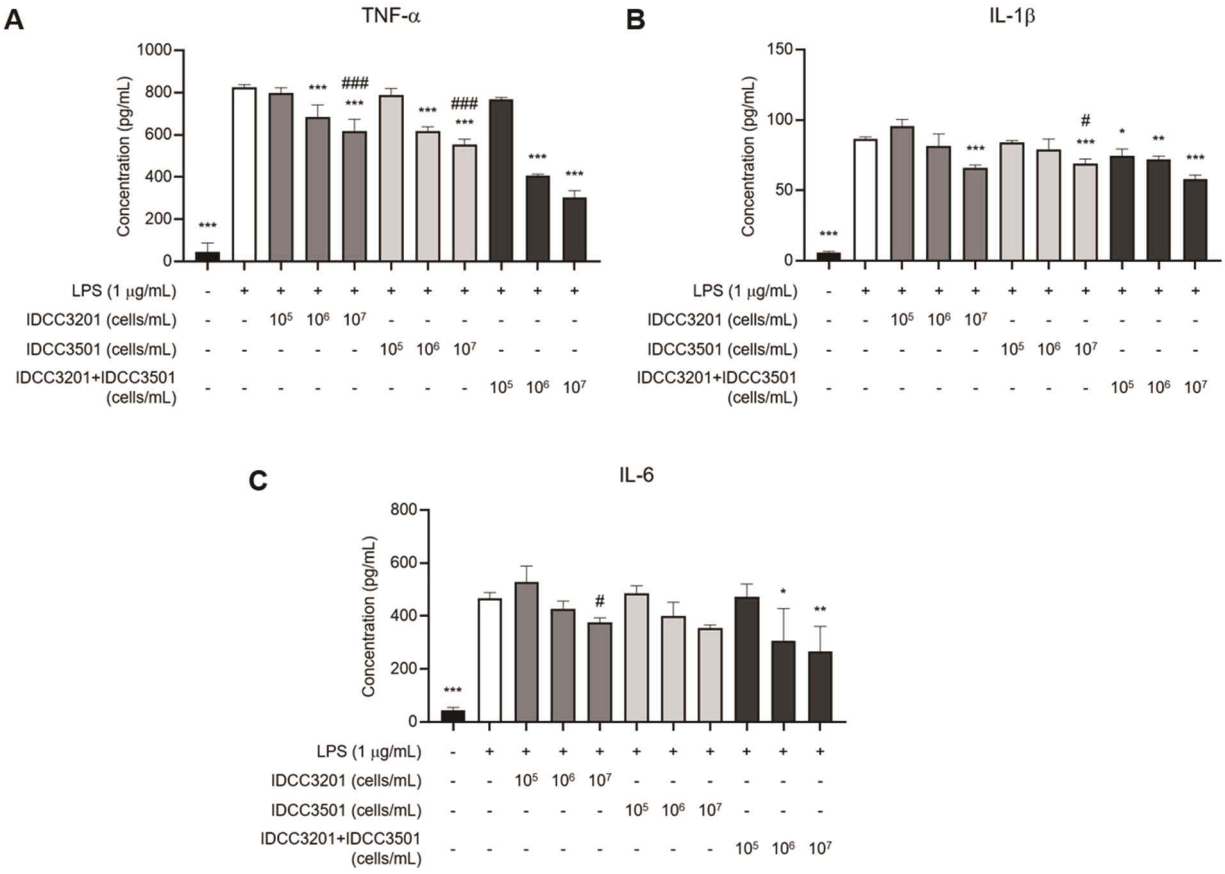

Fig. 4. Effects of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture on inflammatory cytokine secretion in LPS-stimulated BV-2 cells. BV-2 cells were pretreated with heat-killed bacterial samples at 10^5–10^7 cells/mL for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. The levels of secreted (A) TNF-α, (B) IL-1β, and (C) IL-6 were measured by ELISA. Data are presented as the mean ± SD from three independent experiments. Statistical significance versus the LPS-treated control was determined by one-way ANOVA followed by Dunnett's multiple comparisons test. Direct comparisons between the IDCC 3201+IDCC 3501 mixture and each single-strain treatment at 10^7 cells/mL were performed using Šídák's multiple comparisons test for selected pairwise comparisons. $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ versus the LPS-treated control; #$p < 0.05$, ###$p < 0.001$ between the indicated groups.

respectively. The mixture reduced IL-1β secretion to 57.86 ± 3.10 pg/mL, corresponding to a 33.1% decrease relative to the LPS control ($p < 0.0001$).

IL-6 secretion increased from 43.70 ± 12.23 pg/mL in the non-LPS control to 468.30 ± 20.86 pg/mL in the LPS control. Although IDCC 3201 and IDCC 3501 reduced the mean IL-6 level at 10^7 cells/mL, these reductions did not reach statistical significance. In contrast, the mixture significantly reduced IL-6 secretion to 265.67 ± 95.17 pg/mL, corresponding to a 43.3% decrease relative to the LPS control ($p = 0.0019$). At 10^7 cells/mL, direct comparisons showed significant differences between the mixture and the single-strain treatments for selected cytokines, as indicated in Fig. 4.

These results indicate that the IDCC 3201+IDCC 3501 mixture suppressed not only LPS-induced inflammatory mRNA expression but also extracellular inflammatory cytokine release.

The IDCC 3201+IDCC 3501 Mixture increased BDNF Secretion and Intracellular BDNF Protein Expression

To further evaluate the neurotrophic biomarker response to bacterial treatment, BDNF levels in culture supernatants were measured by ELISA, and intracellular BDNF protein expression was evaluated by western blotting. LPS stimulation reduced BDNF secretion from 590.20 ± 17.81 pg/mL in the non-LPS control to 421.70 ± 57.87 pg/mL in the LPS control, indicating that inflammatory activation decreased the BDNF-related neurotrophic biomarker response in BV-2 cells (Fig. 5A). Among the single-strain treatments, IDCC 3501 at 10^7 cells/mL increased secreted BDNF to 548.98 ± 43.53

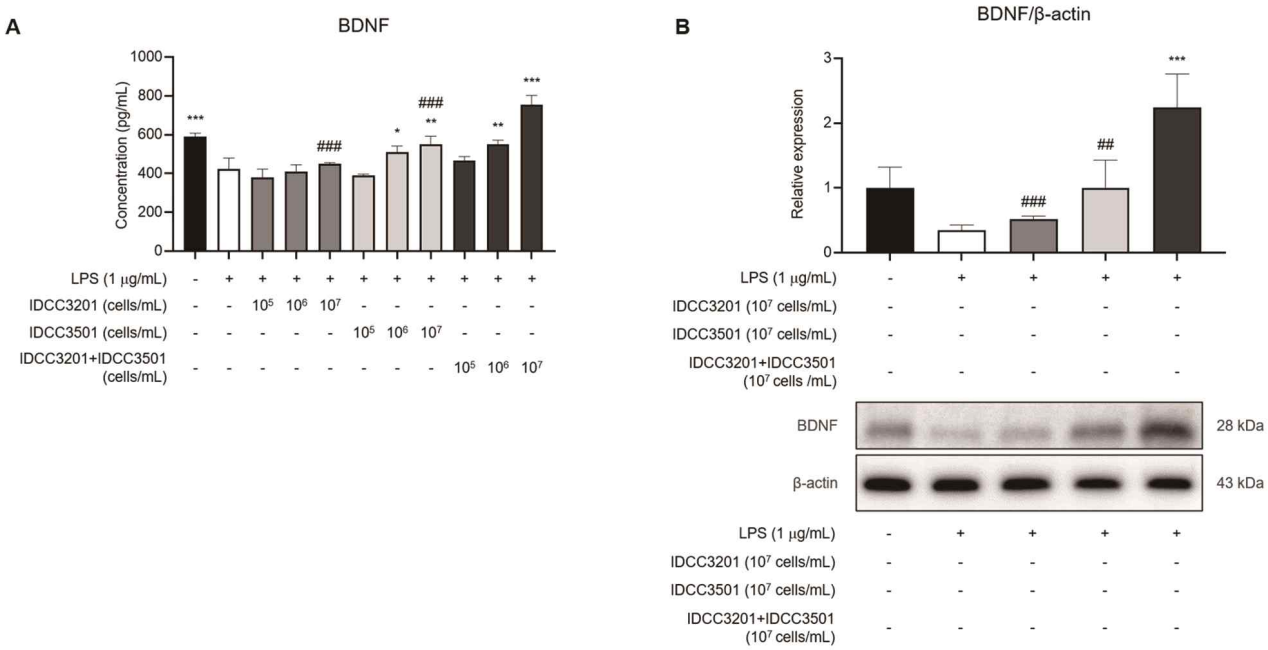

Fig. 5. Effects of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture on BDNF secretion and intracellular BDNF protein expression in LPS-stimulated BV-2 cells. BV-2 cells were pretreated with heat-killed bacterial samples for 1 h and then stimulated with LPS (1 μg/mL) for 24 h. (A) BDNF secretion was measured by ELISA following treatment with the bacterial samples at 10^5–10^7 cells/mL. (B) Intracellular BDNF protein expression was analyzed by western blotting following treatment with the bacterial samples at 10^7 cells/mL. Data are presented as the mean ± SD from three independent experiments. Statistical significance versus the LPS-treated control was determined by one-way ANOVA followed by Dunnett's multiple comparisons test. Direct comparisons between the IDCC 3201+IDCC 3501 mixture and each single-strain treatment at 10^7 cells/mL were performed using Šídák's multiple comparisons test for selected pairwise comparisons. $^{***}p < 0.001$ versus the LPS-treated control; ##$p < 0.01$, ###$p < 0.001$ between the indicated groups.

pg/mL, whereas IDCC 3201 did not significantly increase BDNF secretion. The IDCC 3201+IDCC 3501 mixture increased BDNF secretion to 753.60 ± 48.27 pg/mL, corresponding to a 78.7% increase relative to the LPS control ($p < 0.0001$).

Western blotting also supported the BDNF-restoring response of the mixture (Fig. 5B). LPS reduced intracellular BDNF protein expression from 1.00 ± 0.32 in the non-LPS control to 0.35 ± 0.08 in the LPS control. IDCC 3201 increased the mean BDNF protein expression level to 0.52 ± 0.05, and IDCC 3501 increased it to 1.00 ± 0.43; however, neither single-strain treatment reached statistical significance. In contrast, the mixture increased intracellular BDNF protein expression to 2.24 ± 0.52, corresponding to an approximately 6.4-fold increase relative to the LPS control ($p = 0.0002$).

Taken together, the IDCC 3201+IDCC 3501 mixture suppressed LPS-induced inflammatory cytokine responses while increasing both BDNF secretion and intracellular BDNF protein expression. Direct comparisons at 10^7 cells/mL further identified significant differences between the IDCC 3201+IDCC 3501 mixture and the single-strain treatments for BDNF-related measurements, as indicated in Fig. 5. Taken together, the mixture was associated with reduced inflammatory cytokine responses and increased BDNF-related biomarker responses in LPS-stimulated BV-2 cells.

Integrated Biomarker Profiling Shows coordinated Anti-Inflammatory and BDNF-Related Marker Responses by the IDCC 3201+IDCC 3501 Mixture

The integrated biomarker profile is presented in Fig. 6. Fig. 6A shows the inflammatory biomarker profile, including the inflammatory transcripts *Tnf*, *Il1b*, *Il6*, and *Ptgs2*, as well as the secreted cytokines TNF-α, IL-1β, and IL-6. Fig. 6B summarizes the BDNF- and *Dlg4*-related profile, including *Bdnf* mRNA expression, BDNF secretion, intracellular BDNF protein expression, and *Dlg4* mRNA expression.

Compared with the LPS control, the IDCC 3201+IDCC 3501 mixture showed an overall reduction in inflammatory markers. Specifically, the mixture reduced the mRNA expression of *Tnf*, *Il1b*, *Il6*, and *Ptgs2*, together with the secretion of TNF-α, IL-1β, and IL-6. In contrast, BDNF- and *Dlg4*-related markers showed an increasing pattern, indicating that the mixture restored LPS-suppressed BDNF-related biomarkers and *Dlg4* mRNA expression.

In the integrated profile, IDCC 3201 showed a relatively

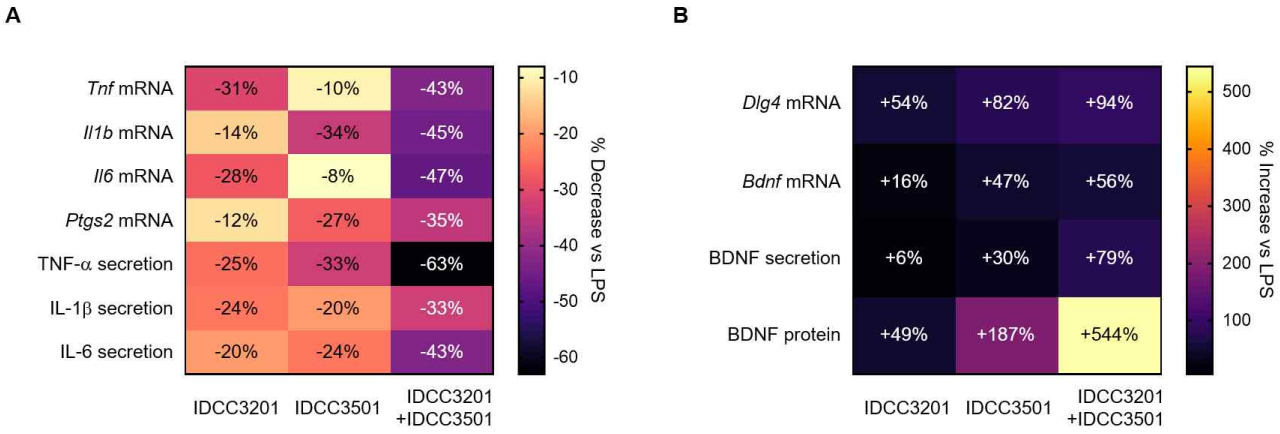

Fig. 6. Integrated biomarker profile of heat-killed IDCC 3201, IDCC 3501, and the IDCC 3201+IDCC 3501 mixture in LPS-stimulated BV-2 cells.

The integrated profiles summarize changes in (A) inflammatory biomarkers, including *Tnf*, *Il1b*, *Il6*, and *Ptgs2* mRNA expression and TNF-α, IL-1β, and IL-6 secretion, and (B) BDNF/*Dlg4*-related biomarkers, including *Dlg4* and *Bdnf* mRNA expression, BDNF secretion, and intracellular BDNF protein expression. Biomarker changes at 10^7 cells/mL were visualized relative to the LPS-treated control.

selective reduction in inflammatory markers, whereas IDCC 3501 showed a more evident increase in BDNF- and *Dlg4*-related markers. The IDCC 3201+IDCC 3501 mixture combined these partial response patterns into a broader biomarker profile characterized by reduced inflammatory markers and increased BDNF/*Dlg4*-related markers. These results suggest that the IDCC 3201+IDCC 3501 mixture may shift LPS-stimulated BV-2 cells toward a less inflammatory and more BDNF-related biomarker state.

Discussion

In this study, we evaluated whether heat-killed IDCC 3201, heat-killed IDCC 3501, and the IDCC 3201+IDCC 3501 mixture modulate inflammatory mediators, BDNF-related biomarkers, and *Dlg4* mRNA expression in LPS-stimulated BV-2 microglial cells. The major finding of this study was that the IDCC 3201+IDCC 3501 mixture reduced the expression of inflammation-related mRNAs and the secretion of inflammatory cytokines, while increasing BDNF secretion, intracellular BDNF protein expression, and *Dlg4* mRNA expression. These results suggest that the heat-killed IDCC 3201+IDCC 3501 mixture may attenuate the LPS-induced inflammatory biomarker profile and promote the recovery of BDNF-related neurotrophic marker responses.

LPS induces a pro-inflammatory response in microglia through TLR4-dependent signaling and increases the expression of inflammatory mediators such as TNF-α, IL-1β, IL-6, and COX-2 (Fang *et al.*, 2022; Li *et al.*, 2022; Skrzypczak-Wiercioch and Sałat, 2022). In the present study, LPS stimulation increased the mRNA expression of *Tnf*, *Il1b*, *Il6*, and *Ptgs2*, as well as the secretion of TNF-α, IL-1β, and IL-6. In contrast, the IDCC 3201+IDCC 3501 mixture broadly reduced these inflammation-related transcripts and cytokines. The fact that the mixture showed a consistent inhibitory pattern across a wider range of markers than either single strain suggests that its effect was not limited to a specific cytokine, but rather reflected broader modulation of the LPS-induced inflammatory biomarker profile.

The differences in responses between the single strains and the mixture are also noteworthy. IDCC 3201 showed relatively pronounced inhibitory effects on several inflammation-related markers, whereas IDCC 3501 showed relatively clearer effects on BDNF- and *Dlg4*-related markers. The IDCC 3201+IDCC 3501 mixture showed a broader biomarker-modulating profile across inflammatory and BDNF/Dlg4-related markers, suggesting that the two strains may contribute differently to the overall response pattern. Previous studies have discussed that multi-strain probiotic or postbiotic preparations may provide broader biological effects through combined or complementary strain-specific properties (Chapman *et al.*, 2011; Kwoji *et al.*, 2021). In the present study, additional direct comparisons at 10^7 cells/mL identified significant differences between the mixture and the single-strain treatments for selected biomarkers. However, these selected pairwise comparisons were limited to the highest tested concentration and do not establish the overall statistical superiority or synergistic activity of the mixture. Therefore, the mixture response should be interpreted as a broad and coordinated biomarker-modulating profile rather than definitive evidence of synergy.

The recovery of BDNF-related markers is an important feature of this study. BDNF is a representative neurotrophin associated with neuronal survival, synaptic plasticity, and memory-associated processes. In this study, LPS reduced *Bdnf* mRNA expression, BDNF secretion, and intracellular BDNF protein expression, whereas the IDCC 3201+IDCC 3501 mixture increased these markers at multiple levels. In particular, intracellular BDNF protein expression was significantly increased only in the mixture-treated group, supporting the possibility that the mixture more effectively restored BDNF-related biomarker responses. These findings suggest that, under inflammatory microglial activation, the mixture may be involved not only in the suppression of inflammatory responses but also in the recovery of the neurotrophic biomarker profile.

The changes in *Dlg4* mRNA expression further suggest that synapse-related biomarkers may be affected under

LPS-induced inflammatory conditions (Badshah *et al.*, 2016; Sheppard *et al.*, 2019). *Dlg4* encodes PSD-95, a postsynaptic scaffold protein involved in excitatory synapse organization and postsynaptic scaffolding (Levy *et al.*, 2022). In this study, LPS decreased *Dlg4* mRNA expression, whereas the IDCC 3201+IDCC 3501 mixture increased its expression. However, because PSD-95 protein expression and synaptic function were not directly evaluated, this result should be interpreted at the level of a synapse-related biomarker.

The samples used in this study were heat-killed bacterial cells prepared by treatment at 100°C for 30 min. Therefore, the observed effects are likely to have been mediated by bacterial surface structures, cell wall components, or heat-stable bacterial components rather than by bacterial proliferation or newly produced metabolites during treatment (Bron *et al.*, 2013; Piqué *et al.*, 2019). Unlike viable bacteria, heat-killed bacterial cells can reduce confounding factors associated with bacterial growth, acid production, and changes in the culture medium composition. Thus, they are useful for evaluating host-cell responses induced by bacterial cellular components in cell-based models (Geraldo *et al.*, 2020; Piqué *et al.*, 2019). In addition, postbiotic-type preparations may have advantages over viable bacteria in terms of stability, safety, and applicability, which is meaningful from the perspective of functional material development (Aguilar-Toalá *et al.*, 2018). In this context, the heat-killed IDCC 3201+IDCC 3501 mixture can be considered a postbiotic-type candidate material capable of modulating inflammatory and BDNF-related biomarker responses in BV-2 cells.

Several limitations should be considered when interpreting the findings of this study. First, BV-2 cells are an immortalized microglial cell line and do not fully represent primary microglia or the complexity of the *in vivo* brain microenvironment (Henn *et al.*, 2009; Stansley *et al.*, 2012). Second, although this study evaluated changes in inflammatory mediators and BDNF/*Dlg4*-related biomarkers, it did not directly assess neuronal protection, synaptic function, or cognitive function. Third, the upstream mechanisms underlying the observed changes remain unclear because related signaling pathways, such as NF-κB/MAPK and TrkB/CREB/Akt, were not directly analyzed. Therefore, the present findings should be interpreted as phenotypic modulation of inflammatory and BDNF/ *Dlg4*-related biomarkers rather than direct evidence of pathway-level regulation. Fourth, PSD-95 was evaluated only at the *Dlg4* mRNA level, not at the protein level, and the specific active components of the heat-killed bacterial cells responsible for the observed effects were not identified. Fifth, this study did not include a known anti-inflammatory or microglial-modulating positive control, which limits the ability to benchmark the magnitude of the observed effects against a reference treatment. Future studies should investigate inflammatory signaling, BDNF-related signaling, PSD-95 protein expression, and functional validation using neuron–microglia interaction models.

In conclusion, this study demonstrates that the IDCC 3201+IDCC 3501 mixture reduced inflammatory transcripts and cytokine secretion while increasing BDNF-related biomarkers and *Dlg4* mRNA expression in LPS-stimulated BV-2 microglial cells. The mixture showed a broad and coordinated biomarker-modulating pattern across inflammatory and BDNF-related markers, suggesting its potential as a postbiotic-type candidate material for further investigation. However, these findings provide preliminary evidence at the in vitro biomarker level, and further pathway-level studies and *in vivo* validation are required before linking these biomarker responses to actual neuroprotective or cognitive efficacy.

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