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Randomized Control Trials

# Gut microbiota indole-3-propionic acid mediates neuroprotective effect of probiotic consumption in healthy elderly: A randomized, double-blind, placebo-controlled, multicenter trial and *in vitro* study☆

Chong-Su Kim [a], Sunhee Jung [b], Geum-Sook Hwang [b], Dong-Mi Shin [a, c, *]

[a] Department of Food and Nutrition, College of Human Ecology, Seoul National University, Seoul 08826, Republic of Korea
[b] Integrated Metabolomics Research Group, Western Seoul Center, Korea Basic Science Institute, Seoul 03759, Republic of Korea
[c] Research Institute of Human Ecology, Seoul National University, Seoul 08826, Republic of Korea

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SUMMARY

*Background & aims:* The beneficial effects of probiotic consumption on age-related decline in cerebral function have been previously reported in the literature; however, the mechanistic link between gut and brain interactions has not yet been fully elucidated. Therefore, this study aimed to identify the role of gut microbiota-derived metabolites in gut-brain interactions via blood metabolomic profiling analysis in clinical trials and in vitro mechanistic studies.

*Methods:* A randomized, double-blind, placebo-controlled, multicenter clinical trial was conducted in 63 healthy elderly individuals (≥65 years of age). Participants were administered either placebo (placebo group, N = 31) or probiotic capsules (*Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI; probiotics group, N = 32) for 12 weeks. Global and targeted metabolomic profiling analyses of their blood samples were then performed using $^{1}$H nuclear magnetic resonance and liquid chromatography-mass spectrometry methods, both at baseline and at the end of the trial. Gut microbial analysis was conducted using the 16S ribosomal ribonucleic acid gene sequencing method. Subsequently, microglial BV2 cells were treated in vitro with indole-3-propionic acid (IPA) following lipopolysaccharide stimulation, and neuronal SH-SY5Y cells were treated with conditioned media from the BV2 cells. Finally, the levels of pro-inflammatory cytokines in BV2 cells and neurotrophins in SH-SY5Y cells were quantified using a real-time polymerase chain reaction or enzyme-linked immunosorbent assay.

*Results:* The metabolomic profiling analyses showed that probiotic consumption significantly altered the levels of metabolites involved in tryptophan metabolism ($P < 0.01$). Among these metabolites, gut microbiota-produced IPA had a 1.91-fold increase in the probiotics group ($P < 0.05$) and showed a significant relation to gut bacterial profiles ($P < 0.01$). Elevated IPA levels were also positively associated with the level of serum brain-derived neurotropic factor (BDNF) in the probiotics group ($r = 0.28$, $P < 0.05$), showing an inverse trend compared to the placebo group. In addition, in vitro treatment with IPA (5 μM) significantly reduced the concentration of proinflammatory TNF-α in activated microglia ($P < 0.05$), and neuronal cells cultured with conditioned media from IPA-treated microglia showed a significant increase in BDNF and nerve growth factor production ($P < 0.05$).

*Conclusions:* These results show that gut microbiota-produced IPA plays a role in protecting the microglia from inflammation, thus promoting neuronal function. Therefore, this suggests that IPA is a significant mediator linking the interaction between the gut and the brain in the elderly with probiotic supplementation.



## 1. Introduction

Biological functions such as cognition decline with age, but maintaining an optimal cognitive health is key to a sustained quality of life throughout the years. A healthy diet has the potential

☆ This study was registered with the Clinical Research Information Service (http://cris.nih.go.kr; registration ID: KCT0003929).

* Corresponding author. Department of Food and Nutrition, College of Human Ecology, Seoul National University, Seoul 08826, Republic of Korea.
*E-mail address:* shindm@snu.ac.kr (D.-M. Shin).

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of helping maintain brain health during aging [1,2], and the modification of dietary habits has been associated with lower frailty and enhanced cognitive ability in elderly populations [3]. Moreover, supplementation with anthocyanin-rich foods improves cognitive performance in older adults with mild-to-moderate dementia [4], and we previously demonstrated that dietary supplementation with probiotics improves age-related cognitive changes and mental stress in healthy older adults [5]. Therefore, the use of these compounds to modulate the gut microbiota and improve brain health has been at the center of research in the last decades.

The human gut contains diverse microbial communities [6] that can impact the host's physiology and biological functions [7]. Commensal microbes can interact with the brain via the gut-brain axis, thus affecting the host's emotions, cognition, and behaviors [8–12]. For example, antibiotic-induced gut dysbiosis in mice has been shown to contribute to cognitive impairment [13], while some studies have demonstrated that the administration of microbiota-accessible carbohydrates improves cognitive function in obese mice through the gut-brain axis [14]. Recent studies have demonstrated that circulating metabolites produced by intestinal bacteria constitute one of the mechanisms via which the gut microbiota and the brain interact [7,15,16].

Indole-3-propionic acid (IPA) is a metabolite produced exclusively from dietary tryptophan by the bacteria *Clostridium sporogenes* and *Peptostreptococcus anaerobius* [15,17]. It is absorbed by intestinal epithelial cells and circulates in the bloodstream of the host [18]. There is evidence of its anti-inflammatory properties in an animal model of steatohepatitis [18], and circulating IPA has also been shown to reduce ischemia-induced neuronal injuries by reducing DNA damage and lipid peroxidation [19]. However, its regulatory roles in brain functions have not been fully studied yet.

Brain is made up of different cell types, including neurons and glial cells (such as microglia, astrocytes, and oligodendrocytes). Neurons act as messengers that send electrical and chemical signals between neuronal cells. Glial cells supply nutrients to neurons, provide them with support and protection, and are involved in phagocytosis. The interactions between neurons and microglia are important in maintaining homeostasis in the brain, and the disruption of such interaction can result in neurological disorders and mental diseases [20]. In response to an immune stimulus, the microglia are activated by the M1 phenotype, which produces pro-inflammatory chemokines and cytokines such as tumor necrosis factor-α (TNF-α) and interleukin-1β (IL-1β) [21]. Signals from activated microglia lead to abnormal neuronal activity, neurogenesis, and synaptogenesis [22]. For example, activation of microglia by lipopolysaccharide (LPS), standardized inflammatory agents, results in neuronal cell death [23] and neurite dystrophy [24], which are implicated in age-related neurodegenerative disorders such as Alzheimer's disease and mild cognitive impairment [22,25]. Therefore, the regulation of microglia-mediated neuroinflammation promotes a healthy brain function due to its neuroprotective effects.

We previously demonstrated that 12 weeks of probiotic consumption had beneficial impacts on cerebral functions associated with changes in the gut microbiota through a randomized controlled trial in independently-living elderly individuals [5]. This previous finding prompted us to unravel the mechanistic actions underlying gut-brain interactions mediated by probiotics. We hypothesized that gut-derived metabolites induced by probiotic consumption could have a neuroprotective effect through the regulation of microglia-mediated inflammation in the brain. To test our hypothesis, we profiled host metabolome after their intervention with probiotics in a randomized controlled trial (RCT) involving healthy elderly individuals, and performed an in vitro study to identify the mechanisms underlying the neuroprotective effects of probiotic supplementation. This study contributes to the further exploration of such mechanisms, which are not yet fully understood, and could aid in the search for biomarkers related to the prevention and treatment of mental illnesses.

## 2. Materials & methods

### 2.1. Study design and participants

This study was a randomized, double-blind, placebo-controlled, multi-center clinical trial performed on 63 healthy elderly individuals (≥65 years old). Details about subject recruitment and eligibility have been described elsewhere [5]. Briefly, participants were recruited in Seoul and Seongnam City in the Republic of Korea and screened for eligibility based on overall health status (health history, health-related behavior, dietary history, daily habits, and cognitive and mental health status). The study was approved and monitored by the Institutional Review Boards of Seoul National University (IRB No. 1801/002–015) and Bundang Jesaeng Hospital (IRB No. IMCN18-01). This study was registered with the Clinical Research Information Service (http://cris.nih.go.kr; registration ID: KCT0003929).

### 2.2. Probiotic intervention

The details about probiotic intervention have been already been described in our previous study about the effects of probiotics [5]. Briefly, participants were randomly assigned to one of two groups based on a random sequence that was stratified by sex with a 1:1 allocation. The random sequence was generated by a staff member who was not involved in the study using GraphPad Prism (version 6.05; GraphPad Software, San Diego, CA, USA). Participants were administered either placebo (2 g of soybean oil/day) or probiotic capsules ($1 \times 10^9$ CFU of *Bifidobacterium bifidum* BGN4 and *Bifidobacterium longum* BORI in soybean oil/day) for 12 weeks. All procedures were double-blinded, as all participants, study coordinators, and researchers did not know which intervention participants were receiving until all data analyses was completed.

### 2.3. Blood metabolome profiling analysis

Twelve-hour fasting blood samples were collected and placed in serum-separating tubes at each visit (baseline and 12th week). The serum samples were aliquoted and immediately stored at −80 °C for further analysis. For the untargeted metabolomic profiling analysis by nuclear magnetic resonance (NMR), 200 μL of serum was mixed with 400 μL of 0.9% w/v sodium chloride in deuterium oxide and then transferred to 5-mm NMR tubes. One-dimensional $^1$H NMR spectra were acquired using a Bruker Avance III HD 800 MHz NMR spectrometer (Bruker BioSpin, Germany) with a Bruker 5 mm CPTCI Z-GRD probe. A water-suppressed CPMG spin-echo pulse sequence was used to attenuate broad signals from proteins and lipoproteins. A total of 128 transients were acquired with 64 k data points, a spectral width of 16,025.641 Hz, a relaxation delay of 4 s, and an acquisition time of 2.045 s. Free induction decays were weighed by an exponential function with a 0.3 Hz line-broadening factor prior to the application of the Fourier transform. All acquired $^1$H NMR spectra were processed using the software TopSpin 3.1 (Bruker BioSpin, Germany) and Chenomx NMR Suite version 7.1 (Chenomx, Canada). The synthetic electronic reference signal (ERETIC, electronic reference to access in vivo concentration) was used as a known reference signal peak to quantify the metabolites [26].

Subsequently, the targeted profiling of metabolites related to tryptophan metabolism was carried out: 30 μL of serum was mixed

with 60 μL of ice-cold acetonitrile (ACN) with 1% formic acid and then vortexed. The sample solution was incubated at 4 °C for 10 min and centrifuged at 4 °C and 13,000 rpm for 10 min. Fifty microliters of the supernatant was transferred to a new 1.5 mL tube and dried in a vacuum centrifuge. For the liquid chromatography-mass spectrometry (LC/MS) analysis, the dried samples were dissolved in 50 μL of 20% aqueous ACN (v/v).

The targeted profiling of bile acids was performed by mixing 20 μL of bile with 1000 μL ice-cold methanol, which was then vortexed. The sample solution was centrifuged at 4 °C and 13,000 rpm for 10 min. For the LC/MS analysis, samples were diluted with a methanol/water mixture (5:5, v/v). To quantify the levels of metabolites, an ultra-performance liquid chromatography/triple-quadrupole mass spectrometry (UPLC/TQ-MS) analysis was performed using an Acquity UPLC I-class system equipped with a Xevo TQ-XS mass spectrometer and an electrospray ionization (ESI) source (Waters Corporation, Milford, MA). The software TargetLynx Application Manager (version 4.2, Waters) was used for data acquisition and analysis.

For the targeted metabolic profiling of tryptophan metabolism, LC separation was carried out using a CORTECS UPLC T3 column (2.7 μm, 2.1 mm × 100 mm, Waters). The binary gradient system was made up of 0.1% formic acid in water (solvent A) and 0.1% formic acid in ACN (solvent B). The linear gradient used for the elution and equilibrium of the initial gradient for subsequent runs were as follows: 1% solvent B, 0–0.67 min; 13% solvent B, 1.33 min; 15% solvent B, 3.67 min; 95% solvent B, 4.33–5 min; and 1% solvent B, 5.1–7 min. Column temperature and flow rate were 45 °C and 0.4 mL/min, respectively.

Finally, for the targeted metabolic profiling of bile acids, LC separations were carried out on an ACQUITY UPLC HSS T3 column (50 × 2.1 mm, particle size 1.7 μm, Waters) with a binary gradient at a flow rate of 0.5 mL/min. The mobile phases were 5 mM ammonium acetate in water (solvent A) and an acetonitrile/methanol mixture (50:50, v/v) (solvent B). The linear gradients were as follows: 40% solvent B, 2 min; 45% solvent B, 2.5 min; 50% solvent B, 3.5 min; 55% solvent B, 4.6 min; 80% solvent B, 5.7 min; 85% solvent B, 5.9 min–6.5 min; and 35% solvent B, 2 min to equilibrate to the initial condition. Column temperature and flow rate were 60 °C and 0.6 mL/min, respectively. The auto-sampler temperature was maintained at 10 °C and the injection volume of the sample was 5 μL. The samples were analyzed in single reaction monitoring (SRM) mode. The SRM transitions were performed with the following operational parameters: capillary voltage, 2 kV; cone voltage, 20 V; source temperature, 150 °C; desolvation temperature, 650 °C; cone gas flow, 150 L/h; desolvation gas flow, 900 L/h; collision gas flow, 0.15 mL/min; and nebulizer gas flow, 7 bars.

Fold changes in metabolite concentration were calculated by dividing the concentration of the sample after the intervention by the concentration of the sample before the intervention (after/before) in each group.

### 2.4. Gut microbiota analysis

Participants were instructed to collect their fecal samples in a stool collection tube that contained DNA-stabilizing preservative reagent (Norgen Biotek, Thorold, ON, Canada) within the 48-h period before the date of visit. Stool samples were collected at each visit (baseline and 12th week) and aliquoted for immediate storage at −80 °C for further analysis. The detailed method for gut microbiota analysis has been described in a previous study [5]. Genomic DNA was isolated from fecal samples using the QIAamp fast DNA Stool Mini Kit (QIAGEN, Hilden, Germany) according to the manufacturer's instructions. Hypervariable regions (V3–V4) of the 16S ribosomal ribonucleic acid (rRNA) gene were amplified for sequencing analysis on an Illumina MiSeq sequencing system (Illumina, San Diego, CA, USA). The sequencing outputs were then processed for sequence quality control, phylogenetic tree building, diversity and taxonomic analysis, and differential abundance analysis at various taxonomic levels, using QIIME2 (version 2019.1).

### 2.5. Cell culture and treatment

Murine microglial BV2 cells were provided by Professor Chung-Hyun Cho (School of Medicine, Seoul National University). Human neuroblastoma SH-SY5Y cells were obtained from the Korean Cell Line Bank (Seoul, Republic of Korea). BV2 cells and SH-SY5Y cells were cultured in a Dulbecco's modified Eagle's medium (DMEM, Welgene, Daegu, Republic of Korea) and a minimum essential medium (MEM, Welgene, Daegu, Republic of Korea), respectively. They were also supplemented with 10% fetal bovine serum (FBS, Welgene, Daegu, Republic of Korea) and 1% antibiotics/antimycotics (Sigma, MO, USA) under a humidified atmosphere containing 95% air and 5% $CO_2$ at 37 °C. BV2 cells were seeded at $1 \times 10^5$ cells/well in 24-well culture plates and pretreated with IPA (Bioclone, Seoul, Republic of Korea) at various concentrations (0 μM, 2.5 μM, 5 μM, 10 μM) for 6 h, followed by lipopolysaccharide (50 ng/mL; Sigma, MO, USA) for 24 h at 37 °C. The conditioned media were collected from BV2 cells and centrifuged at 2000 rpm for 10 min to remove any cell debris. Subsequently, the conditioned media were diluted (1:4) in a neuronal culture medium, which was placed with SH-SY5Y cells seeded at $1 \times 10^5$ cells/well in 24-well culture plates. SH-SY5Y cells were then incubated for 24 h.

### 2.6. Cell viability assay

Cell viability was evaluated using a 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay kit (Abcam, MA, USA) following the manufacturer's instructions. BV2 cells were seeded in 24-well plates at $1 \times 10^5$ cells/well for cell viability assays, and were pretreated with IPA (Bioclone, Seoul, Republic of Korea) at various concentrations (0 μM, 2.5 μM, 5 μM, and 10 μM) for 6 h at 37 °C. The medium was then replaced with an MTT solution diluted in the same volume of serum-free medium. After 3 h of incubation, the MTT solvent was added to each well and the absorbance was measured at 590 nm after 15 min.

### 2.7. Real-time polymerase chain reaction (RT-PCR) analysis

Cells were harvested and total RNA was extracted using an RNA isolation kit (RNAqueous-4PCR kit; Ambion Inc., TX, USA). Total RNA integrity and quantity were assessed using a NanoDrop 2000 spectrophotometer (Thermo Fisher Scientific, DE, USA). cDNA was synthesized by a two-step procedure using a MessageSensor RT kit (Ambion, TX, USA), and mRNA levels were quantified by the SYBR Green qPCR method (Applied Biosystems, CA, USA). The mRNA expression levels of IL-1β, TNF-α, brain-derived neurotropic factor (BDNF), and nerve growth factor (NGF) were calculated using the $\Delta\Delta C_T$ method. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were used as housekeeping genes for the normalization of mRNA expression. The primer sequences are listed in Table 1.

### 2.8. Enzyme-linked immunosorbent assay (ELISA)

The supernatant from the cell culture was collected and centrifuged at 1500 rpm for 10 min at 4 °C. The concentrations of IL-1β and TNF-α (DY401-05 and DY410-05, respectively R&D Systems, Minneapolis, MN, USA) in the supernatant were measured using an ELISA kit following the manufacturer's instructions.

**Table 1**
Primer sequence for RT-PCR.

| Gene | Forward primer sequence (5′-3′) | Reverse primer sequence (5′-3′) |
|---|---|---|
| IL-1β | CTGTGACTCATGGGATGATGATG | CGGAGCCTGTAGTGCAGTTG |
| TNF-α | CCCTCACACTCAGATCATCTTCT | GCTACGACGTGGGCTACAG |
| BDNF | TGCAGGGGCATAGACAAAAGG | CTTATGAATCGCCAGCCAATTCTC |
| NGF | CAACAGGACTCACAGGAGCA | ACCTCTCCCAACACCATCAC |
| β-actin | GGGAAATCGTGCGTGACATTAAG | TGTGTTGGCGTACAGGTCTTTG |
| GAPDH | AGGTCGGTGTGAACGGATTTG | TGTAGACCATGTAGTTGAGGTCA |

### 2.9. Statistical analysis

Data from participants who completed the study was used for statistical analysis as previously described [5]. The study variables' normality assumption and homogeneity of variance were tested using a Kolmogorov–Smirnov test. An unpaired t-test or Mann–Whitney U test was performed for between-group analysis, and a paired t-test or the Wilcoxon signed-rank test was used for within-group analysis. Pearson's correlation analysis was executed to assess associations between variables, and the correction for multiple testing was performed based on the false discovery rate (FDR), or Bonferroni correction. Metabolites with a fold change $\geq 1.5$ or $\leq -1.5$ were considered statistically significant. In vitro data analyses were carried out using ANOVA, followed by Dunnett's or Tukey's test for multiple comparisons. *P*-value <0.05 and FDR <0.05 were considered statistically significant in all statistical analyses. All statistical analyses were performed using R (version 1.3.1093, PBC, Boston, MA, USA), SPSS (version 25.0; SPSS Inc., Chicago, IL, USA), or GraphPad Prism (version 9.0.0; GraphPad Software, San Diego, CA, USA).

## 3. Results

### 3.1. Changes in host metabolome by probiotic intervention in healthy elderly

A total of 53 participants (with an average age of 72 and 71.11 years in the Placebo and Probiotics groups, respectively) completed the study (Placebo group, N = 26; Probiotics group, N = 27), and 24 and 26 serum samples from the placebo and probiotics group, respectively, were used for metabolomic profiling (Supplementary Fig. 1). First, we performed an untargeted metabolomic profiling analysis to provide a global snapshot of the metabolic changes induced by probiotic intervention. The global profiling of serum metabolites helped identify 12 differential metabolites, including those involved in aromatic amino acid (tyrosine, phenylalanine), and glycine and serine metabolism (serine, glycine, betaine, and methionine) (Fig. 1A). To understand the metabolic pathways regulated by these differential metabolites, we conducted a pathway enrichment analysis with a set of these metabolites. Using the KEGG pathway database, we found that the top five pathways, including tyrosine and tryptophan biosynthesis, as well as D-glutamine and D-glutamate metabolism, were enriched in the group that was administered probiotics (Fig. 1B). The pathway related to tryptophan metabolism, mediated by the gut microbiota, showed the most significant changes after the intervention with probiotics. Therefore, we sought to identify potential key metabolites in tryptophan metabolism using a targeted metabolic profiling analysis.

Notably, we found that the gut microbiota-produced metabolite IPA, involved in tryptophan metabolism, was greatly increased in the group that was given probiotics (fold change = 1.91, $P < 0.05$, Fig. 1C). We also discovered that metabolites in bile acid metabolism, which are affected by the gut microbiota, significantly shifted in the probiotics group. For instance, chenodeoxycholic acid (CDCA), a primary bile acid, significantly increased in the group that received probiotics (fold change = 2.18, $P < 0.05$, Supplementary Fig. 2). Additionally, secondary bile acids metabolized by the microbiota showed a significant increase in the probiotics group: deoxycholic acid (DCA; fold change = 1.51), ursodeoxycholic acid (UDCA; fold change = 1.51), and taurodeoxycholic acid (TUCDA; fold change = 1.91) (all $P < 0.05$, Supplementary Fig. 2).

### 3.2. Elevated levels of serum IPA after probiotic intervention are associated with gut bacterial profiles and serum brain-derived neurotropic factor in healthy elderly

To examine the link between the changes in metabolites and gut bacterial profiles, we performed a correlation analysis between both. When assessing the correlation between metabolites in bile acid metabolism and gut bacterial profiles, there were no significant associations between them (data not shown). However, among the metabolites involved in tryptophan metabolism, only gut microbiota-produced IPA showed a significant correlation with the gut bacterial profiles. At phylum level, changes in the relative abundances of Synergistetes and Tenericutes were positively associated with elevated levels of IPA ($P < 0.05$, Table 2). In addition, we identified the top 20 genera that were significantly associated with IPA ($P < 0.05$, Table 2), which revealed that global changes in gut bacterial profiles at genus level were significantly associated with metabolic changes. We further investigated the link between metabolic features and cognitive responses by conducting a correlation analysis between both. The heatmap in Fig. 2 shows the correlation coefficient between the change in the concentration of serum BDNF, which is a well-known marker of cognitive function, and metabolites. Notably, from the metabolites in the tryptophan pathway, elevated IPA levels were positively associated with changes in BDNF levels after probiotic intervention (r = 0.28, $P < 0.05$), which is the opposite trend to that observed in the placebo group. Collectively, we identified that IPA metabolized by gut microbiota might be a target molecule in the gut-brain axis, which is mainly affected by probiotic intervention.

### 3.3. Gut microbiota-produced IPA has anti-inflammatory effects on microglial cells

To determine whether IPA is a key mediator of gut-brain interactions, we investigated the neuroprotective effects of IPA in microglial cells, which are responsible for local responses to injuries, such as brain inflammation, as well as the maintenance of brain tissues [25]. First, we tested the cytotoxicity of IPA using a MTT assay, which showed that IPA was not significantly toxic (at 1 μM, 2.5 μM, 5 μM) in BV2 cells treated with IPA compared to the no-treatment group (cell viability >90%, all $P > 0.05$, Fig. 3A). However, BV2 cell viability decreased significantly, compared to the no-treatment group (69.35% vs. 100%, $P < 0.05$, Fig. 3A), at a concentration of 10 μM.

Next, we assessed mRNA and protein levels of proinflammatory cytokines (IL-1β and TNF-α) in cells pretreated with IPA at different concentrations (0 μM, 2.5 μM, 5 μM, and 10 μM) for 6 h, followed by LPS (50 ng/mL) for 24 h mRNA expression of IL-1β showed dose-dependent reductions in IPA-treated BV2 cells compared to the LPS-alone treatment group (−5.81% at 1 μM, −12.93% at 2.5 μM, −11.92% at 5 μM, −29.23% at 10 μM; all $P > 0.05$, Fig. 3B), but mRNA expression of TNF-α did not show significant changes (Fig. 3C). At protein level, the concentration of IL-1β slightly decreased at a concentration of 2.5 μM and 5 μM of IPA compared to the LPS-alone treatment group (−5.28% at 2.5 μM, −1.21% at 5 μM, all $P > 0.05$, Fig. 3C). Moreover, the concentration of TNF-α showed

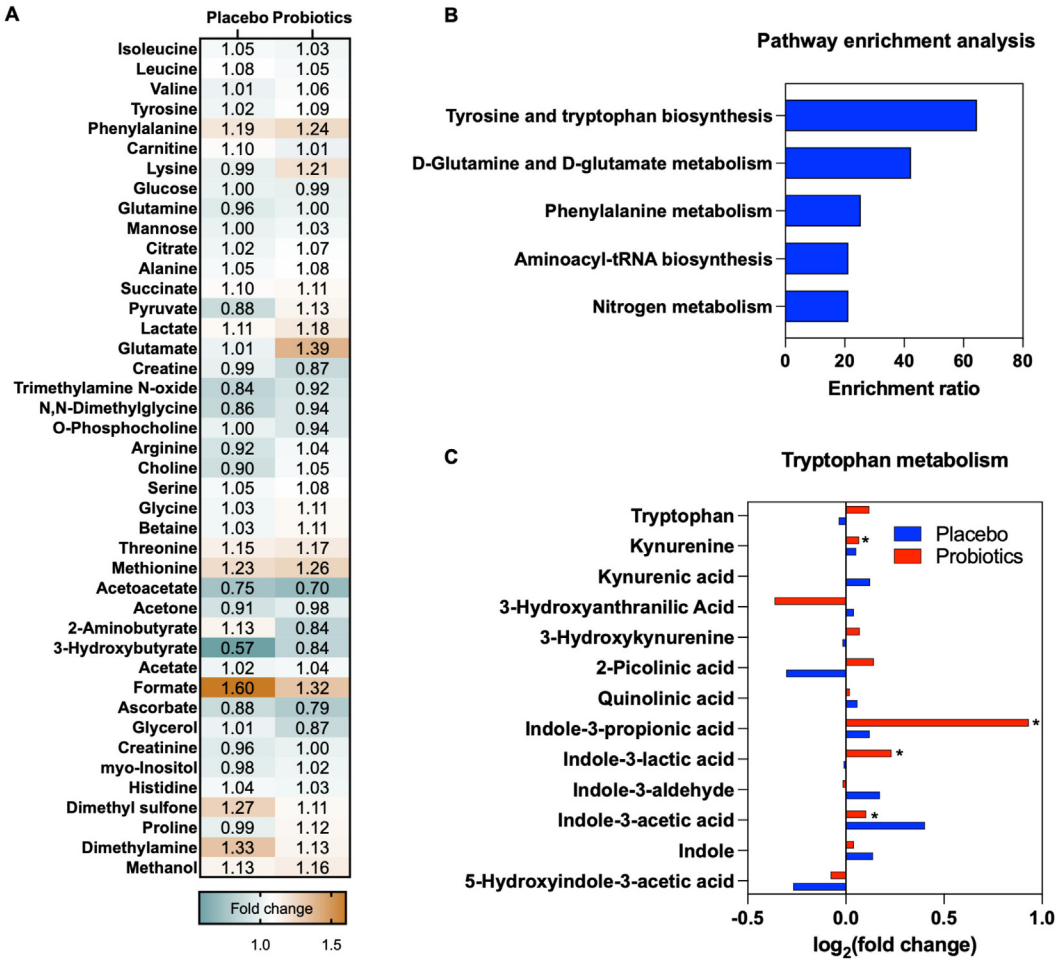


**Fig. 1. Changes in host metabolome through probiotic intervention in healthy elderly.** (A) The global profiling of serum samples was determined by $^{1}$H NMR. This heatmap shows fold changes in metabolite concentration, which is the ratio between the concentration of the sample before the intervention and of the sample after the intervention (after/before) in each group. Metabolites that were significantly altered in the group that was administered probiotics are marked in red. (B) The top 5 enriched pathways from the set of significantly altered metabolites were selected based on the enrichment ratio, which was calculated by observed/expected hits. (C) The targeted profiling of metabolites in tryptophan metabolism was determined by LC/MS. The axis shows $\log_2$ (fold change) of metabolite concentration. *$P < 0.05$ based on the Wilcoxon signed-rank test.

significant reductions in IPA-treated BV2 cells compared to the LPS-alone treatment group in a dose-dependent manner (−7.66% at 1 μM, −9.44% at 2.5 μM, −10.55% at 5 μM, −17.34% at 10 μM; all $P < 0.05$, Fig. 3D). Thus, these results indicate that IPA exhibits anti-inflammatory effects by inhibiting the production of TNF-α in microglial cells.

### 3.4. Gut microbiota-produced IPA has neuroprotective effects via the regulation of microglia–neuron interactions

Next, we tested our hypothesis about how the anti-inflammatory effect of IPA might have neuroprotective effects in neurons. Based on the results of the cytotoxicity assay and the anti-inflammatory effects measured at various concentrations of IPA, a conditioned medium was prepared with 5 μM of IPA. As shown in Fig. 4A, human neuroblastoma SH-SY5Y cells were incubated in this medium for 24 h, and we then measured mRNA levels in neurotrophins, including BDNF and NGF, to evaluate the neuroprotective effect of IPA in SH-SY5Y cells. BDNF and NGF at mRNA level increased by 46.12% and 37.94% in the IPA-treated conditioned medium compared to the LPS-alone treatment group, respectively ($P < 0.05$, Fig. 4B). Collectively, these results suggest that the enhancement of cerebral function after probiotic intervention found in the previous study was mediated by the neuroprotective effect of gut microbiota-produced IPA through the regulation of microglia–neuron interactions.

## 4. Discussion

### 4.1. Principal findings

Gut bacterial metabolites play pivotal roles as major mediators in gut microbiota and brain interactions. Our previous study found that probiotic intervention improves age-related decline in cerebral function by modulating gut microbiota; therefore, we tested whether the modulation of gut-brain interactions by probiotic consumption might benefit from changes in bacterial metabolites. The main finding of the present study is that probiotic intervention in healthy elderly individuals increases the level of gut microbial-derived IPA, which exerts neuroprotective effects through the regulation of inflammatory signals in microglial BV2 cells.

### 4.2. Roles of IPA in the regulation of gut microbiota

IPA is known to have positive effects on gut microbiota. For example, Zhao et al. reported that IPA reduces HFD-induced gut

**Table 2**
Correlation between the relative abundance of gut microbiota and the concentration of IPA.

| | Indole-3-propionic acid, uM | |
|---|---|---|
| **Phylum, relative abundance** | ***R*** | ***P*** |
| p_Actinobacteria | 0.0083 | 0.9642 |
| p_Bacteroidetes | −0.2084 | 0.2524 |
| p_Cyanobacteria | 0.0377 | 0.8376 |
| p_Fusobacteria | −0.0456 | 0.8045 |
| p_Lentisphaerae | −0.0577 | 0.7539 |
| p_Patescibacteria | −0.1075 | 0.5581 |
| p_Spirochaetes | 0.0209 | 0.9098 |
| p_Epsilonbacteraeota | −0.0066 | 0.9713 |
| p_Proteobacteria | −0.1036 | 0.5726 |
| p_Firmicutes | 0.2027 | 0.2660 |
| p_Synergistetes | 0.4670 | 0.0071 |
| p_Tenericutes | 0.5912 | 0.0004 |
| p_Verrucomicrobia | 0.2310 | 0.2034 |
| **Genus, relative abundance** | ***R*** | ***P*** |
| g_Solobacterium | −0.3698 | 0.0373 |
| g_Alloprevotella | −0.4786 | 0.0056 |
| g_Lactobacillus | −0.5078 | 0.0030 |
| g_Eggerthella | −0.3645 | 0.0402 |
| g_Lachnospiraceae UCG-004 | −0.3758 | 0.0340 |
| g_Ezakiella | −0.3619 | 0.0418 |
| g_Angelakisella | −0.4315 | 0.0137 |
| o_Mollicutes RF39 | 0.5401 | 0.0014 |
| g_Family XIII AD3011 group | 0.6391 | 0.0001 |
| g_Senegalimassilia | 0.5395 | 0.0014 |
| g_UBA1819 | 0.5223 | 0.0022 |
| g_Oscillospira | 0.5018 | 0.0034 |
| o_DTU014 | 0.5001 | 0.0036 |
| o_Izimaplasmatales | 0.4919 | 0.0042 |
| g_Rikenellaceae RC9 gut group | 0.4860 | 0.0048 |
| g_Ruminococcaceae NK4A214 group | 0.4767 | 0.0058 |
| g_Pyramidobacter | 0.4625 | 0.0077 |
| g_Actinomyces | 0.4156 | 0.0180 |
| g_Ruminococcaceae UCG-008 | 0.4015 | 0.0228 |
| g_[Eubacterium] eligens group | 0.3855 | 0.0293 |

The values in the table are Pearson's r value and p-value from correlation analysis on the delta values (wk12-wk0).

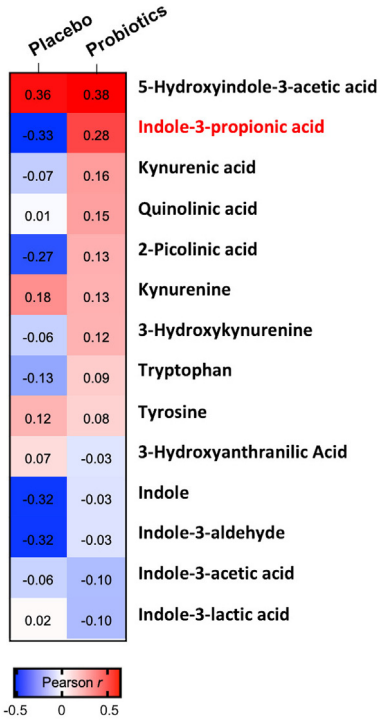


**Fig. 2. Association between elevated gut microbiota-produced IPA levels and brain-derived neurotropic factor in healthy elderly.** The heatmap represents the correlation coefficient from Pearson's correlation analysis, which measures the association between the changes in metabolites and the brain-derived neurotropic factor (BDNF) in each group. Metabolites that were significantly associated with BDNF levels are marked in red.

dysbiosis in rats, and that the beneficial effect of IPA on gut microbiota was mediated by a reduction in the production of endotoxins (lipopolysaccharide) and the endotoxin-derived activation of NF-$\kappa$ B signals [18]. Moreover, IPA has been shown to have antibiotic activity against a broad spectrum of mycobacteria, which suggests that it modulates gut microbial composition [27]. In line with these findings, this study showed that elevated levels of IPA after probiotic treatment are associated with changes in gut bacterial profiles. For example, we found that the populations of the phylum Tenericutes and the genus *Mollicutes* RF39, which have also been previously studied with regards to IPA levels [28], increased in abundance with the levels of IPA. In addition, abundance of *Eggerthella*, which has been demonstrated to increase in multiple sclerosis through the gut-brain axis [29], showed a negative association with the levels of IPA. Although IPA-producing strains were not detected due to the lack of resolution of 16S rRNA data, we assume that these bacteria flourish in the intestine where IPA-producing bacteria thrive. This may be partially due to the increased level of IPA, which is closely associated with intestinal epithelial homeostasis [18], thus contributing to the overall improvement of the gut's ecosystem.

### 4.3. Roles of IPA in neuroinflammation

*Bifidobacterium* is one of the most widely investigated probiotics related to modulation of the gut-brain axis. Particularly, the specific strains of *Bifidobacterium* (*B. bifidum* BGN4 and *B. longum* BORI) used in this study have been shown to have immunomodulatory effects in immune cells by an in vitro study and to suppress allergic responses in infants with eczema and adults with irritable bowel syndrome [34,35]. These results suggest that BGN4 and BORI have modulatory roles in the inflammatory pathway that is linked to the gut-brain axis; this prompted us to study their potential and underlying mechanisms for the regulation of the gut-brain axis.

Tryptophan metabolism in the gut occurs through three main pathways: the serotonin (5-hydroxytryptamine) pathway, the kynurenine pathway, or conversion into indole and its derivatives [30]. Each pathway and bioactive molecule derived from tryptophan has unique implications for the host's physiology [30]. There have been studies reporting the antioxidative properties [19] and chemical chaperone activity [31] of IPA, an indole derivative. In addition, IPA seems to decrease with age, which suggests it plays a possible role in age-related diseases. As evidenced by our findings, older adults in this study showed one-tenth concentration of plasma IPA compared to healthy young adults [32] (0.09 μM *vs.* 0.92 μM). We also demonstrated that IPA levels in older adults increased with probiotic intervention (1.91-fold change), which was associated with improved brain function. Based on these observations, we further examined whether gut microbiota-produced IPA has implications for the gut-brain axis. IPA has recently received attention as it is a signaling molecule characterized by its neuromodulatory properties: there is evidence of its protective effects on amyloid-beta (A $\beta$) fibril formation, which damages and kills neurons during progressive pathogenic events in Alzheimer's disease [33]. Furthermore, an in vitro study showed that A $\beta$ fibril formation was reduced in the presence of IPA by approximately 50% [33]. Additionally, the expression of *Ccl2* (a pro-inflammatory cytokine) from astrocytes (the most abundant cell type involved in neuronal transmission and immune response in the brain) was reduced by

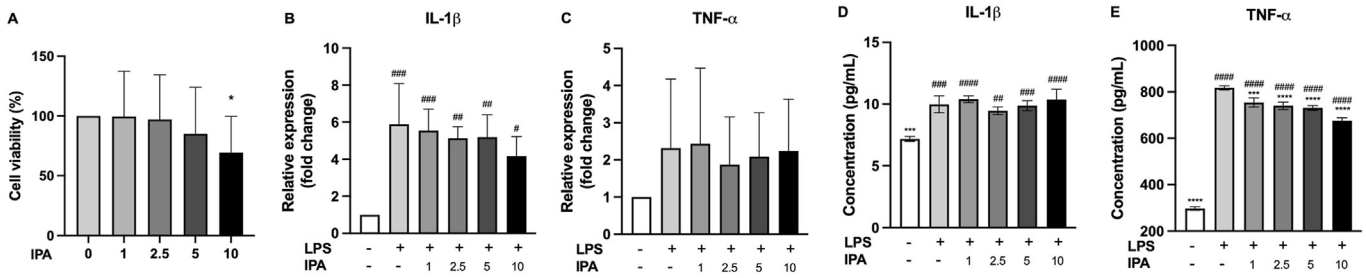


**Fig. 3. Gut microbiota-produced IPA's anti-inflammatory effects on microglial cells**. (A) The cytotoxicity of IPA (0 μM, 1 μM, 2.5 μM, 5 μM, and 10 μM) was measured in BV2 cells. $^{*}P < 0.05$ (versus the no-treatment group) based on ANOVA followed by Dunnett's multiple comparison analysis. (B–C) The relative expression levels of pro-inflammatory cytokines (IL-1β and TNF-α) were assessed in BV2 cells and compared to the no-treatment group. (D–E) Concentrations of proinflammatory cytokines (IL-1β and TNF-α) were measured in BV2 cells. $^{\#}P < 0.05$ (versus the no-treatment group), and $^{*}P < 0.05$ (versus the LPS-alone treatment group) based on ANOVA followed by Tukey's multiple comparison analysis. Data are expressed as the mean ± standard error. Abbreviations: IPA, indole-3-propionic acid; IL-1β, interleukin-1β; LPS, lipopolysaccharide; TNF-α, tumor necrosis factor-α.

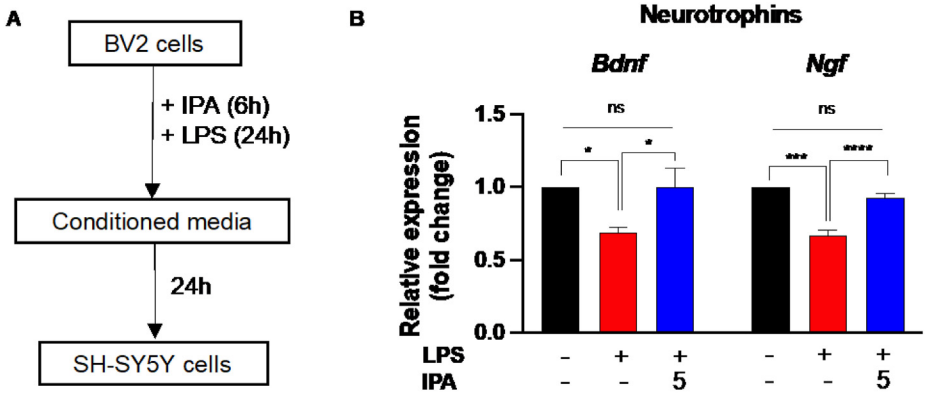


**Fig. 4. Gut microbiota-produced IPA's neuroprotective effects through the regulation of microglia–neuron interactions**. (A) Experimental scheme for the conditioned medium assay. (B) The relative expression levels of BDNF and NGF were assessed in SH-SY5Y cells and compared to the no-treatment group. $^{*}P < 0.05$, $^{***}P < 0.0005$, and $^{****}P < 0.0001$ based on ANOVA and followed by Tukey's multiple comparison analysis. NS indicates no statistically significant difference. Data are expressed as the mean ± standard error. Abbreviations: BDNF, brain-derived neurotropic factor; IPA, indole-3-propionic acid; LPS, lipopolysaccharide; NGF, nerve growth factor.

IPA administration in antibiotic-treated mice, suggesting that IPA influences astrocyte-mediated neuroinflammation [17]. Along with astrocytes, the microglia also plays an important role in the immune response of the central nervous system. The regulatory roles of IPA in microglia-mediated immune responses are not yet fully understood; however, we found that conditioned media from IPA-treated microglial cells had a beneficial effect on the productions of neuroprotective BDNF and NGF in neuronal cells by inhibiting the production of pro-inflammatory factors from microglia. These results suggest that the increased IPA levels after probiotic consumption helped avoid the functional loss of neurons through the regulation of microglia-mediated inflammation, thereby improving cognitive function and mental stress in healthy elderly individuals.

### 4.4. Mechanisms of neuroprotective effects

Neurotrophins, including BDNF and NGF, regulate the survival, development, function, and plasticity of neurons [36]. Both BDNF and NGF act in an autocrine and paracrine manner to protect neurons, suggesting that increased expression of BDNF and NGF in neuronal cells may protect themselves and surrounding neurons from inflammatory stimuli derived from activated microglia [37]. Accumulated evidence supports the involvement of BDNF in neuroinflammation and cognitive disorders [38]; therefore, attempts have been made to improve cognitive function by enhancing BDNF through diet and physical exercise as therapeutic targets for Alzheimer's disease and mild cognitive impairment [39]. After a 16-week physical exercise intervention, levels of peripheral BDNF have been shown to increase with a reduction in pro-inflammatory cytokines in older adults with mild cognitive impairment [40]. In addition, NGF has been demonstrated to exert neuroprotective effects by steering microglia toward an anti-inflammatory phenotype [41]. An in vitro study showed that NGF's anti-inflammatory effect was effective in reverting the pro-inflammatory status of microglia primed with amyloid-β peptide, which is a well-known in vitro model for Alzheimer's disease [41]. These results support our findings about how the anti-inflammatory effects of IPA act as neuroprotectors by increasing neurotrophins in neuronal cells.

This study also found that metabolites involved in bile acid metabolism were altered by probiotic intervention. Primary bile acid is secreted into the gut, where the microbiota converts it into secondary bile acids, including deoxycholic acid and ursodeoxycholic acid (UDCA). These secondary bile acids pass through the blood–brain barrier, which in turn influences the physiology of the brain [42]. UDCA, which was increased by probiotic intervention in this study, has also been shown to protect brain endothelial cells by reducing apoptosis in an in vitro study [43]. In addition, we showed that the levels of tauroursodeoxycholic acid (TUDCA), a taurine-conjugated form of UDCA metabolized by the gut microbiota, also increase with probiotic intervention. It has been demonstrated that TUDCA has a neuroprotective effect as it reduces neuroinflammation in microglial cells in mice [44]. Therefore, it seems

that there is a more complex biological mechanism regulating the role of probiotics in cerebral function, as shown by the multiple metabolic pathways involved in gut microbiota–brain interactions.

### 4.5. Strengths and limitations

Limitations of this study include the fact that it cannot provide functional insights into gut microbial profiles due to the lack of resolution of 16S rRNA data. Thus, future studies using shotgun sequencing analyses require further exploration. In addition, the findings of our in vitro study about IPA's neuroprotective effect require further evaluation in animal and clinical studies. To the best of our knowledge, and despite these limitations, this is the first study that provides evidence to support the claim that gut microbial-derived IPA elevated by probiotic intervention has neuroprotective effects through the regulation of microglial–neuronal interactions, thus acting as a link for communication in the gut-brain axis. These results also suggest that dietary intervention with IPA or targeting the gut microbiota with probiotic supplementation may help prevent age-related brain disorders.

## 5. Conclusion

Our study described one of the mechanisms by which probiotic supplementation can improve cognition in elderly adults through changes in gut microbiota. The results presented herein reveal that IPA mediates the interaction between the gut and brain, and metabolite has protective effect on the neuron against inflammation through the regulation of inflammatory signals in microglial cells when probiotics are incorporated into diets. Therefore, these findings can contribute to the prevention and treatment of mental illnesses as they can aid in the search for biomarkers that could be used to such ends.

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## Author contributions

Chong-Su Kim and Dong-Mi Shin: conceptualization; Chong-Su Kim, Sunhee Jung, Geum-Sook Hwang, and Dong-Mi Shin: methodology; Chong-Su Kim and Sunhee Jung: investigation; Chong-Su Kim and Sunhee Jung: data curation, formal analysis, software; Chong-Su Kim: visualization; Chong-Su Kim: writing–original draft preparation; Dong-Mi Shin: validation, supervision, writing–review and editing. Dong-Mi Shin was primarily responsible for the final content. All authors have read and approved the final manuscript.

## Data statement

The data are available upon request from the corresponding author.

## Conflicts of interest

The authors declare no conflicts of interest.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.clnu.2023.04.001.

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