

RESEARCH ARTICLE

The probiotic strain *Bacillus subtilis* CUI primes antimicrobial innate immune response and reduces low-grade inflammation: a clinical study

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Abstract

LifeinU™ *Bacillus subtilis* CUI (BSCUI) has been previously shown to be effective in stimulating mucosal immune responses and supporting resistance to common infectious disease episodes in the elderly. The current clinical study aimed at exploring potential pathways by which BSCUI could beneficially modulate the immune system and contribute to protection against infection in the general population. A total of 88 participants from three different age groups were supplemented with BSCUI (2×10^9 cfu/day) for 4 weeks. The effect of the intervention on mucosal immunity was assessed by faecal sIgA levels. In addition, a series of complementary immunoassays were selected, including immune phenotyping, gene expression, basal cytokine levels, cytokine levels in lipopolysaccharide (LPS)-stimulated whole blood and phagocytosis assay. Although no significant effect was observed on faecal sIgA levels after intervention, BSCUI showed a positive effect on a consistent set of markers of the peripheral innate immune system in adults and the elderly. Percentages of peripheral blood myeloid cells as well as the expression of the activation marker CD69 on monocytes were significantly increased after probiotic intervention. BSCUI supplementation resulted in significant enrichment of clusters of genes involved in response to type I interferon and phagocytosis pathway. Consistently, *ex vivo* stimulation of whole blood with LPS resulted in a statistically significant increase in pro-inflammatory cytokines (interleukin (IL)-1beta, IL-6, interferon-gamma, IL-12, tumour necrosis factor (TNF)-alpha, macrophage inflammatory protein (MIP)-1alpha, IL-8) and phagocytosis assays showed increased capacity of monocytes to engulf bacteria as well as higher phagosome maturation. BSCUI supplementation also had a positive effect on low-grade inflammation as significant reduction in basal levels of several serum cytokines (IL-10, TNF-alpha, MIP-1alpha, IL-8) were observed in the elderly subgroup. Overall, BSCUI primed immune cells for a better response to microbial challenges and reduced low-grade inflammation associated with aging.

Registered at ClinicalTrials.gov with the identifier NCT05403398

Keywords

probiotic – immunomodulation – immune defence – phagocytosis – inflammaging

1 Introduction

The human gastrointestinal tract hosts a highly diverse microbial ecosystem, collectively referred to as gut microbiota, that plays a crucial role in human health and disease (Flint *et al.*, 2012). It affects numerous aspects of host physiology from digestion and metabolism to neurobiology (Fan and Pedersen, 2021; Sharon *et al.*, 2016). The human gut microbiota strongly influences development and homeostasis of the immune system and this interplay has a major role in the control and response to infection (Belkaid and Harrison, 2017; Gensollen *et al.*, 2016; Pickard *et al.*, 2017). It contributes to the resistance and clearance of infection, both directly through mechanisms of colonisation resistance (e.g. competition for resources and space, production of antimicrobial substances or stimulation of mucus production) and indirectly through modulation of mucosal immunity (Perez-Lopez *et al.*, 2016). The role of the gut microbiota in immunity also extends to systemic compartments and distant organs such as the lung (Budden *et al.*, 2017; McAleer and Kolls, 2018). The symbiotic relationship between the gut microbiota and the host is a fragile equilibrium that can be affected by several factors (e.g. lifestyle habits, diet or antibiotics use). A divergence from the normal microbiota composition, generally referred to as dysbiosis, can substantially affect mucosal and systemic immunity (Levy *et al.*, 2017). Owing to the well documented impact of the gut microbiota on immune health, there is an interest in understanding the role of microbiota modulation in health and disease. Of the available solutions, probiotics, which are defined as 'Live microorganisms that, when administered in adequate amounts, confer a health benefit on the host' (Hill *et al.*, 2014), have been shown to modulate immune system function (Lehtoranta *et al.*, 2020). Probiotic effects on immune health are strain-dependent and different lactic acid bacteria and bifidobacteria have been described to induce very different maturation and cytokine production patterns in dendritic cells (DCs) and even to elicit opposing T-cell responses. Certain probiotics have been shown to induce the production of interleukin (IL)-12 by macrophages and DCs, promote interferon (IFN)- γ secretion, and favour inflammatory Th1 responses (Fujisawa *et al.*, 2004; Mohamadzadeh *et al.*, 2005; Pochard *et al.*, 2005), whereas other reports show that probiotic-induced IL-10 levels play an important role in limiting Th1-mediated proinflammatory responses (Hart *et al.*, 2004; Zeuthen *et al.*, 2006). There are few published reports addressing the health benefits of probi-

otic spore-formers, mostly belonging to the genus *Bacillus*. Bacteria of this genus can form spores that enable them to survive harsh environmental conditions. This ability gives them a significant advantage over the most common lactic acid probiotics (*Lactobacillus* and *Bifidobacterium*), because in their spore form, *Bacillus* probiotics can survive the acidic conditions of the stomach and spread safely throughout the nutrient-rich small intestinal tract. In addition, *Bacillus* probiotics are of growing interest because they are stable in commercial foods for long periods of time (Cutting, 2011). The efficacy of *Bacillus* spp. as probiotics has been studied in several *in vitro* and *in vivo* animal models, and a few have also been validated in human clinical trials. Available data from these studies have demonstrated the beneficial effects of various strains of *Bacillus* spp. on human health. For example, several researchers have recognised the preventive role of *Bacillus* probiotics in intestinal disorders (Lopetuso *et al.*, 2016; Zhang *et al.*, 2016). *Bacillus subtilis* species have long been safely consumed as dietary supplements and food ingredients in various parts of the world (Lefevre *et al.*, 2017). A specific strain of *B. subtilis*, CUI (CNCM I-2745) has been described as an effective probiotic in stimulating immune responses of healthy elderly subjects (Lefevre *et al.*, 2015). Probiotic supplementation with *B. subtilis* CUI was found to decrease the occurrence of infectious episodes in healthy free-living elderly subjects during a period of common infectious disease (i.e. the winter season in France). These effects were attributed to the ability of *B. subtilis* CUI to enhance peripheral as well as intestinal and respiratory mucosal immune responses, as indicated by the increased concentrations of faecal and salivary secretory IgA and serum interferon-gamma. Postulated mechanisms of actions of *B. subtilis* CUI include stimulation of the immune system, production of antimicrobials and modulation of the composition of gut microbiota (Dudonné *et al.*, 2015; Lefevre *et al.*, 2015; Urdaci *et al.*, 2004; Villéger *et al.*, 2014). However, the precise mechanisms by which *B. subtilis* CUI exert its positive probiotic effects remains relatively unexplored.

In the current study, we aimed to investigate whether *B. subtilis* CUI can positively modulate the mucosal and peripheral immune system in three target populations representing the general population. A series of complementary immunoassays were selected, including immune phenotyping, gene expression, cytokine levels in LPS-stimulated whole blood and phagocytosis assay.

2 Materials and methods

Study design

This exploratory study was designed as a single-arm study with repeated measures, involving three different populations, in which each subject served as its own control. This clinical trial was conducted between June 2022 and July 2022. The duration of the intervention was 4 weeks, with biomarker assessments at baseline, after 2 weeks, and after 4 weeks of intervention. Participants visited the study site (NIZO, Ede, the Netherlands) four times, once for information on the trial and subjects' eligibility assessment (screening visit), once for baseline measurements (visit 1, V1), after two weeks of probiotic supplementation (visit 2, V2) and at the end of the four-week intervention (visit 3, V3).

Participants, ethics and trial registration

This human intervention study was approved by the Medical Ethics Committee of Brabant, Tilburg, the Netherlands and registered as protocol NL79890.000.21. In addition, the clinical trial was registered in a clinical trials registration system at ClinicalTrials.gov with the identifier NCT05403398. The clinical trial was performed according to the ethical principles of the Declaration of Helsinki. The study included three separate subgroups, primarily defined by age: healthy children between 3 and 6 years; healthy adults between 30 and 49 years; healthy elderly between 65 and 79 years. Participants were excluded if they had an acute respiratory or gastrointestinal tract infection, any vaccination, antibiotic treatment, or regular intake of probiotics within 1 month prior to the start of the study. The use of anti-inflammatory or immunosuppressive medications was also an exclusion criterion. For complete inclusion and exclusion criteria lists for the three subgroups, see Supplementary Data S1. Signed written informed consent for the study was obtained from all adults and elderly subjects and from parents/caretakers of the children before protocol-specific procedures were carried out.

Investigational product

The probiotic product consists of *Bacillus subtilis* CUI (BSCUI) mixed with excipients (maltodextrin and magnesium stearate). Each probiotic capsule contains 2 billion spores of BSCUI (LESAFFRE, Marcq en Baroeul, France). BSCUI is a specific strain exclusive to Lesaffre and registered in the CNCM collection under the number I-2745. Capsules were taken orally, as a food supplement. The supplement was provided in a formulation

for clinical study use in a daily dosage (2×10^9 cfu/day). The capsules were supplied to the study subjects and parents/caretakers, respectively, during study visit 1, and participants were requested to consume 1 capsule daily in the morning (just before breakfast). For the children, caretakers were asked to empty the capsule into a small glass of a cold drink (e.g. water or milk) or yogurt and make sure that all of it was consumed. Compliance was determined through the assessment of returned packaging and interviews of the subjects at each visit. Subjects were instructed to maintain their habitual lifestyle and the consumption of any other probiotic was forbidden during the entire study period.

Study endpoints

The primary endpoint was the change after 4 weeks of probiotic supplementation in faecal secretory immunoglobulin A (sIgA) concentration. Secondary endpoints were the change from baseline to 4 weeks in circulating levels of cytokines, in *ex vivo* production of cytokines in a control and lipopolysaccharide (LPS) stimulated condition, and in *ex vivo* phagocytic capacity of monocytes and granulocytes. Additionally, the effect of 4 weeks supplementation with BSCUI was assessed on immune cell phenotypes and activation markers and on gene expression in peripheral blood mononuclear cells (PBMC). The effect of probiotic intervention was also assessed on gut microbiota composition and activity.

Biological samples collections

Faecal samples were collected at V1, V2 and V3 in the 3 subgroups. Faecal samples were collected within 24 h before each visit. The adult subjects and the children's caregivers were given all the materials and instructions to collect a faecal sample. Approximately 1–2 g of stool were collected at each visit. Of the stool samples, 100 mg was used for sIgA analysis and 250 mg for DNA isolation. Immediately after collection, the stool sample was frozen at -20°C in the subject's home freezer. For this purpose, an envelope and materials for transport were provided to prevent the sample from thawing during transport.

Fasting blood samples were collected at V1 and V3 in adults and elderly only (not performed in the healthy children subgroup for ethical reason).

Immune markers

Faecal sIgA

Faecal sIgA levels were measured using the IDK sIgA ELISA kit (Immundiagnostik, Bensheim, Germany). A faecal sample of 100 mg was diluted in 10 ml ELISA

buffer and further processed according to the manufacturer's protocol.

Serum cytokine quantification

The following cytokines were analysed IL-1 β , IL-6, IL-8, IL-10, IL-12, IFN- γ , macrophage inflammatory protein (MIP)-1 α , and tumour necrosis factor (TNF)- α . Cytokine concentration was assessed with Bio-Plex Pro™ Human Cytokine Assay (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Fluorescence was measured with Bio-Plex 200 Array reader and Bio-Plex Manager software 6.1.

BD Vacutainer CAT 2 ml PET tube with clot activator (BD Biosciences, Vianen, the Netherlands, BD 368271) were used for serum preparation. Samples were incubated for 30 minutes at room temperature and centrifuged for 10 min at 2,000 $\times g$ and room temperature. The serum was collected and stored at -80 °C for cytokine analysis.

Ex vivo cytokine quantification

Heparinized whole blood was collected and used within 1 h. Blood was diluted 1:5 in Roswell Park Memorial Institute (RPMI) 1640 Medium (ThermoFisher, Bleiswijk, the Netherlands) in a 96 wells plate and incubated for 20 h with and without 100 ng/ml LPS (LPS from *Escherichia coli* O111:B4, Merk Life Sciences NV, Amsterdam, the Netherlands) at 37 °C in a humidified atmosphere containing 5% CO₂. After incubation, samples were centrifuged at 2,000 $\times g$ for 10 min at room temperature. Four technical repeats (wells in a plate) were performed. The supernatant of two wells was mixed after which these samples were stored at -20 °C for further analysis as described above.

Ex vivo phagocytosis assay

Ex vivo phagocytosis in whole blood was studied using pHrodo™ Green *E. coli* BioParticles™ (ThermoFischer Scientific, Waltham, MA, USA) according to the manufacturer's instructions. Fluorescence was measured using FACSCanto II (BD, Franklin Lakes, NJ, USA). Samples were analysed using FlowJo™ Version 10 (FlowJo LLC, Ashland, OR USA).

Immune cell phenotyping

This analysis was performed at Wageningen University, Cell Biology and Immunology group (the Netherlands). Blood samples were collected in BD Vacutainer CPT tubes. After blood withdrawal, tubes were stored at room temperature and transported to Wageningen University. Tubes were centrifuged for 25 min at 1,800 $\times g$

and room temperature in a swinging bucket rotor (with breaks). The cell fraction was poured into 50 ml tubes. The cells were washed twice with phosphate buffered saline (PBS). The washed PBMCs were diluted in 1 ml PBS for cell counting. PBMCs were stained with fluorochrome-conjugated antibodies against extra-cellular markers for cell phenotyping (see antibodies in Table 1). Isolated PBMCs (1.0 $\times 10^6$ per well) were added to a 96-well NUNC plate (Thermo Fisher Scientific), and cells were washed twice by adding 100 μ l PBS followed by plate centrifugation for 4 min at 400 $\times g$ and room temperature. For live/dead staining, Zombie Aqua™ viability solution (Biolegend, US) was 500 $\times$ diluted in PBS, 100 μ l was added per well and incubated for 30 min at room temperature. The cells were washed with FACS buffer (200 μ l) containing PBS (1 $\times$) supplemented with BSA (0.5% v/v), ethylenediaminetetraacetic acid (EDTA, 2.5 mM), and sodium azide (NaN₃, 10% v/v) after which the plate was centrifugated for 4 min at 400 $\times g$ (room temperature), and fluid was discarded. The mixture of antibodies (Table 1) was diluted in FACS buffer and added to the cells. The plate was incubated for 30 min at 4 °C in the dark. Cells were washed three times by adding cold FACS buffer (200 μ l) and centrifuging for 4 minutes at 400 $\times g$ (4 °C). Supernatants were discarded, the cell pellet was resuspended in FACS buffer (300 μ l) and measured on CytoFLEX LX (Beckman Coulter, Brea, CA, USA). The generated flow cytometry data was analysed using FlowJo v10. Results are presented as relative abundance for the cell populations and mean fluorescent intensity (MFI) for the activation markers (CD69, NKG2D). The gating strategy for selecting different immune cell subsets is described in the supplementary data (Supplementary Figures S1 and S2).

PBMC gene expression assessment

Blood was collected in BD Vacutainer 10 ml PET heparin tubes. PBMCs were isolated using Leucosep® tubes (Greiner Bio-One, Kremsmünster, Austria), prefilled with separation medium according to the manufacturer's instructions. Heparinized blood (10 ml) was 1:2 diluted with Dulbecco's phosphate-buffered saline (DPBS) and poured into the Leucosep® tube. The sample was centrifugated for 10 min at 1000 $\times g$ (brakes switched off). The enriched cell fraction was poured out of the tube and washed twice with DPBS for 10 minutes at 250 $\times g$. Cells were counted using a TC20 Auto Cell Counter. After the final wash, the cells were suspended in RLT buffer (Qiagen RNease kit, Qiagen, Hilden, Germany) with 1% β -mercaptoethanol accord-

TABLE 1 Antibodies used for immune phenotyping

Panel	Marker	Fluorochrome	Company	Cat#
Adaptive	CD3	AF700	Biolegend	344822
Adaptive	CD4	BUV395	BD	563550
Adaptive	CD8	FITC	Biolegend	344704
Adaptive	CD14	PerCPCy5.5	Biolegend	325622
Adaptive	CD19	BV785	Biolegend	302240
Adaptive	CD25	BV421	Biolegend	302630
Adaptive	CD45	APC-Fire750	Biolegend	304062
Adaptive	CD56	BV605	Biolegend	318334
Adaptive	CD69	PE-Cy7	Biolegend	310912
Adaptive	CD127	PE/Dazzle594	Biolegend	351336
Adaptive/innate	HLA-DR	PerCPCy5.5	Biolegend	307630
Adaptive	NKG2D	PE	Biolegend	320806
Adaptive/innate	Human TruStain FcX		Biolegend	422302
Adaptive/innate	True-Stain Monocyte Blocker		Biolegend	426101
Innate	CD3	FITC	Biolegend	344804
Innate	CD11c	PE	Biolegend	337206
Innate	CD14	APC-Fire750	Biolegend	301854
Innate	CD16	BV785	Biolegend	302046
Innate	CD19	FITC	Biolegend	302206
Innate	CD56	FITC	Biolegend	318304
Innate	CD68	PE-Cy7	Biolegend	333816
Innate	CD69	BV605	Biolegend	310938
Innate	CD86	APC	Biolegend	305412
Innate	CD123	PE-Dazzle594	Biolegend	306034
Innate	CD206	BV421	Biolegend	321126

ing to the manufacturer's instruction. The samples were stored at -80°C until RNA isolation.

RNA isolation was performed with RNeasy Mini Kit (Qiagen, US) according to the manufacturer's instructions. After lysis and precipitation, the samples were loaded onto the RNeasy column and subjected to DNase treatment to remove DNA. RNA was eluted in RNase-free water and stored at -80°C in multiple aliquots for quality control and library preparation. RNA concentration was measured by the NanoDrop ND-1000 (Thermo Scientific). Further, 10% of the samples were analysed at the Agilent 2100 Bioanalyzer (Agilent Technologies, Waldbronn, Germany).

RNA samples were shipped on dry ice to Novogene UK (Milton, UK). Directional mRNA libraries were prepared by Novogene (Poly A enrichment) and sequenced on an Illumina NovaSeq 6000 PE150 platform (Illumina, San Diego, CA, USA) to obtain at least 6 Gb raw PE150 data per sample. Quality-controlled data files in FASTQ format were transferred to NIZO for bioinformatics analysis.

For RNAseq analysis, a total of 118 RNA samples were collected from two distinct populations, adults and the elderly. These samples were analysed using the nf-core/rnaseq pipeline version 3.12.0 (<https://nf-co.re/rnaseq/3.12.0>), which encompasses a series of steps. Initially, a preprocessing step was performed using tools such as FastQC, TrimGalore, BBsplit, and SortMeRNA to ensure quality control. Then, reads were aligned to the reference genome and gene expression levels were quantified using the STAR tool. Post-processing steps included count generation using featureCounts and Kalisto, followed by additional processing and a final quality control assessment using RSeqQC, DESeq2, and MultiQC. The resulting data were then subjected to a differential analysis using DESeq2 version 1.38.3, where genes with a log fold change greater than 1.5 or less than -1.5 and a P -value of 0.05 were considered differentially expressed. A Gene Set Enrichment Analysis (GSEA) was also conducted using the fgsea package in R version 4.1.0.

Faecal microbiota composition

The faecal DNA isolation method is based on mechanical disruption. An aliquot of 250 mg of faecal material was placed in 700 µl STAR buffer (Roche) with 0.5 g of 0.1 mm Zirconia beads. Lysis was performed with the FastPrep at 5.5 ms and room temperature for 3 × 1 min followed by heat treatment (95 °C for 15 min shaken at 100 rpm). Samples were centrifuged for 10 min at 4 °C and 21,000×g. Supernatants were subjected to one more round of mechanical disruption and heat treatment by adding another 300 µl of STAR buffer. The supernatant (250 µl) thereof was further purified with Maxwell (Promega, Madison, WI, USA) according to the manufacturer's protocol (Maxwell 16 Tissue LEV total RNA purification kit, cat#ASI220). DNA quantity was measured with Quant-iT™ dsDNA Assay Kit BR (ThermoFischer Scientific) and shipped on dry ice to Novogene for metagenomic sequencing.

DNA libraries were prepared by Novogene and sequenced on an Illumina NovaSeq 6000 PE150 platform to obtain at least 2 Gb raw PE150 data per sample. Quality-controlled data files in FASTQ format were transferred to NIZO for bioinformatics analysis.

Raw sequencing data was quality controlled using fastqc (v0.11.9). Reports for quality controlled reads were combined using multiQC (v1.12). Quality controlled reads were then subjected to the Humann pipeline (v3.6). Multivariate analyses consisted in Principal Coordinate Analysis (PCoA) on Bray Curtis beta-diversity. Statistical significance and the proportion of microbial composition variance explained by different effects and covariates was obtained using PERMANOVA. Statistics were performed with R (v4.2.3). Multivariate analysis was performed using the Maaslin2 (v1.8.0) and FactoMineR (v1.34) packages. Graphics were produced using ggplot2 (v3.4.0). Alpha and Beta-diversity were analysed using the vegan package (v2.6-4).

Safety

Adverse events were recorded during the study by investigators.

Statistical analysis

Sample size

The primary outcome of this study was faecal sIgA level. With a sample size of $n = 30$ (with 20% drop-out), it is possible to detect an increase of 54% in the levels of faecal sIgA with a power of 80%, in both the adult and elderly populations (Lefevre *et al.*, 2015). For children, a sample size of 21 subjects enables the detection of an

increase of 50% in the levels of faecal sIgA with a power of 80% (Béghin *et al.*, 2021).

Efficacy analyses

Analyses were performed on the per-protocol population (PP). PP population was defined per timepoint. Decisions about the allocation of subjects to the PP were taken during a data review meeting. The database was locked after the completion of the data review meeting and decisions were documented in the minutes of the data review meeting. All data were analysed with Linear Mixed Models (LMMs) with adjustment of analysis on age, gender and body mass index (BMI) at baseline. Model goodness-of-fit were assessed using the Akaike Information Criterion and model assumptions (linearity, homoscedasticity, linearity of residuals, non-collinearity of parameters) were checked. In case of non-compliance to the model assumptions, log transformations were performed. Hochberg adjustments were used to correct for multiple testing.

Statistical analyses were performed using SAS® software version 9.4 (TSIM7) (SAS Institute Inc., Cary, NC, USA) and R software version 4.2.2 (R Foundation).

3 Results

Study flowchart and baseline characteristics of the population

One hundred and eight (108) subjects were screened for study eligibility and a total of eighty-eight (88) participants were included in this study. The flowchart of subjects throughout the study is presented in Figure 1. A total of 28 children, 29 adults and 30 elderly completed the entire study. A total of 24 children, 24 adults and 27 elderly were included in per-protocol (PP) population at baseline. The baseline characteristics of the subjects are presented in Table 2. The main reasons for exclusion of subjects from the PP population were missed biological samples, occurrence of infectious diseases or use of medication that may have interfered with the outcome parameters of the study. Excellent mean compliance was observed (>99%).

BSCUI intake does not alter the composition and function of the gut microbiota in children, adults, and the elderly

Effects of oral treatment with BSCUI on the gut microbiota composition and functionality are shown in Figure 2 and Figure 3, respectively. As expected, the relative abundance of *B. subtilis* was significantly increased after

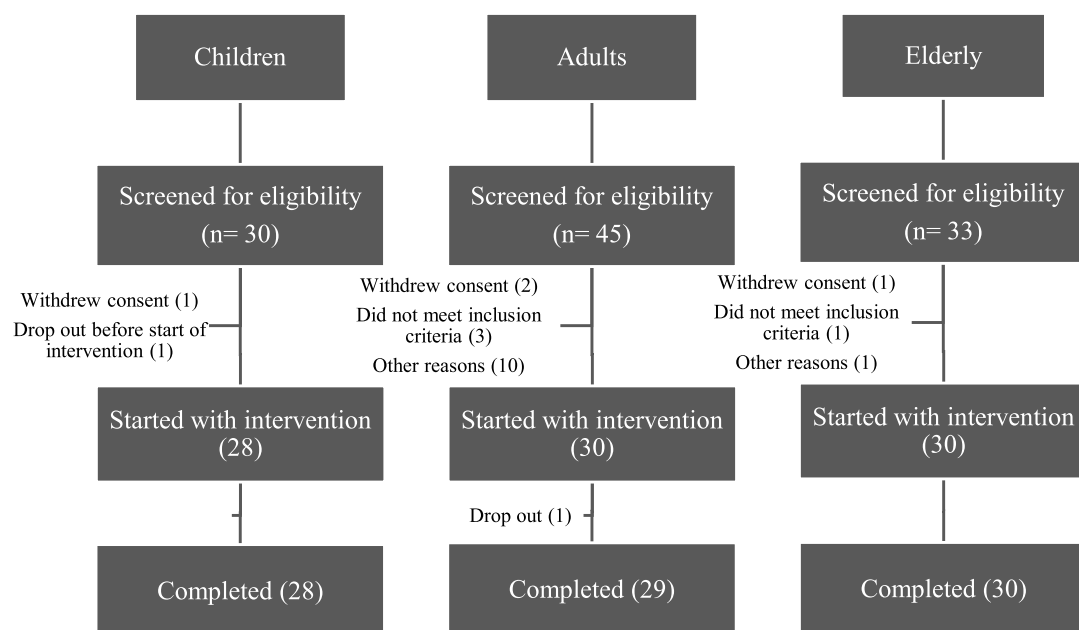


FIGURE 1 Study flow chart.

TABLE 2 Baseline characteristics of participants included in the per protocol group¹

	Children (n = 24)	Adults (n = 24)	Elderly (n = 27)
Age (years)	4.8 ± 1.2	40.6 ± 5.2	70.1 ± 3.4
Gender (female/male)	12/12	19/5	15/12
Height (cm)	112 ± 10	171 ± 9	172 ± 9
Weight (kg)	19.0 ± 3.1	67.3 ± 9.7	76.1 ± 9.3
BMI (kg/m ²)	15.1 ± 0.8	22.8 ± 1.8	25.5 ± 1.5

¹ Quantitative variables are shown as mean ± standard deviation. BMI = body mass index.

treatment. However, when correcting for false discovery rate then only adults showed a significant increase in this bacterium (Figure 2A). No significant differences were observed in microbiota composition (Figure 2B-G) nor functionality (Figure 3A-F) in any groups and any time point comparison.

Oral supplementation with BSCUI tends to increase faecal sIgA content in children, adults, and the elderly

The effect of oral supplementation with BSCUI on faecal sIgA levels is presented in Figure 4 and Table 3. There was a trend toward increased faecal sIgA production in children and the elderly 2 weeks after BSCUI ingestion, with increases from baseline of 24% and 29%, respectively. This trend was also observed 4 weeks after the start of the probiotic supplementation in the three groups studied, with increases of 24%, 36%, and 20% in children, adults, and the elderly group, respectively, as compared to the baseline. BSCUI ingestion

thus seemed to increase faecal sIgA but these trends did not reach statistical significance.

BSCUI supplementation reduces low-grade inflammation in the elderly

A chronic systemic inflammatory state occurs to varying degrees during aging (Chung *et al.*, 2019). Therefore, in the current study, the effect of BSCUI supplementation on low-grade inflammation was assessed only in the elderly group (Figure 5). Levels of IL-8, TNF-alpha, and MIP-1alpha significantly decreased by 21%, 22%, and 17%, respectively, after supplementation with BSCUI. IFN-gamma, IL-1beta, and IL-6 were not affected. A statistically significant reduction in IL-10 level was also observed after intervention with BSCUI. IL-12 was under detection threshold.

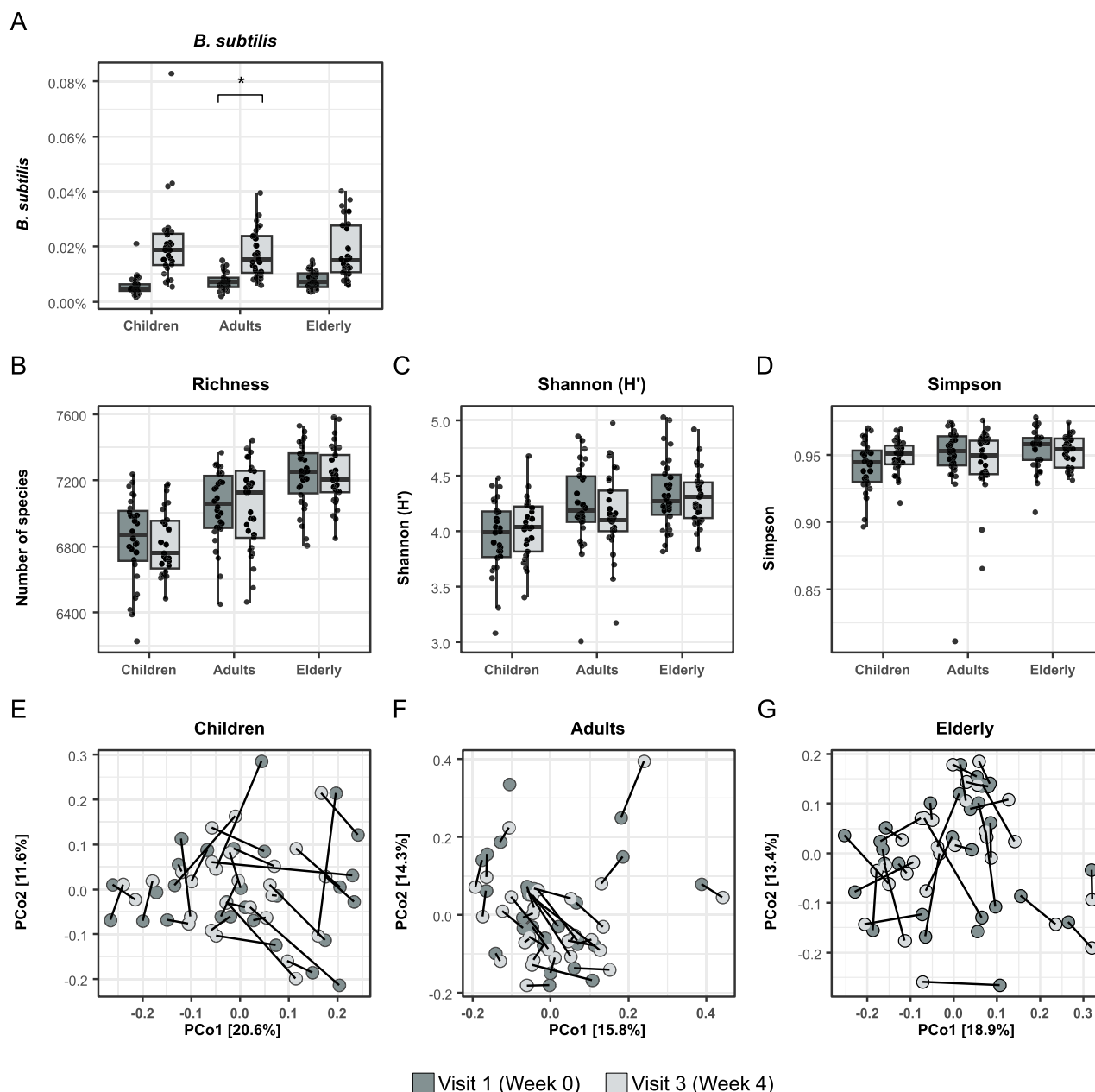


FIGURE 2 Effect of BSCU1 on microbiota composition. (A) *Bacillus subtilis* abundance in faecal samples before and after treatment, (B-D) Diversity indices and (E-G) PCoA analysis (*P < 0.05).

BSCU1 uptake modulates immune cell relative abundance in adults and the elderly

We further investigated by flow cytometry whether treatment with BSCU1 influenced the relative abundance of innate (Figure 6) and adaptive immune cells (Figure 7) in adult and elderly PBMCs. CD14⁺ monocytes were the predominantly affected cell types (Figure 6A). A significant increase of 6.6 and 8.2 percentage points were observed in the adult and elderly groups, respectively. The increase in the relative abundance of monocytes was accompanied by a significant decrease of 4 and 5.2 percentage points in conventional den-

dritic cells (DC) (Figure 6B) and 1 percentage point in plasmacytoid dendritic cells (pDC) in the adult and elderly groups, respectively (Figure 6C). Importantly, we observed an increase in the mean fluorescence intensity of the activation marker CD69 expression at the cell surface of CD14⁺ monocytes by 74.2% and 73.9% in adult and elderly groups, respectively (Figure 6D). The relative abundance of natural killer (NK) cells and their activation status were not affected by treatment with BSCU1 (Figure 6E-F). A significant decrease in CD25⁺ CD127⁺ regulatory T cells (Tregs) and CD4⁺ T cells was observed in the elderly consuming BSCU1 (Figure 7A-B).

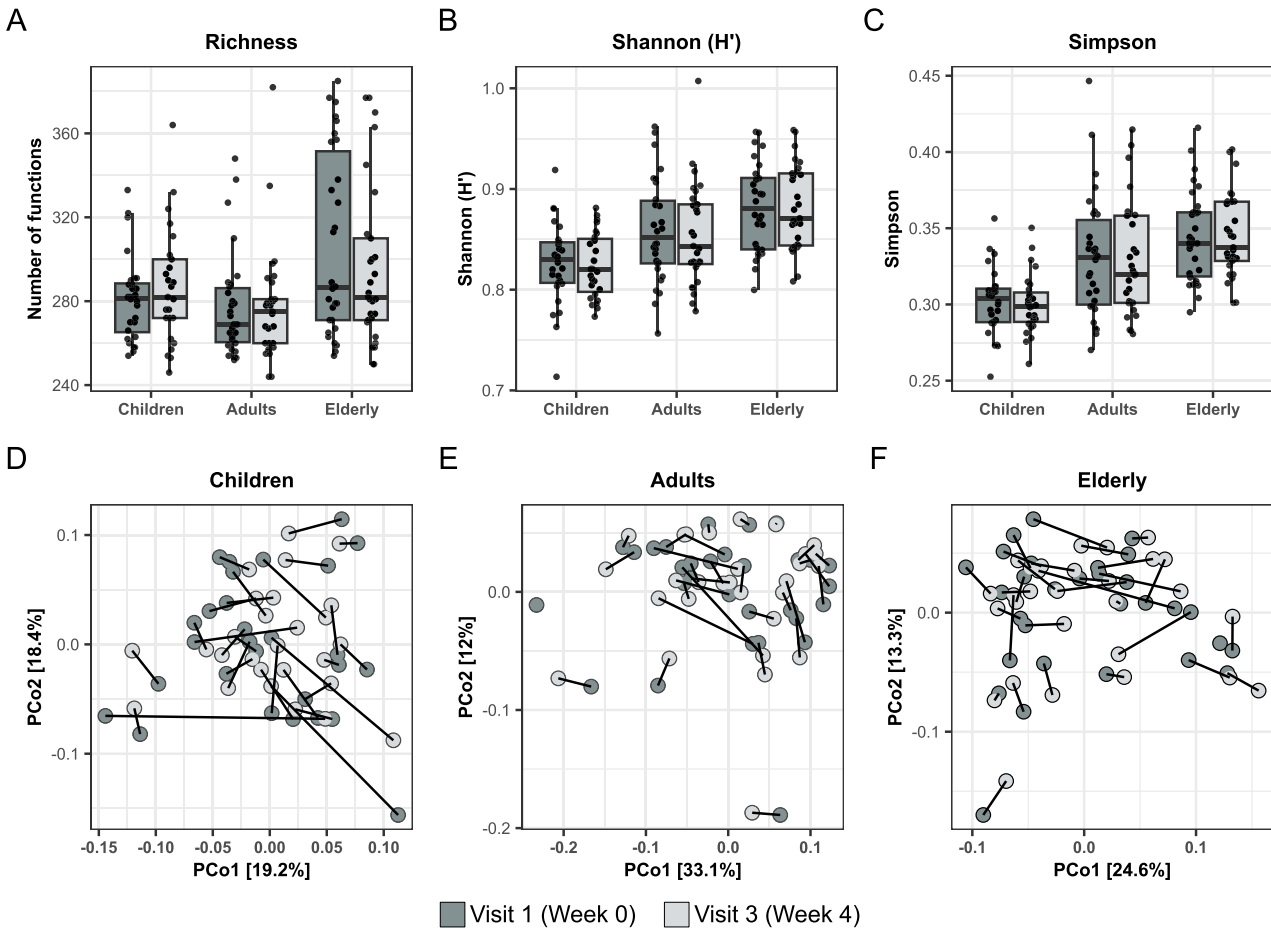


FIGURE 3 Effect of BSCUI on microbiota functionality. (A-C) Diversity indices and (D-F) PCoA analysis before and after treatment.

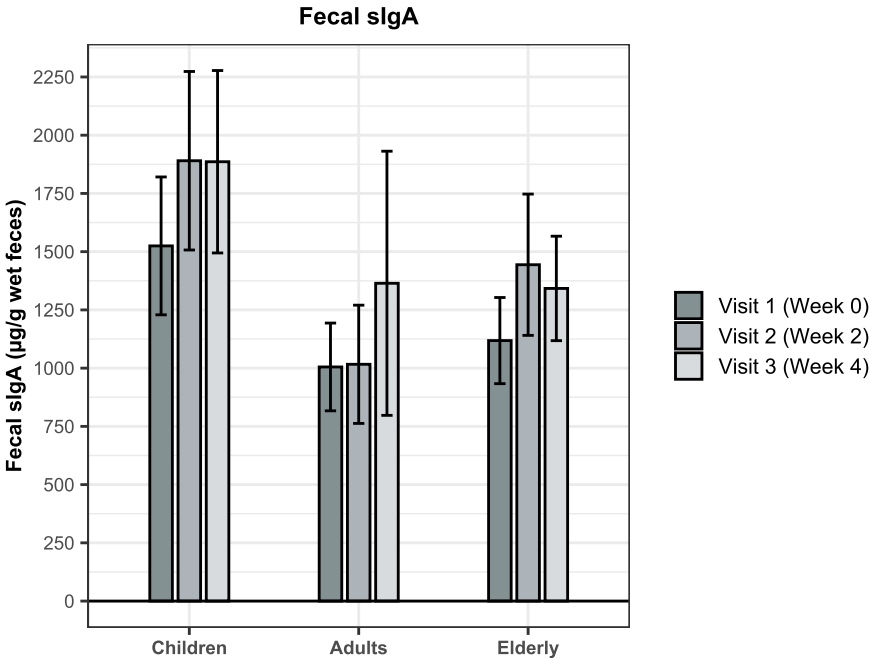


FIGURE 4 Faecal sIgA levels in children, adults and the elderly. The sIgA content was determined at baseline and 2 and 4 weeks after the first intake of BSCUI in each group. Data show means values $\pm$ standard error.

TABLE 3 Effects of BSCUI on faecal sIgA levels after 4 weeks of intervention

Population	Change from baseline to week 4 ($\mu\text{g/g}$ wet faeces)	Standard error	P-value
Children	292.77	469.28	0.5354
Adult	390.76	528.30	0.4625
Elderly	164.79	313.47	0.6008

There were no biologically significant changes in circulating B cells and CD8⁺ T cells (Figure 7C-D). Expression of NKG2D, an activating receptor mainly found on cytotoxic immune cells, was decreased at the cell surface of CD8⁺ T cells in elderly patients (Figure 7E). Taken together, these results suggest an immunomodulation by BSCUI affecting mainly T cells and myeloid cells.

BSCUI supplementation modulates gene expression program in immune cells

To further explore how supplementation with BSCUI affects the gene expression program of immune cells, bulk RNA sequencing on PBMCs obtained at baseline and after 4 weeks of treatment has been performed in adult and elderly groups (Figure 8). Principal component analysis (PCA) revealed that 29% of the information was in PC1 and PC2, with more individuals segregating with PC2 in the adult group than in the elderly group, suggesting a more profound change in the PBMC gene expression program in adults compared to elderly (Figure 8A-B). The volcano plots for both the adult and elderly groups showed moderate modulation of gene expression, still a log2 fold change superior of 1.5 for upregulation of the inflammatory IL-8 cytokine, EGR1 (a zinc finger transcription factor essential for monopoiesis (Trizzino *et al.*, 2021)) and G0S2 (a gene recently suggested to be involved in the inflammatory response of mononuclear cells (Kobayashi *et al.*, 2008)) transcripts (Figure 8C-D). Gene Ontology (GO) term analysis in the adults showed enrichment in several pathways including 'response to type I IFN', 'complement activation', 'oxidative phosphorylation', 'phagocytosis recognition' and 'phagocytosis engulfment' upon BSCUI intake (Figure 8E). Thus, supplementation with BSCUI resulted in changes in the gene expression program related to monocytes and their activation status.

BSCUI supplementation primes innate immune cells to better respond to an inflammatory challenge

GO term 'response to type I IFN' was enriched in the RNAseq dataset after supplementation with BSCUI. *Ex vivo* cytokine release was measured in whole blood after lipopolysaccharide (LPS) stimulation to indicate the potential or efficiency of immune cells to fight infec-

tions. Whole blood obtained at baseline (V1) and after 4 weeks of supplementation (V3) in adults and the elderly was stimulated with LPS for 24 hours. The change in *ex vivo* cytokines levels between V1 and V3 is presented in Figure 9. A significant increase in the production of IFN-gamma, IL-12, IL-1beta, IL-6, IL-8, MIP-1alpha, and TNF-alpha in both the adult and elderly groups were observed after LPS stimulation. The most affected cytokines were IFN-gamma, IL-1beta, and TNF-alpha, with respective increases of 178%, 72%, and 46% in the adult group and 143%, 56%, and 51% in the elderly. IL-12 was also strongly modulated with a 75% increase in adults and an increase of 27% in the elderly. The change in *ex vivo* cytokines levels between V1 and V3 was also assessed in the absence of LPS stimulation (Supplementary figure 3). In the absence of LPS stimulation, BSCUI ingestion did not promote basal secretion of proinflammatory cytokines, although there was an increase in modest basal levels of IL-1beta in adults and MIP-1alpha in the elderly. Thus, supplementation with BSCUI did not trigger basal inflammation but prepared cells to respond better to an inflammatory challenge.

Ingestion of BSCUI promotes phagocytosis capacity in adults and the elderly

GO term 'phagocytosis recognition' and 'phagocytosis engulfment' were enriched in the RNAseq dataset after supplementation with BSCUI. Phagocytosis capacity of granulocytes and monocytes was examined from whole blood collected at baseline and 4 weeks after the first ingestion in adults and elderly using pHrodo™ Green *Escherichia coli* BioParticles™ (Figure 10 and Table 4). The pHrodo Green dye is not fluorescent outside the cell, but fluorescence increases as pH decreases. The percentage of monocytes that phagocytosed fluorescently labelled *E. coli* increased significantly in the elderly with similar trend in adults (Figure 10A). No significant change was observed in the percentage of granulocytes that phagocytosed fluorescently labelled *E. coli* (Figure 10B). The mean fluorescent intensity (MFI) of monocytes, respectively, increased by 75.4% and 69.7% in adults and the elderly (Figure 10C). Increase in mean fluorescent intensity indicates higher pH acidification and thus a faster maturation of phagosomes. A sig-

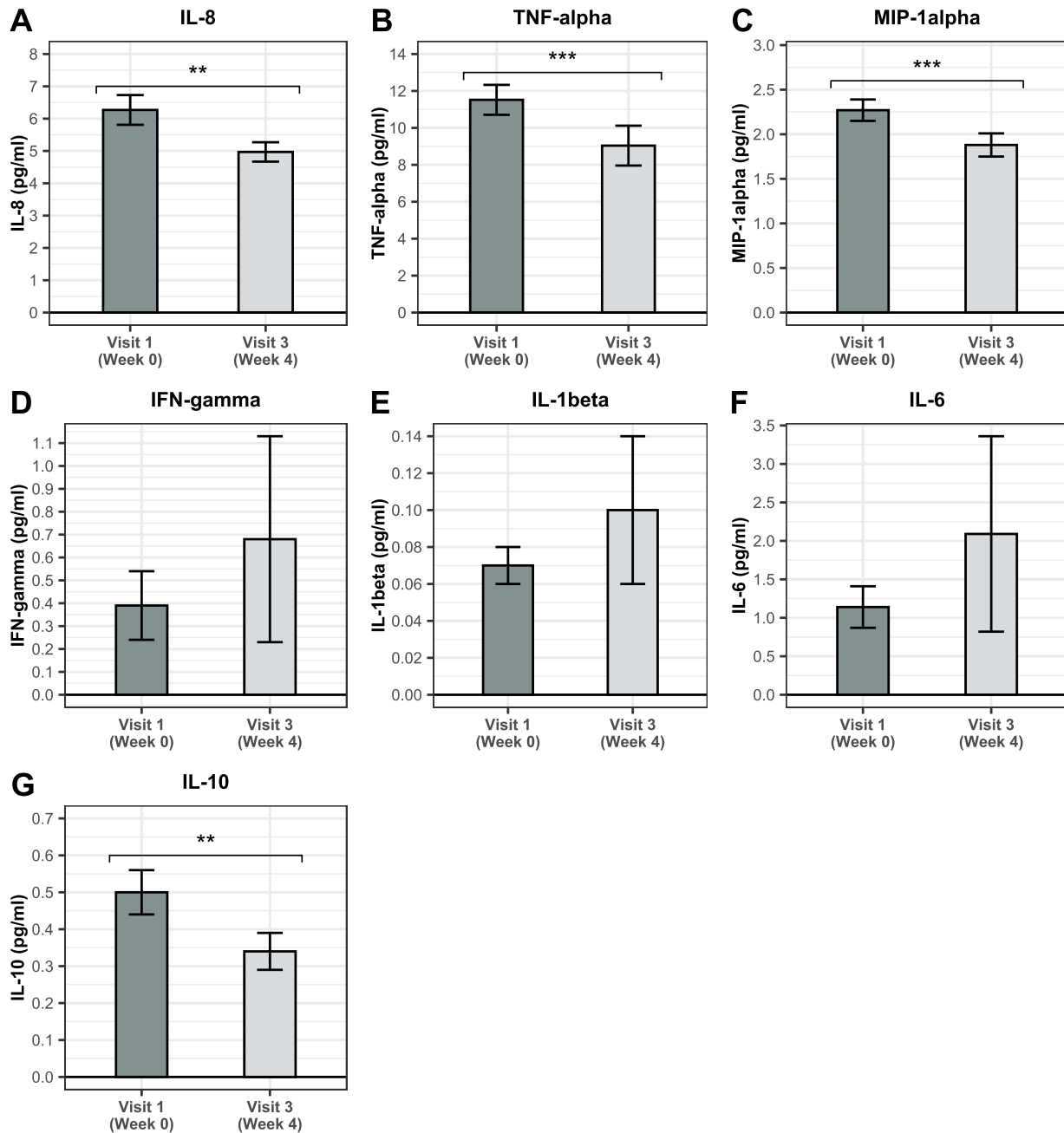


FIGURE 5 Change in circulating cytokines in the elderly. Data show means $\pm$ standard error. *P*-values: ** [0.001 to 0.01]; *** <0.001.

nificant difference was also observed for granulocytes with a 20% and 19.2% respective increase in the mean fluorescence intensity in adults and the elderly (Figure 10D). Thus, BSCUI supplementation promoted the phagocytic capacity of myeloid cells, especially monocytes, in adults and the elderly.

4 Discussion

Oral supplementation of BSCUI has been shown previously to increase mucosal sIgA and improved the

resistance to common infectious diseases in elderly subjects (Lefevre *et al.*, 2015). The current study has been designed to provide insights into the mechanisms of action through which BSCUI could modulate the immune system and support protection against infections.

The microbiota was not globally altered by treatment, except for an increased abundance of *B. subtilis* following dietary supplementation with BSCUI. This is consistent with previous report on probiotics, including spore-forming probiotics (Freedman *et al.*, 2021; Khalesi *et al.*, 2019).

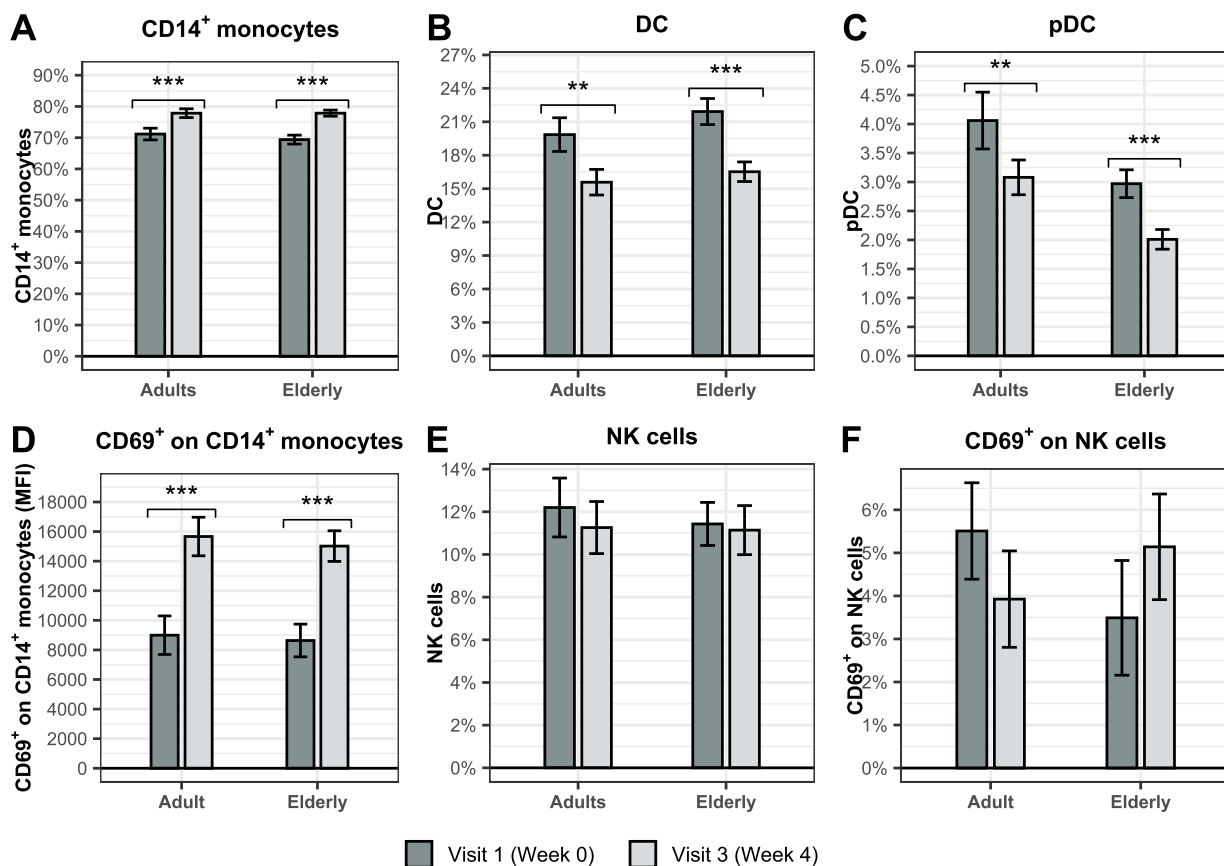


FIGURE 6 Effect of BSCU1 on relative abundance and activation status of innate immune cells. Data show means $\pm$ standard error. *P*-values: ** [0.001 to 0.01]; *** <0.001.

Despite the present study was not able to reproduce previous findings regarding the effect of BSCU1 on sIgA (Lefevre *et al.*, 2015), a trend was observed toward an increase in faecal sIgA concentration in children, adults and the elderly. The interindividual variation of sIgA levels in the current study was higher than expected. As the sample size calculation was performed with a lower variation in sIgA levels, the current study may not have had sufficient power to detect differences in faecal sIgA. The absence of significant effect of BSCU1 on faecal sIgA in the present study could be explained by differences in the study duration, demographic characteristics of the subjects and analytical methods. Indeed, there are no standardised methods known for the measurement of faecal IgA. Differences in faecal sample collection, sIgA extraction, and quantification might partly account for the divergent findings between the studies. Also, the gut microbiota influences intestinal sIgA production and secretion, and in turn, influences faecal sIgA concentrations (Bunker and Bendelac, 2018). Importantly, the gut microbiota varies in different countries and ethnic groups (Deschasaux *et al.*, 2018). It has been shown that individuals with high microbial diversity have relatively stable intestinal ecosystem and are

therefore less susceptible to the introduction of new microbes (Xiao *et al.*, 2020). Therefore, differences in the origin of the studied population may also account for the variability of the effect of BSCU1 on sIgA. Indeed, healthy Dutch individuals have been shown to display one of the highest microbial diversities, and are thereby less susceptible to changes in the gut microbiota.

Low-grade inflammation, often referred to as ‘inflammaging’ when associated with aging, is thought to contribute to many of the chronic diseases of the elderly (Baechle *et al.*, 2023). A variety of stimuli induces inflammaging, including pathogens, endogenous cellular debris and misdirected molecules and nutrients, and the gut microbiota (Franceschi *et al.*, 2018). Indeed, the gut microbiota plays a central role in the development of inflammation due to its ability to release inflammatory mediators. Probiotics can help restore a healthy balance of the gut microbiota and improve barrier function. While there is evidence that certain probiotic strains can reduce low-grade inflammation in the elderly, results are inconsistent and further research is needed (Jukic Peladic *et al.*, 2021). The current study showed that BSCU1 intake lowered basal plasma levels of IL-8, TNF- α , and MIP-1 α . In addition, a signif-

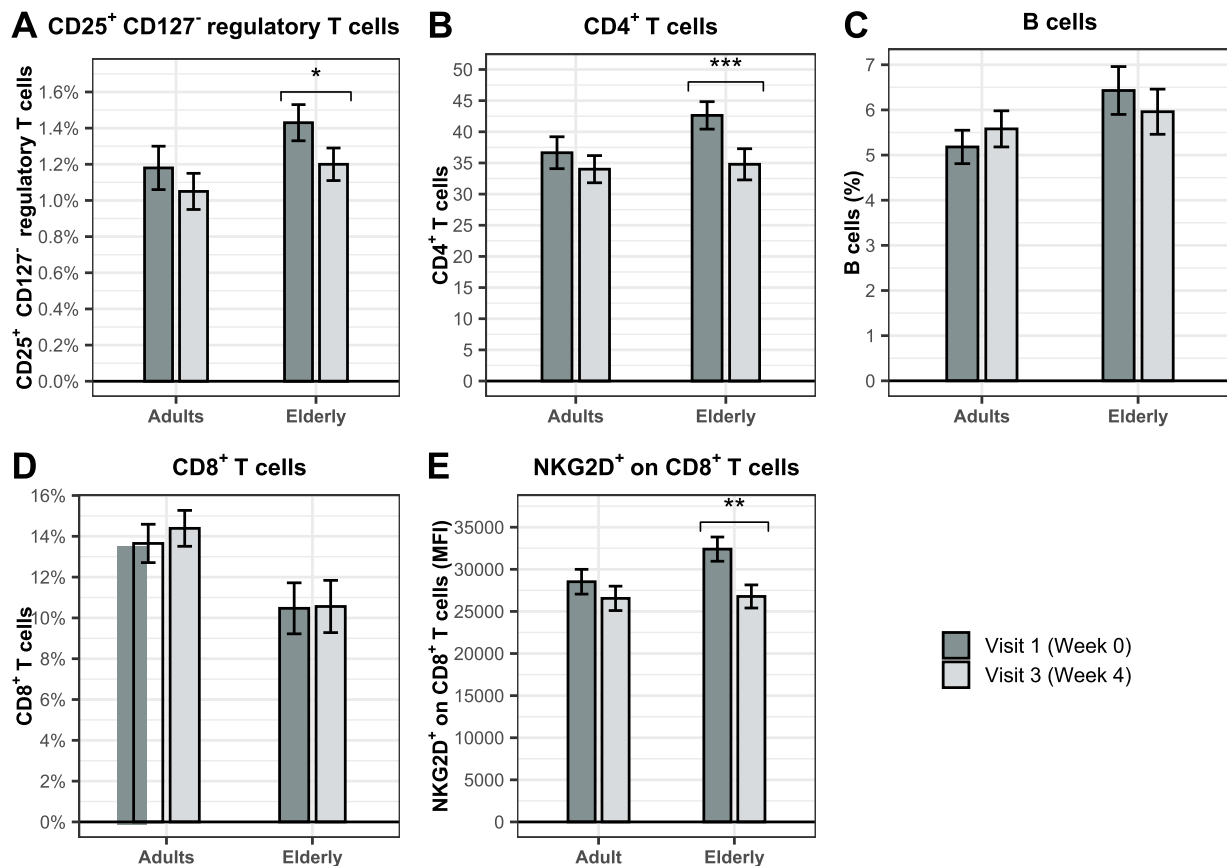


FIGURE 7 Effect of BSCUI on relative abundance and activation status of adaptive immune cells. Data show means $\pm$ standard error. P-values: * [0.01 to 0.05]; ** [0.001 to 0.01]; *** < 0.001.

icant decrease in IL-10 was also observed in elderly following BSCUI supplementation. Dysregulation of this cytokine has been suggested to play a role in the susceptibility of aging individuals toward infections (Almanan *et al.*, 2020) and a reduction in IL-10 may therefore be beneficial in this particular age group. No statistically significant change in IFN-gamma level was observed. These results were inconsistent with our previous findings showing increased IFN-gamma levels and no differences in other proinflammatory cytokines (Lefevre *et al.*, 2015). The administration protocol may be responsible for these discrepancies. Because we observed in the current study that BSCUI probiotic intake primed immune cells to respond better to an inflammatory challenge, it is unlikely that this reduction in low-grade inflammation was related to overall immunosuppression. Rather, alteration of intestinal epithelial cells, e.g. improvement of barrier function, could help reduce the translocation of proinflammatory components of the gut microbiota to the periphery, thereby reducing the inflammatory cascade in the elderly (Untersmayr *et al.*, 2022). It would be interesting to study the markers of gut barrier function in BSCUI-treated individuals. A placebo-controlled study with *B. subtilis* had pre-

viously shown that supplementation for four weeks decreased the number of total T cells, CD8 + T cells, and Tregs (Freedman *et al.*, 2021). We observed a significant decrease in the frequency of circulating CD4⁺ T cells and Tregs in the elderly who consumed BSCUI but no differences in the relative frequency of CD8⁺ T cells. Previous trial on *B. subtilis* DE111 reported no effect of the intervention on myeloid cells (Freedman *et al.*, 2021). In this trial, probiotic intervention with BSCUI was associated with a significant increase in the frequency of monocytes in both the adult and elderly groups, confirming probiotic strain-dependent efficacy.

We found that BSCUI ingestion modulated the gene expression program in adult and elderly immune cells, indicating immune stimulation. We did not detect major changes by ingestion of BSCUI on cytokine production of immune cells without stimulation, suggesting that BSCUI intake did not cause systemic inflammation. However, probiotic treatment promoted upregulation of genes related to monocytes and their activation. Indeed, Gene Ontology (GO) term analysis showed enrichment in several pathways including 'phagocytosis recognition' and 'phagocytosis engulfment' upon BSCUI intake. In addition, BSCUI intake promoted

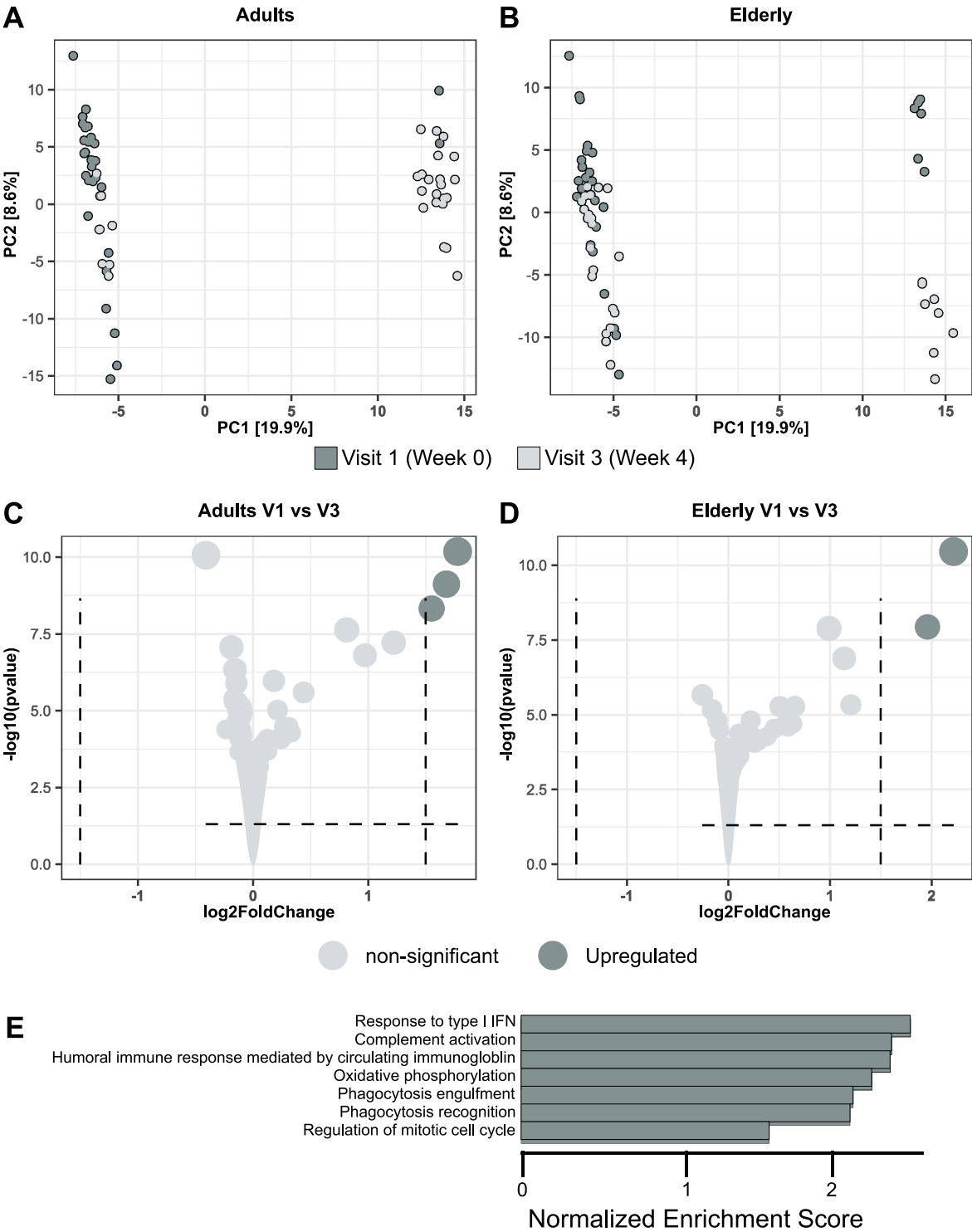


FIGURE 8 BSCU1 supplementation modulates gene expression program in immune cells. Differential gene expression (DGE) analysis of the effect of BSCU1 treatment on peripheral PBMCs in adults and elderly. PCA analysis in adults (A) and elderly (B). Volcano plot in adults (C) and elderly (D). Selected GO terms enriched in adult PBMCs after 4 weeks of BSCU1 treatment compared with baseline (E).

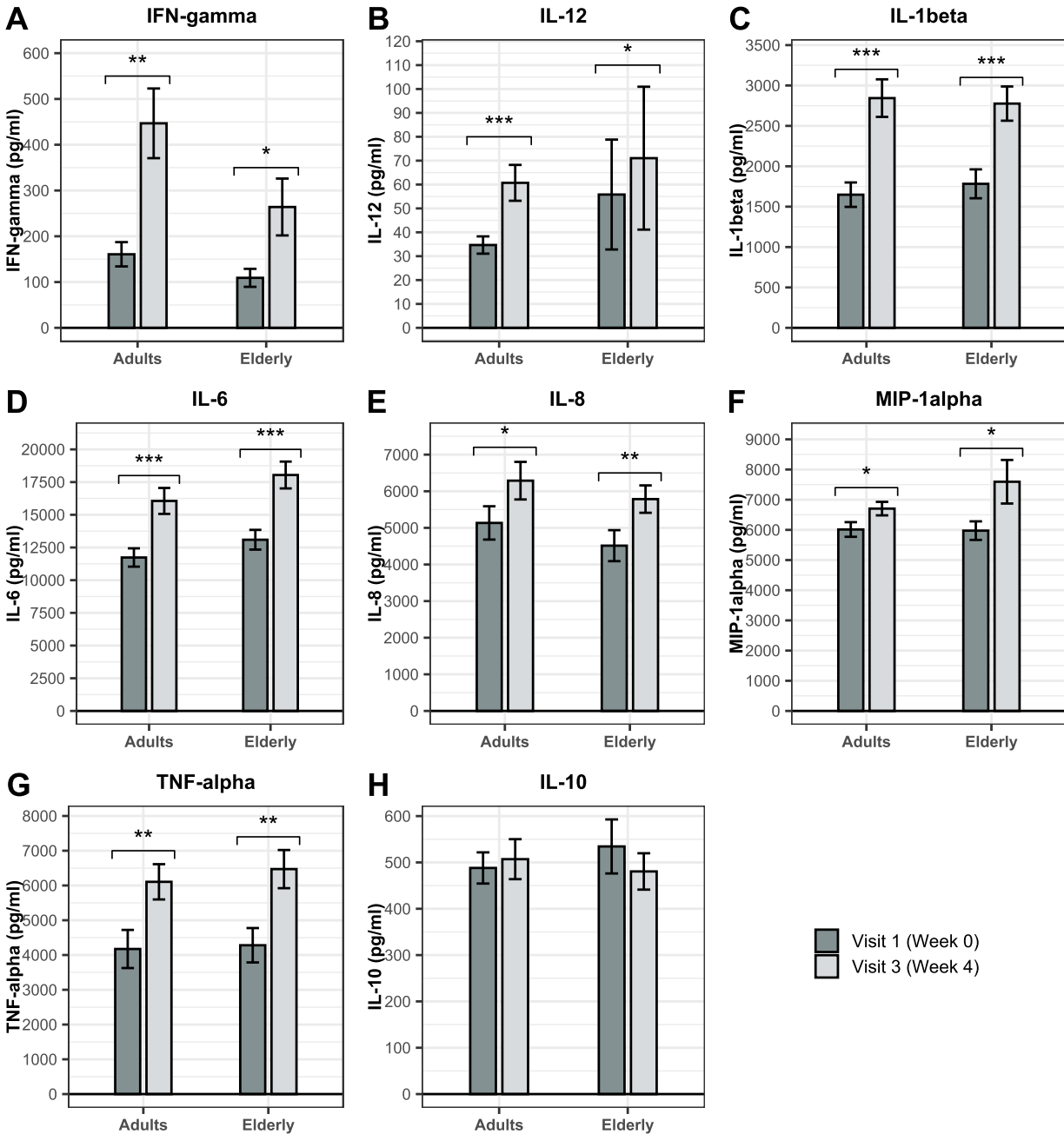


FIGURE 9 Effect of BSCUI on *ex vivo* cytokine production after stimulation with lipopolysaccharides (LPS). Data show means $\pm$ standard error. *P*-values: * [0.01 to 0.05]; ** [0.001 to 0.01]; *** <0.001.

TABLE 4 Effect of BSCUI on *ex vivo* phagocytosis

Population	Parameter	Change from baseline to week 4	Standard error	<i>P</i> -value
Adult	FITC + Monocytes (% points)	+4.52	2.37	0.070
	FITC + Granulocytes (% points)	-0.61	1.06	0.57
	Monocytes (MFI)	+6028.74	559.02	<0.001
	Granulocytes (MFI)	+1864.26	545.63	0.002
Elderly	FITC + Monocytes (% points)	+7.00	1.87	<0.001
	FITC + Granulocytes (% points)	-0.2	1.25	0.87
	Monocytes (MFI)	+5331.77	553.57	<0.001
	Granulocytes (MFI)	+1814.15	581.03	0.004

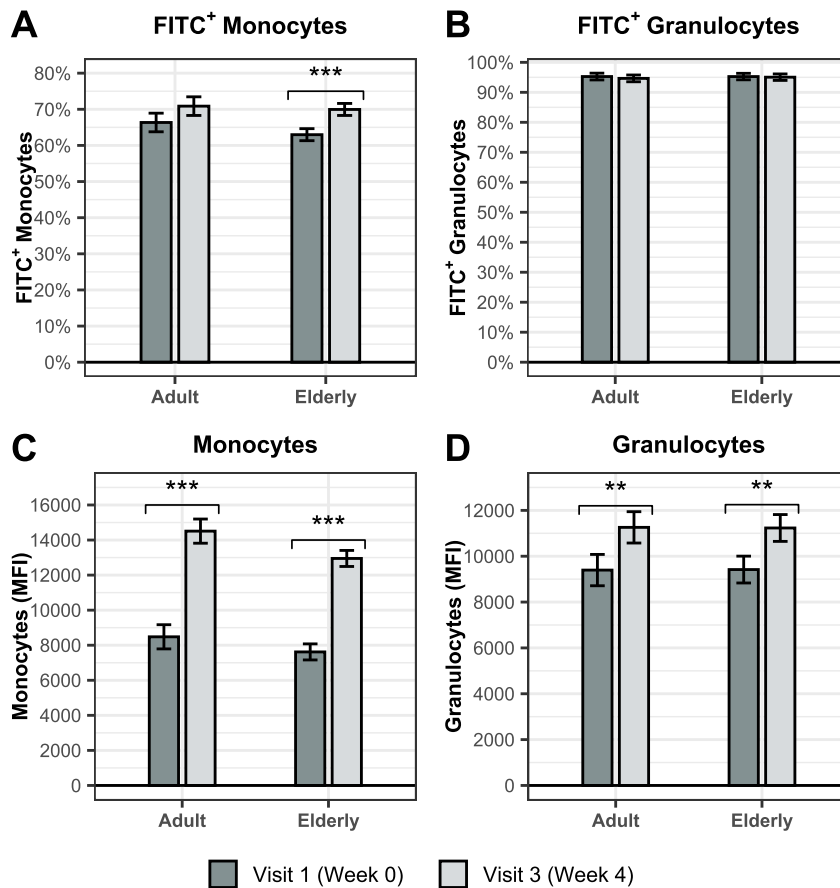
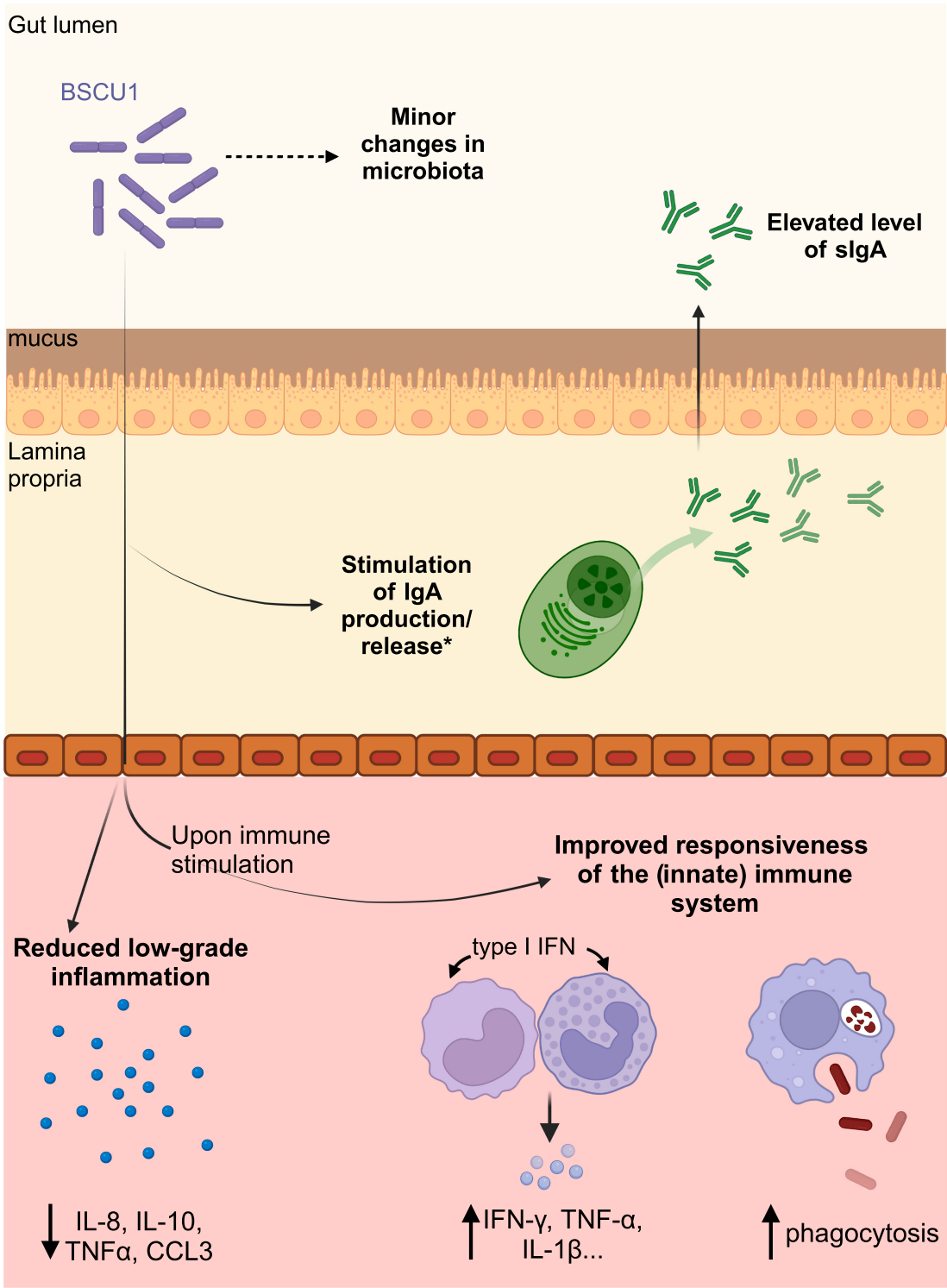


FIGURE 10 BSCU1 promotes phagocytosis capacity in adults and the elderly. Percentage of monocytes (A) and granulocytes (B) that had engulfed bacteria in adult and elderly. Mean fluorescence intensity of engulfed bacteria in monocytes (C) and granulocytes (D) in adult and elderly. Data show means $\pm$ standard error. *P*-values: ** [0.001 to 0.01]; *** <0.001.

pro-inflammatory cytokine secretion upon LPS stimulation, consistent with the GO term enrichment for 'response to type I IFN'. The fact that cytokine release was increased following LPS stimulation may indicate a potential beneficial effect on the ability to fight infection. We also found enrichment for the GO terms 'phagocytosis recognition' and 'phagocytosis engulfment' in adults who consumed BSCU1. Phagocytosis is an important function of the innate immune system that aims to fight and eliminate microbes (Uribe-Querol and Rosales, 2020). We measured increased phagocytotic activity of monocytes and granulocytes in both the adult and elderly groups that had taken the probiotic. This effect could be, at least partly, mediated by cell envelope molecules such as lipoteichoic acid (LTA). LTA is a major constituent of Gram-positive cell envelopes with immunomodulatory activity, such as macrophages activation (Mueller *et al.*, 2006). Villéger *et al.* (2014) have investigated the strain-level structural diversity of LTA from different *Bacillus* strains and highlighted the immunostimulatory properties of the LTA from *B. subtilis* CUI strain.

5 Conclusions

The spore-forming probiotic bacteria BSCU1 has been shown previously to stimulate immune response and improve the resistance to common infectious diseases in elderly subjects. This clinical study provided further insights into the mechanisms of action of BSCU1 (Figure 11). Ingestion of the probiotic BSCU1 reduced the low-grade chronic inflammation in the elderly and primed the immune system to better respond to microbial challenges. Immune profiling combined with transcriptomic analysis and *ex vivo* functional assay provided consistent evidence of increased phagocytic capacity following BSCU1 supplementation. The combination of improved responsiveness to microbial challenges and enhanced phagocytic activity are potential mechanisms of action by which BSCU1 may support defence mechanisms against pathogens.



*Lefevre et al., 2015 DOI:<http://dx.doi.org/10.1186/s12979-015-0051-y>.

FIGURE 11 Schematic representation of the effects of BSCU1 on the immune system.

Supplementary material

Supplementary material is available online at:
<https://doi.org/10.6084/m9.figshare.26324023>

Materials and methods S1. Selection criteria.

Figure S1. The gating strategy to identify monocytes and DCs.

Figure S2. The gating strategy to identify T cells, B cells, and NK cells.

Figure S3. Effect of BSCUI on ex vivo cytokine production without stimulation with LPS.

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Authors' contribution

Design of the study, FM, JFJ, PS, AK; interpretation of the data, AK, TS, MP, ENM, AC; supervision of statistical analysis, FMa; writing – original draft preparation, ENM, FM; writing – review & editing, JFJ, BR, AD, AK, TS, AC; project administration, PS, FM. All authors have read and agreed to the published version of the manuscript.

Conflict of interest

FM, JFJ, BR, AD, FMa and A.C. are employees of Lesaffre. FM, JFJ, BR, AD, FMa and AC were involved in the design of the study, the interpretation of the data and the writing of the manuscript. PS, AK and TD are employees of NIZO Ltd.

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