

Interactions of Probiotics with the Immune Cells of Patients with COVID-19 Pneumonia

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Keywords

Probiotics · COVID-19 · Toll-like receptor 2 · Toll-like receptor 4

Abstract

Introduction: In severe COVID-19, excessive cytokine release may be driven by SARS-CoV-2. We investigated the modulatory effect of probiotics taking into consideration direct interaction with the immune gut cells. **Methods:** Fifty-five patients with confirmed COVID-19 infection were classified by the presence of acute respiratory distress syndrome (ARDS) or not. Peripheral blood mononuclear cells (PBMCs) were isolated and stimulated with lipopolysaccharide (LPS), a preparation of four probiotics (LactoLevure® containing *Saccharomyces boulardii*, *Bifidobacterium lactis* BB-12, *Lactobacillus acidophilus* LA-5, and *L. plantarum*) and/or recombinant human interferon-gamma (rhIFN γ) and tocilizumab. Cytokine concentrations were measured in cell supernatants. Gene expression of Toll-like receptors 2 (TLR2) and 4 (TLR4) was performed by quantitative real-time polymerase chain reaction (RT-PCR). Results were associated with the level of viremia. **Results:** Probiotics

decreased tumor necrosis factor-alpha (TNF α) production by the PBMCs of both ARDS and non-ARDS patients. LPS stimulated the production of interleukin (IL)-1 β , IL-6 in non-ARDS patients. IL-6 production was maintained in the presence of probiotics. rhIFN γ enhanced LPS-stimulated cytokine production by PBMCs; this was not the case when PBMCs were stimulated by probiotics. Probiotics upregulated TLR2 and LPS downregulated TLR4 in the PBMCs of patients with ARDS. PBMCs from patients with viremia had more cytokine production by probiotic stimulation. **Conclusion:** Probiotics interact with the immune system of COVID-19 patients by modulating the production of TNF α , IL-1 β , and IL-6 in an IFN γ -independent mechanism.

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Introduction

Severe host deterioration in COVID-19 has been closely associated with dysregulated cytokine release by immune cells [1, 2]. The Toll-like receptor (TLR) family is involved in the recognition and response to microbial infections. TLRs have a dual role in viral infections through interaction with viral

Table 1. Patient demographics

	No-ARDS (<i>n</i> = 16)	ARDS (<i>n</i> = 39)	Comparators (<i>n</i> = 20)
Age, mean (range), years	61.7 (27–82)	62.8 (28–89)	50.3 (18–92)
Sex			
Female	6 (37.5%)	17 (43.6%)	11 (55%)
Male	10 (62.5%)	22 (56.4%)	9 (45%)
Most common comorbidities, <i>n</i> (%)			
Hypertension	10 (62.5)	14 (35.9)	2 (10)
Dyslipidemia	3 (18.7)	8 (20.5)	2 (10)
Diabetes mellitus	2 (12.5)	5 (12.8)	1 (5)
Obesity	1 (6.2)	2 (5.1)	
Coronary artery disease	1 (6.2)	7 (17.9)	1 (5)
Chronic obstructive pulmonary disease	0 (0)	2 (5.1)	
Hypothyroidism	1 (6.2)	3 (7.7)	1 (5%)
Solid tumor malignancy	0 (0)	2 (5.1)	
CCI, mean (range)	2.56 (0–5)	2.56 (0–6)	1.10 (0–4)
CRP, median (range), mg/L	21.20 (1.5–318)	56.50 (5–574)	<1.5
Ferritin, median (range), ng/mL	500.75 (34.3–1,604)	1,007.14 (99–1,676)	
Mortality at 28 days	0	13 (33.3%)	0

CRP, C-reactive protein; CCI, Charlson's comorbidity index.

surface proteins and activation of JAK/STAT signaling pathways, which may lead to macrophage activation syndrome [3]. Spike glycoprotein, the major infectious surface protein of SARS-CoV-2, has recently been found to bind to human *TLR4* [4]. There is also evidence for a bacterial counterpart in immune stimulation during COVID-19 with bacteria originating from the gut. Indeed, many patients experience gastrointestinal symptoms like nausea, diarrhea, and vomiting [5]. It may be hypothesized that probiotics, commonly taken by the patients, may interact with the immune system and particularly with dendritic cells and macrophages modulating the production of cytokines or chemokines.

Probiotics are defined as nonpathogenic bacteria which, when consumed, may confer to homeostasis. They enhance the function of the mucosal barrier and modulate the antiviral innate and adaptive leukocyte function [6, 7]. This mode of action may be of clinical benefit for patients infected by SARS-CoV-2. A systematic review and meta-analysis indicated that probiotic supplementation in COVID-19 patients led to improvement in overall symptoms and shorter hospital stay [8]. Certain probiotic strains have demonstrated the ability to induce the expression of genes involved in antiviral immunity, potentially offering protective effects against SARS-CoV-2 infection. For example, *Lactobacillus paracasei* DG enhances antiviral responses in intestinal cells, suggesting a potential role in mitigating COVID-19 severity

[9]. A retrospective study also suggested that intake of probiotics could reduce secondary infections in COVID-19 patients and act as modulators of the immune response [10]. The complex interactions of SARS-CoV-2 infection and the innate immune system may provide room for co-infections where comorbidities play a major role [11]. This is also highlighted by the greater risk comorbidities bring for severe COVID-19 in children [12]. Booster vaccination decreases the risk of SARS-CoV-2 infection and hospitalization [13] but also elaborates the importance of immune stimulation for infection prevention. The strengthened immune responses elicited by probiotics, may even serve as an “adjunct-to-vaccination” like strategy for COVID-19.

These findings collectively reinforce the importance of understanding host-microbe interactions in COVID-19 pathogenesis. Given the growing evidence on probiotics' immunomodulatory properties and their potential role in mitigating SARS-CoV-2 severity, we sought to investigate their direct interaction with immune cells in infected patients.

Patients and Methods

Participants

Adult patients with confirmed COVID-19 infection by PCR were eligible to participate. Patients were included in the study from March 2020 to March 2022.

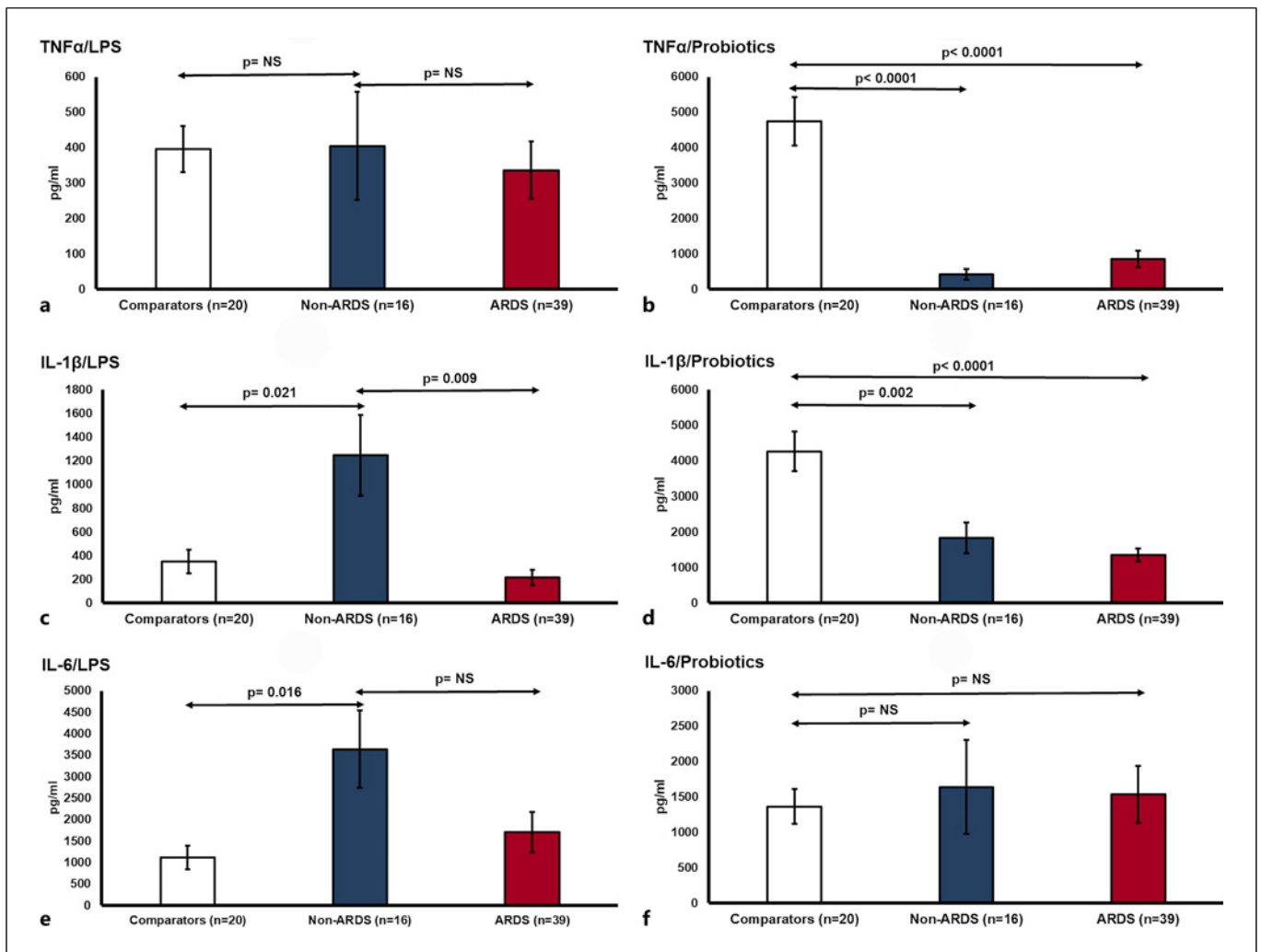


Fig. 1. Cytokine production by PBMCs following stimulation. **a** TNFα of PBMCs supernatants stimulated with LPS. **b** TNFα of PBMCs supernatants stimulated with probiotics. **c** IL-1β of PBMCs supernatants stimulated with LPS. **d** IL-1β of PBMCs supernatants stimulated with probiotics. **e** IL-6 of PBMCs supernatants stimulated with LPS. **f** IL-6 of PBMCs supernatants stimulated with probiotics. IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells.

Inclusion criteria were (a) both sexes, (b) age ≥ 18 years old, (c) positive polymerase chain reaction (PCR) test for COVID-19, and (d) new lung chest X-ray infiltrate. Exclusion criteria were (a) neutropenia defined as an absolute neutrophil count less than $1,000/\text{mm}^3$, (b) any stage IV solid tumor or hematologic malignancy, and (c) medical history of infection by the human immunodeficiency virus. Comparators matched for age, sex, and comorbidities were also enrolled which were tested negative for SARS-CoV-2. Comorbidities were expressed by the Charlson's comorbidity index (CCI). Patients were followed-up for 28 days. This study makes part of an overall study protocol on the inter-

action of gut function with probiotics which was approved by the Ethics Committee of Attikon University Hospital (approval A14, 4th/March 17, 2020). Patients were enrolled after written informed consent provided by themselves or first-degree relatives.

PBMC Isolation

Patients were classified according to the severity of the disease into acute respiratory distress syndrome (ARDS) and non-ARDS according to the Berlin definition [14]. Ten ml of whole blood was collected and peripheral blood mononuclear cells (PBMCs) were isolated after gradient centrifugation over Ficoll.

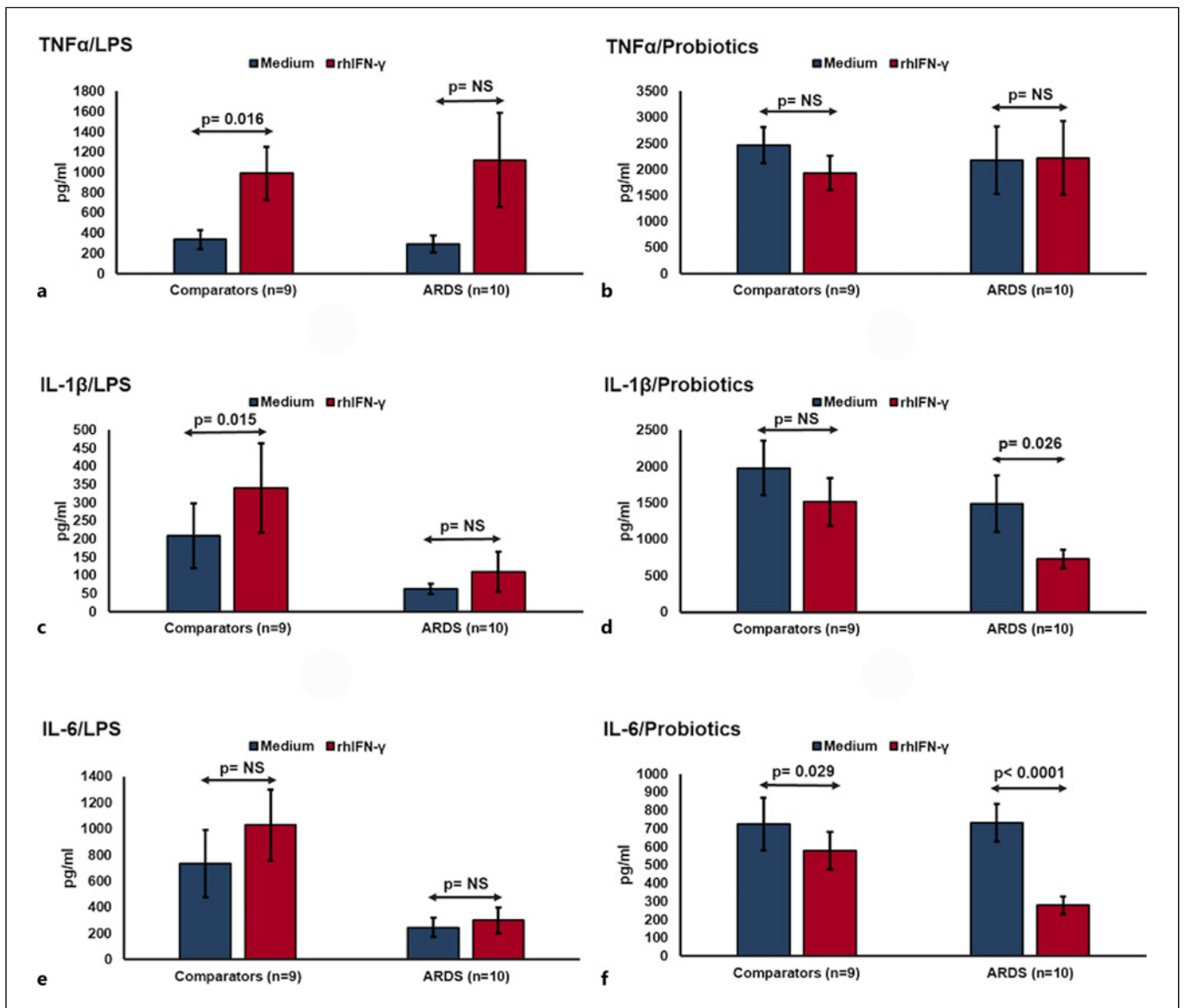


Fig. 2. Cytokine production by PBMCs. **a** TNFα of PBMCs supernatants stimulated with LPS and medium or rhIFNγ. **b** TNFα of PBMCs supernatants stimulated with probiotics and medium or rhIFNγ. **c** IL-1β of PBMCs supernatants stimulated with LPS and medium or rhIFNγ. **d** IL-1β of PBMCs supernatants stimulated with probiotics and medium or

rhIFNγ. **e** IL-6 of PBMCs supernatants stimulated with LPS and medium or rhIFNγ. **f** IL-6 of PBMCs supernatants stimulated with probiotics and medium or rhIFNγ. IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells; rhIFNγ, recombinant human Interferon-γ.

PBMCs were washed 3 times with ice-cold phosphate buffered saline, pH: 7.2 and measured in a Neubauer chamber by exclusion of dead cells using trypan blue staining. PBMCs were dispensed into wells of a 96-well plate in a final volume of 200 μL/well at a concentration of 2.5×10^6 cells/mL. PBMCs were left unstimulated or stimulated with bacterial lipopolysaccharide (LPS) or probiotics, with RPMI 1640 supplemented with 10% fetal calf serum, 2 mM glu-

tamine, 100 U/mL penicillin G, and 0.1 mg/mL streptomycin. All experiments were performed in duplicate.

Probiotic Preparation and Cytokine Measurement

The probiotic preparation consisted of a combination of four probiotics: *Lactobacillus acidophilus* LA-5 1.75×10^9 cfu, *Lactobacillus plantarum* 0.5×10^9 cfu, *Bifidobacterium lactis* BB-12 1.75×10^9 cfu, and

Saccharomyces boulardii 1.5×10^9 cfu per capsule (LactoLevure®, UniPharma, Athens, Greece). Plates were incubated for 48–120 h at 37°C in 5% CO₂. After centrifugation, the supernatants were collected and stored at –70°C until analyzed. The concentrations of tumor necrosis factor-alpha (TNFα), interleukin (IL)-1β, and IL-6 were determined in duplicate by an enzyme-linked immunosorbent assay (Diacclone, Marseille, France). The lower detection limit for TNFα was 4 pg/mL; for IL-1β and IL-6, it was 2 pg/mL.

rhIFNγ, Tocilizumab, and Cytokine Measurement

In separate experiments, PBMCs from patients with ARDS and comparators were left unstimulated or stimulated with LPS, probiotics, recombinant human interferon-gamma, and combinations. The concentration of rhIFNγ was 100 ng/mL [15]. In some experiments, tocilizumab was added at 10 μg/mL [16]. The concentrations of TNFα, IL-1β, and IL-6 were determined in duplicate by the enzyme-linked immunosorbent assay described above.

RNA Extraction and Gene Expression Analysis

The total RNA was extracted from the cell population of 5 COVID-19 patients with ARDS and 5 healthy comparators using an RNeasy Plus Mini Kit (QIAGEN GmbH, Hilden, Germany) according to manufacturer protocol. The concentration of total RNA was measured with a NanoDrop Spectrophotometer ND-1000 (Peqlab, Erlangen, Germany), and 1 μg of total RNA was used for reverse transcription with the QuantiTect Reverse Transcription Kit (QIAGEN, GmbH) including a genomic DNA digestion step. PCR was performed two-fold as duplicates with 1 μL cDNA within a 25 μL reaction volume of QuantiFast SYBR Green PCR Kit (QIAGEN GmbH) using an iQ5 Multicolor Real-Time PCR Detection System (Bio-Rad, USA) for the quantitative PCR thermal cycles in 2 steps. Primers are all commercially available and validated by QIAGEN.

The PCR initial activation step was performed for 5 min at 95°C. The second cycling step started with denaturation for 10 s at 95°C, followed by annealing for 30 s at 60°C; 40 cycles were performed for each sample. Amplification was followed by a melting curve analysis for all genes to verify the formation of specific products. Blanks were subjected to this protocol as well. The *RRN18S* gene was used as an endogenous control. The PCR product was recognized after 3% w/v agarose gel prepared with 0.5x TBE (AppliChem,

GmbH, Germany) and visualized under ultraviolet radiation after ethidium bromide staining (1 μg/mL) (AppliChem, Germany) (online suppl. Table 1; for all online suppl. material, see <https://doi.org/10.1159/000545873>). Gene expression is calculated as normalized fold change compared with that of the house-keeping gene *RRN18S* [17].

Measurement of Viral Load and Cytokines

PCR for the SARS-CoV-2 genes *N38*, *N36*, and *Orf1ab36/N34* was performed in the blood and a threshold of 30 Cts was used to divide the patients into two subgroups (≥ 30 Cts those with low viral load and < 30 Cts those with high viral load). The concentrations of TNFα, IL-1β, and IL-6 were determined in duplicate by the enzyme-linked immunosorbent assay and then compared between the two subgroups.

Endpoints

The study primary endpoint was the impact of the four probiotic preparation on cytokine production by PBMCs from patients with COVID-19 according to the level of severity. The mechanism of action of probiotics was a secondary endpoint.

Data Sources and Measurements

Clinical data were collected from the patients' electronic records, their medical files, and the hospital's electronic system on their admission day in the study. Accordingly, the same data were collected for the comparators.

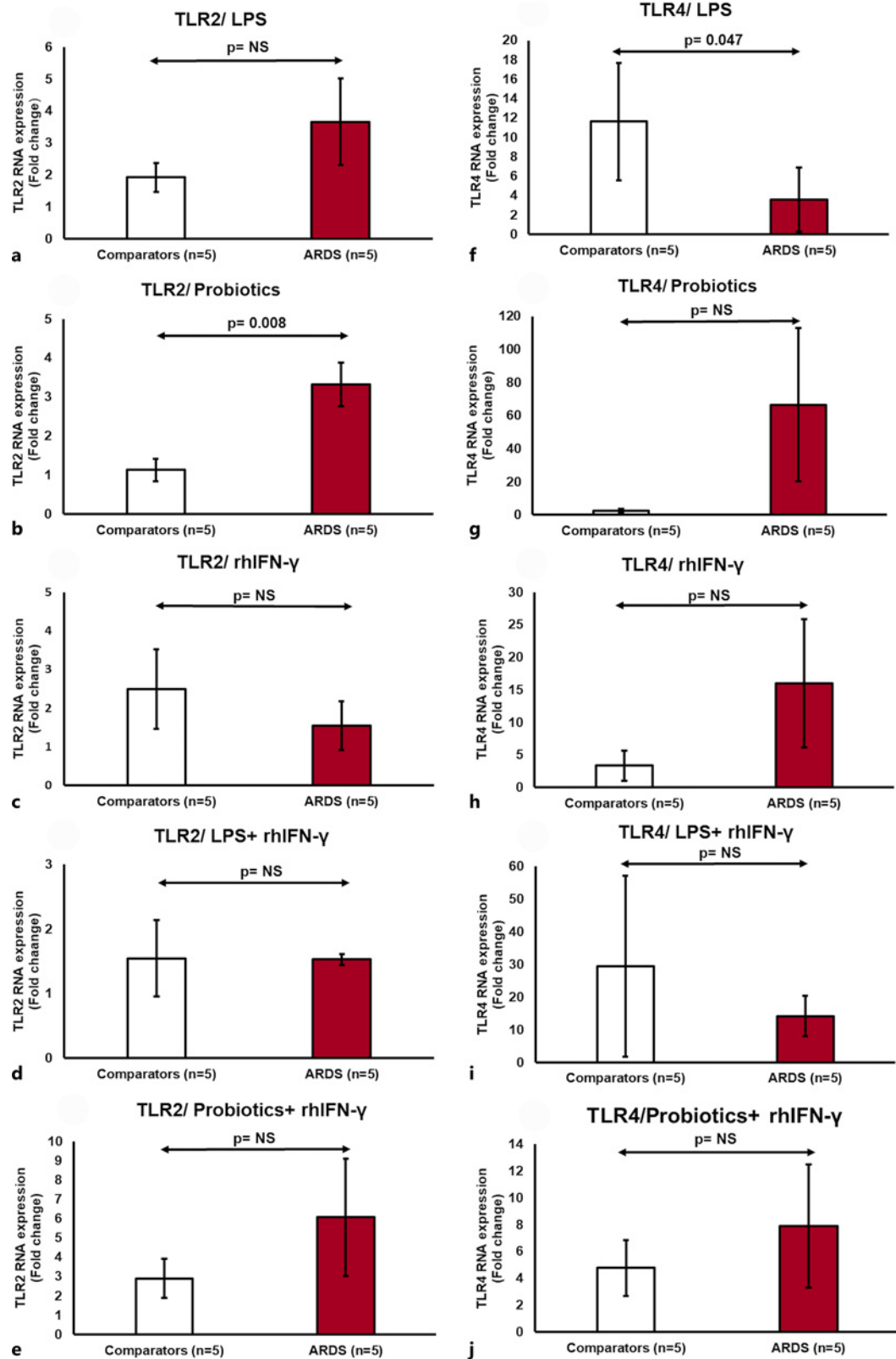
Statistical Analysis

Results were expressed as means and SE. Comparisons were done by the Mann-Whitney U test or by the Wilcoxon's test for paired samples. Patients were classified into two subgroups regarding their blood viral load; those with PCR detecting viral genes at ≥ 30 Cts and reflecting low viral load; and those with PCR detecting viral genes at less than 30 Cts and reflecting high viral load. Any two-tailed $p < 0.05$ was considered significant.

Results

Study Participants

From March 2020 to March 2022, 55 patients suffering from COVID-19 and 20 healthy comparators were enrolled. Table 1 lists the demographic characteristics of patients and comparators.



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(For legend see next page.)

Impact of Probiotics on Cytokine Production by PBMCs

Following stimulation by LPS, no statistically significant difference was found in TNF α production by the PBMCs of patients compared to healthy comparators. However, with the addition of probiotics, the production of TNF α production was decreased both in ARDS and in non-ARDS patients (Fig. 1a, b). The production of IL-1 β and of IL-6, following stimulation of PBMCs with LPS, was higher in non-ARDS patients compared to both patients with ARDS and healthy comparators. The production of IL-1 β was decreased with the addition of probiotics (Fig. 1c, d). However, in the presence of probiotics, the production of IL-6 remained unaltered (Fig. 1e, f).

Impact of Tocilizumab on Cytokine Production by PBMCs

Stimulations of PBMCs from patients with ARDS and comparators were repeated in the presence of the IL-6 receptor inhibitor tocilizumab. The drug did not have any effect on the production of TNF α , IL-1 β , and IL-6 (online suppl. Fig. 1) showing that the LPS and the probiotic effects were not related to an effect on the IL-6 receptor.

Impact of rhIFN γ on Cytokine Production by PBMCs

In separate set of experiments, stimulations of PBMCs were repeated in the presence of the cytokine stimulator rhIFN γ . The addition of rhIFN γ enhanced cytokine production from healthy PBMCs under LPS stimulation and the production of TNF α , but not of IL-1 β and IL-6, from PBMCs of patients with ARDS. However, upon probiotic stimulation, rhIFN γ did not modify cytokine production from healthy PBMCs and decreased the production of IL-1 β and IL-6 from PBMCs of patients with ARDS (Fig. 2). These results point towards a specific downregulation of IL-1 β and IL-6 production from PBMCs of patients with COVID-19 ARDS more pronounced under probiotic stimulation than LPS stimulation and which is independent from rhIFN γ .

Gene Expression

Next, we examined the gene expression of *TLR2* and *TLR4*. We noticed upregulation of *TLR2* expression by the PBMCs of patients with ARDS following stimulation of probiotics and downregulation of *TLR4* expression in the PBMCs of patients with ARDS following stimulation with LPS. With the addition of rhIFN γ , no statistically significant change in the expression of these genes was observed (Fig. 3) showing that the effect of rhIFN γ was independent of gene expression.

Viral Load and Cytokines

Finally, we compared the production of cytokines by the PBMCs of patients with ARDS with more than 30 Cts and less than 30 Cts of the genes *N38*, *N36*, and *Orf1ab36/N34* of SARS-CoV-2 in the blood. Cytokine production was similar between these subgroups of patients following stimulation with probiotics. However, the production of IL-1 β after stimulation of PBMCs with LPS was significantly pronounced from the PBMCs of patients with more than 30 Cts of SARS-CoV-2 genes (Fig. 4).

Discussion

The presented findings suggest a complex dysregulation of cytokine production by the PBMCs of patients with COVID-19 pneumonia. Indeed, PBMCs from patients with ARDS show limited capacity for the production of IL-1 β and of IL-6 which is pronounced when the level of viremia by SARS-CoV-2 is low and which is associated with the downregulation of *TLR4*. When cells are stimulated by the preparation of four probiotics, the production of IL-6 is maintained to levels similar to healthy comparators. This is partly associated with the upregulation of *TLR2* and it is independent of the presence of IFN γ . IFN γ is produced by T-helper 1 (Th1 cells), natural killer, and natural killer T cells as response to viral infection. The independence of the effect of probiotics from the addition of rhIFN γ in the cell medium indicates that probiotics might exert benefit to

Fig. 3. *TLR2* and *TLR4* relative expression by PBMCs following stimulation. **a** *TLR2* relative expression of PBMCs supernatants stimulated with LPS. **b** *TLR2* relative expression of PBMCs supernatants stimulated with probiotics. **c** *TLR2* relative expression of PBMCs supernatants stimulated with rhIFN γ . **d** *TLR2* relative expression of PBMCs supernatants stimulated with LPS + rhIFN γ . **e** *TLR2* relative expression of PBMCs supernatants stimulated with probiotics + rhIFN γ . **f** *TLR4* relative expression of PBMCs supernatants stimulated with

LPS. **g** *TLR4* relative expression of PBMCs supernatants stimulated with probiotics. **h** *TLR4* relative expression of PBMCs supernatants stimulated with rhIFN γ . **i** *TLR4* relative expression of PBMCs supernatants stimulated with LPS + rhIFN γ . **j** *TLR4* relative expression of PBMCs supernatants stimulated with probiotics + rhIFN γ LPS, lipopolysaccharide; PBMCs, peripheral blood mononuclear cells; rhIFN γ , recombinant human interferon- γ ; TLR2, Toll-like receptor 2; TLR4, Toll-like receptor 4.

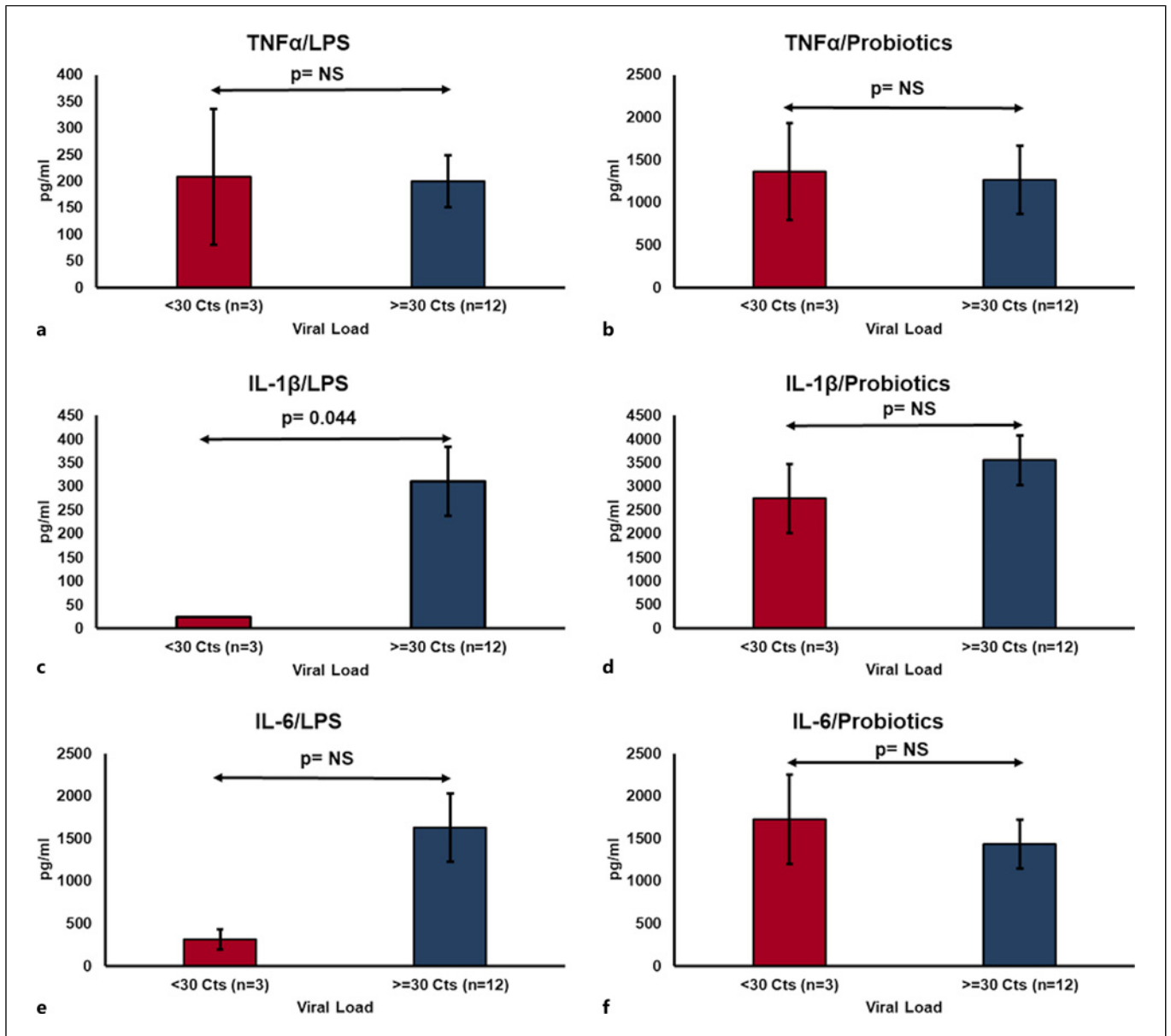


Fig. 4. Cytokine production by PBMCs following stimulation depending on the level of viremia. **a** Comparison of TNFα of PBMCs supernatants stimulated with LPS between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. **b** Comparison of TNFα of PBMCs supernatants stimulated with probiotics between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. **c** Comparison of IL-1β of PBMCs supernatants stimulated with LPS between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. **d** Comparison of IL-1β of PBMCs supernatants

stimulated with probiotics between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. **e** Comparison of IL-6 of PBMCs supernatants stimulated with LPS between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. **f** Comparison of IL-6 of PBMCs supernatants stimulated with probiotics between patients with viral load in the blood <30 Cts and patient who with viral load ≥30 Cts. IL, interleukin; LPS, lipopolysaccharide; TNF, tumor necrosis factor; PBMCs, peripheral blood mononuclear cells; Ct, cycle threshold.

maintain immune responses irrespective of the production of IFNγ which is produced in response to viral infection [18].

The immunomodulatory effects of probiotics are mediated through their interaction with immune cells. Our results suggest that they have a dual effect; they

decrease the production of IL-1 β and TNF α and reduce cytokine storm; and they maintain a basal level of immune function through the production of IL-6.

TLR4 recognizes LPS and TLR2 recognizes several pathogen-associated molecular patterns including structures of Gram-positive bacteria. Interestingly, in intubated COVID-19 patients, our analysis revealed that probiotic stimulation increased *TLR2* expression, while LPS stimulation decreased *TLR4* expression. This can be interpreted as an already existing down-regulation of *TLR4* due to infection of SARS-CoV-2 in ARDS. On the contrary, in the same cells, the TLR2 pathway remains intact, allowing probiotics to act at the level of TLR2.

It is evident from various clinical studies that probiotics have a beneficial effect on COVID-19 infection [18–21]. In a randomized placebo-controlled study designed to evaluate the efficacy and safety of a new probiotic formulation in COVID-19, a significant remission of symptoms was observed in patients who had received probiotics [18]. In particular, it was shown that in the case of COVID-19 infection the use of probiotics improved several clinical symptoms, such as fever, cough, myalgia, dyspnea, nausea, diarrhea, and abdominal pain. Additionally, patients taking probiotics showed a reduction in lung infiltrates and nasopharyngeal viral load and overall had not only symptomatic but also complete viral clearance [19]. In another study, it was found that patients receiving probiotics during COVID-19 treatment had significantly lower levels of inflammatory markers procalcitonin and C-reactive protein compared to the placebo group, while plasma albumin levels and lymphocyte counts were significantly higher [20]. Anwar et al. [21] suggested that the metabolic products of *L. plantarum* block the entry of SARS-CoV-2 in the cells by blocking angiotensin-converting enzyme 2 receptors and inhibit viral transcription by targeting protein S and blocking RNA-dependent RNA polymerase. Given the strongly positive effect of probiotics on the immune response of COVID-19 patients but also the high heterogeneity of the immune response of the virus over time, it is clear that additional studies are needed to determine their mechanism of action at both the cellular and genomic level and their potential benefit in specific patient groups.

Our study indicates that probiotics may modulate the immune response in COVID-19 patients by reducing the production of TNF α and IL-1 β through a mechanism independent of the IL-6 receptor and of the presence of IFN γ . The findings support the use of probiotics as an adjunctive therapy to modulate the inflammatory re-

sponse in COVID-19, particularly in non-ARDS patients or those with moderate symptoms.

Three main limitations of the study need to be acknowledged: (a) the small number of participants makes it difficult to draw firm conclusions; (b) the study design was based on the ex vivo effect of probiotics on PBMCs isolated from patients and not on the cytokine stimulation of PBMCs isolated from patients already on probiotic treatment; and (c) the study used a specific formulation of four probiotics (LactoLevure[®]) and results are not generalizable to other probiotic formulations. Overall, while the study provides insights on the modulation of innate immune responses by probiotics in patients infected by SARS-CoV-2, the precise mechanism by which probiotics exert these immunomodulatory effects requires further study.

Statement of Ethics

This study protocol was reviewed and approved by the Scientific Committee of Attikon General University Hospital of Athens; approval A14, 4th/March 17, 2020. Written informed consent was obtained from participants (or their parent/legal guardian/next of kin) to participate in the study.

Conflict of Interest Statement

Evangelos J. Giamarellos-Bourboulis has received honoraria from Abbott Products Operations, bioMérieux, GSK, UCB, Sobi AB, and Thermo Fisher Brahms GmbH; independent educational grants from Abbott Products Operations, AbbVie, bioMérieux Inc., Johnson and Johnson, Incyte, MSD, Novartis, UCB, Sanofi, Sobi; and the Horizon 2020 European Grants ImmunoSep and RiskinCOVID; and the Horizon Health grant EPIC-CROWN-2, POINT and Homi-LUNG (granted to the Hellenic Institute for the Study of Sepsis). Evangelos Giamarellos-Bourboulis was a member of the journal's Editorial Board at the time of submission. The other authors do not declare any conflict of interest.

Funding Sources

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Author Contributions

Ioannis Mitrou enrolled patients, run the experiments, wrote the first draft of the manuscript, and approved the final version to be submitted. George Dimopoulos enrolled patients, reviewed the manuscript, and approved the final version to be submitted. Konstantina Dakou analyzed the data, reviewed the manuscript, and approved the final version to be submitted. Panagiotis Koufargyris, Georgia Damoraki, and Theologia Gkavogianni run

laboratory experiments, reviewed the manuscript, and approved the final version to be submitted. Evangelos J. Giamarellos-Bourboulis conceptualized the study, contributed to data analysis and drafting of the manuscript, reviewed the manuscript, and approved the final version to be submitted.

Data Availability Statement

All data generated or analyzed during this study are included in this article. Further inquiries can be directed to the corresponding author.

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