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RESEARCH ARTICLE

Immunomodulatory Effects of *Bifidobacterium longum* W11 Produced Exopolysaccharide on Cytokine Production

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ARTICLE HISTORY

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Abstract: ***Background***: Health benefits, including immune modulating capability, exerted by *Bifidobacterium* strains have been attributed to their exopolysaccharides (EPSs).

Objective: The effects of the purified EPS from *B. longum* W11 on cytokine production by peripheral blood mononuclear cells (PBMCs) alone or ConA-stimulated were investigated.

Method: The production of IFN-γ, IL-1β, IL-6 and IL-10 by PBMCs from healthy adult donors was analysed using purified EPS at two different concentrations (100 μg/mL and 200 μg/mL) and ConA, as an immunopotentiating marker. Moreover, the monosaccharide composition of the EPS from *B. longum* W11 was detected using HPLC analysis.

Results: The results demonstrated the ability of purified EPS to increase the production of the tested cytokines, except IL-10, in ConA-stimulated PBMCs. In not-stimulated-PBMCs, EPS increased the production of IL-6 (at 200 μg/mL) and IL-10 (at 100 μg/mL). The HPLC analysis showed the presence of main monomers, galactose and glucose (ratio 1:1 wt/wt), and small amount of rhamnose.

Conclusion: The results of this study demonstrate the ability of the EPS produced by *B. longum* W11 to interact *in vitro* with the human PBMCs, showing an immune-regulatory profile alone and an immune stimulatory profile in ConA-stimulated PBMCs. This suggests putative applications for the EPS from *B. longum* W11 in different pathological conditions.

Keywords: *Bifidobacterium longum*, probiotic, exopolysaccharide, chromatographic analysis, immunomodulatory effects, cytokine production, PBMC, HPLC

1. INTRODUCTION

Bacterial exopolysaccharides (EPSs) are polymers of carbohydrates surrounding the cell wall; they are characterized by a definite primary structure from which some remarkable physicochemical and biological properties derive [1]. Most common monosaccharides that build these polymers are galactose, glucose, fructose, fucose and rhamnose, which are detected in half of *Bifidobacterium longum* strains. Moreover, EPS can be constituted of a single monosaccharide type (homopolysaccharides, HoPSs) or more than one type (heteropolysaccharides, HePSs) [2, 3]. EPSs are not normally used as an energy reservoir for the producing strains, but they play important functional roles as protection against adverse environmental and stressful conditions, cell-cell interaction and improving adherence and thus colonization [1, 4-9]. EPS synthesized by strains belonging to *Bifidobacterium* and *Lactobacillus* genera, with claimed probiotic attributes, are able to interact with specific receptors located on the eukaryotic cells, and could play an important role in the immune modulation capability of the producing strain, through innate and adaptive responses [4, 10-15]. Probiotic strains of *Bifidobacterium* and *Lactobacillus* have shown an interesting physiological function, mainly due to EPS production, which has an important role in host-interaction and in protection against pathogens [5, 7 10-23]. The production and chemical structural diversity of EPS from bifidobacteria and lactobacilli are highly dependent on the genotype of the producing strain; moreover, the resulting

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physicochemical features determine their biological functions [9, 13, 15, 24]. Therefore, it is very important that the strains currently used as probiotics for human application, must be genetically well-characterized, using different molecular methods [25, 26]. *Bifidobacterium longum* W11 is a strain from human origin (feces) widely employed in the most popular probiotic products and it has a long history of safe use and healthy properties [27-29]. Medina *et al.* (2007) [30], using Peripheral Blood Mononuclear cells (PBMCs), demonstrated that *B. longum* W11 cells could have an immunomodulatory activity, stimulating the production of T-helper 1 (Th-1) cytokines. Recent studies have demonstrated that EPS could play a relevant role in the immune modulation capability of the producing strain [2, 7, 11, 14]. However, no studies have been performed on the ability of this strain to synthesize EPS. In our previous studies, we sequenced the genome of *Bifidobacterium longum* W11 to investigate, by genotypic screening, its ability to produce EPS and to identify specific gene *cluster* predicted to be responsible for synthesis [31, 32].

Moreover, studying the biological properties of the EPS produced by *B. longum* W11, we demonstrated that it exhibited *in vitro* antioxidative activity, regulating cellular oxidative stress [32].

The aim of the present work was to investigate the immunomodulatory capability of EPS from *Bifidobacterium longum* W11 on human PBMCs and to analyze the monomer composition of the polymer using HPLC analysis.

2. MATERIALS AND METHOD

2.1. Bacterial Strain and Culture Conditions

The probiotic strain tested in this study was *Bifidobacterium longum* W11, marketed by Alfa Wasserman S.p.A. (Bologna, Italy) and produced by Probiotical (Italy). It was purchased in December 2013 and stored at -80°C in MRS broth (deMan, Rogosa & Sharpe; Sigma Aldrich, Milan, Italy) plus 0.25% L-cystein (MRSc, Sigma Aldrich, Italy) with 20% glycerol.

The strain was restored in MRSc agar at 37°C under anaerobic conditions using the AnaeroGen sachet (Oxoid, Rodano, Italy). After 48 h of incubation a colony was inoculated in 10 ml of MRSc broth at 37°C under anaerobic conditions. The overnight broth culture (200 µL) was spread on MRSc agar plates and incubated for 5 days at 37°C in anaerobic conditions to obtain the biomass for EPS extraction [33].

2.2. Chemical Characterization of EPS from *B. longum* W11

2.2.1. Exopolysaccharide Extraction and Purification

The extraction of EPS produced by *Bifidobacterium longum* W11 was carried out using the method described by Ruas-Madiedo *et al.* (2006) [33]. The cell biomass was collected from MRSc plates using 2 ml of ultrapure water (Biospa, Milan, Italy) and sterile L-shaped handles (Copan, Brescia, Italy). To release the EPS, from the cells surface, one volume of the collected biomass was added to one volume of NaOH solution (2M) and mixed at room temperature for 16 h, under constant stirring. The bacterial cells were removed by centrifugation (10414 × g, 30 min, 5°C) and, the solubilized EPS was precipitated by adding two volumes of absolute ethanol solution (96% V/V, at 4°C for 48-72 h) to one volume of the supernatant. The precipitated EPS was then collected by centrifugation (11063 × g, 30 min, 5°C), resuspended in ultrapure water and dialyzed (3 days at 4 °C), using dialysis tubes, with 12 kDa molecular mass cutoff (Sigma-Aldrich), and the dialysis water was changed twice daily.

The crude EPS solution was purified from protein and small molecules using the Sevag agent (chloroform : n-butanol = 4 : 1, V/V) followed by centrifugation (8000 × g, 10 min, 4°C) as described by Xu *et al.* (2011) [34]. The obtained EPS fraction was further purified of ionic compounds via ion-exchange resin (Dowex 50, Sigma Aldrich) [32]. The retentate purified EPS was collected, dialyzed as previously described and freeze-dried (Telstar LyoQuest-85 lyofilter).

2.2.2. High-Performance Liquid Chromatography

The purified EPS (1 mg) was hydrolysed using 1 mL of an aqueous trifluoroacetic acid (TFA) solution (2M) at 120°C for 3 h, according to the method described by Yang *et al.* (2010) [36]. After evaporating the TFA under nitrogen stream at 60°C, the residue was washed twice with pure water (1 mL), twice with methanol (1 mL), and then evaporated under a nitrogen stream.

The monosaccharide composition analysis of the EPS was carried out by High-Performance Liquid Chromatography (HPLC) using a Prevail Carbohydrate ES column (250 mm x 4.6 mm id; 5 µm; Alltech Associates, Deerfield, IL, USA) [37] coupled with the universal Evaporative Light Scattering Detector (ELSD) [38]. A sample of the purified-EPS (0.1 mg) was solubilized in ultrapure water (1 mL) and 20 µl of the solution was injected into the column for HPLC analysis. An acetonitrile-water (70:30, v/v) solution was used as the mobile phase at a constant flow rate of 0.8 mL/min. The composition of the mobile phase was kept constant during the analysis (isocratic elution). Identification and quantification of monosaccharides were performed by comparison with standards (100ppm) of arabinose, fructose, fucose, galactose, glucose, mannose, rhamnose and xylose (≥99.0%, Sigma Aldrich).

2.3. Effects of the EPSs from *B. longum* W11 on Cytokine Production by Human PBMCs

2.3.1. Isolation of Blood PBMCs

To determine the immune response elicited by the purified EPS of *Bifidobacterium longum* W11, we analyzed the cytokine pattern produced by human PBMCs from five healthy adult donors [13].

Heparinized blood was diluted with an equal volume of Dulbecco's Phosphate Buffered Saline (DPBS - Sigma Aldrich); then, 6 ml of the suspension was added carefully on top of 3 ml of Ficoll (Histopaque-1077, Sigma Aldrich). The gradient was made by centrifugation (500 g × 30 min) and the ring containing the PBMCs was carefully removed with a Pasteur pipette [39].

2.3.2. PBMCs Proliferation and Cytokine Measurement

The isolated PMBCs were washed with Dulbecco's Phosphate Buffered Saline (DPBS - Sigma Aldrich), counted in a Burker chamber and resuspended at a final concentration of 2×10^6 cells/mL in Roswell Park Memorial Institute medium (RPMI-1640, Sigma Aldrich), supplemented with 10% (v/v) heat-inactivated bovine fetal serum, L-glutamine (2 mM, Sigma Aldrich) and antibiotics (penicillin 100 U/ml, and streptomycin 100 µg/ml, Sigma Aldrich) [14]. Concanavalin A (Con A, SIGMA, St. Louis, USA), a T-cell mitogen from the jack bean, was used as an immunopotentiating marker to delineate the immunomodulating abilities of the EPS.

After sterilization (0.22 µm filter, Millipore, Italy) two different concentrations of EPS (100 µg/mL and 200 µg/mL) were added to the PBMC cultures without stimulus (PBMCs with RPMI medium) and to PBMCs stimulated with Concanavalin A (ConA, 4 µg/ml).

After 72 h of incubation, in 24 well plates (Thermo Fisher Scientific, USA), at 37°C in anaerobic atmosphere (5%, CO_2, Thermo Fisher Scientific CO_2 incubator, USA) the supernatant from each well was collected and stored at -80°C until cytokine determination. Every treatment was performed in duplicate.

The concentration of both pro-inflammatory (IFN-γ IL-1β and IL-6) and anti-inflammatory cytokines (IL-10) was analyzed using ELISA kits according to the manufacturer's instructions (eBiosciences, Prodotti Gianni, Milan, Italy). Absorbance of each well was measured at 450 nm using an ELISA reader (Epoch Microplate Spectrophotometer, Bio-Tek) [39].

2.3.3. Statistical Analysis

The results are presented as mean ± SD. The significance of the differences between each treatment and the control was assessed by Student's t Test (GraphPad Prism statistical software). P values < 0.05 were considered to be significant.

3. RESULTS AND DISCUSSION

3.1. Chemical Characterization of the EPS from *B. longum* W11

Chromatographic analysis is frequently used to detect the monomer composition of natural bioactive polysaccharides, but the results could be influenced by the column and the detector chosen for the analysis [40-43]. The HPLC chromatogram of the purified and hydrolyzed EPS, obtained using the ELSD detector and Prevail Carbohydrate ES column, is shown in Fig. (**1**). The chromatogram showed two main very close peaks (Fig. **1**). According to the elution curve of the standards, under identical analytical conditions, these peaks were associated with galactose (retention time 7.8 min) and glucose (retention time 8.3 min). The other peak appeared in the chromatogram may be due to a small amount of rhamnose, which is synthesized in half of *B. longum* HePS. Previously obtained results from this analytical procedure recently confirmed our further chemical analytical analysis performed by size exclusion chromatography (SEC) coupled with a multi-angle laser light scattering (MALLS) detector, gas-chromatography and mass spectrometry (GC-MS) and NMR analysis [32].

Galactose and glucose were present in most of the *B. longum* strains in variable proportions. In this study, by peak integration, the quantitative determination revealed the presence of galactose and glucose in a ratio 1:1 wt/wt.

3.2. Effects of EPS from *B. longum* W11 on Cytokine Production by Human PBMCs

The effects of purified EPS from *B. longum* W11 at two different concentrations (100µg/mL and 200µg/mL) on cy-

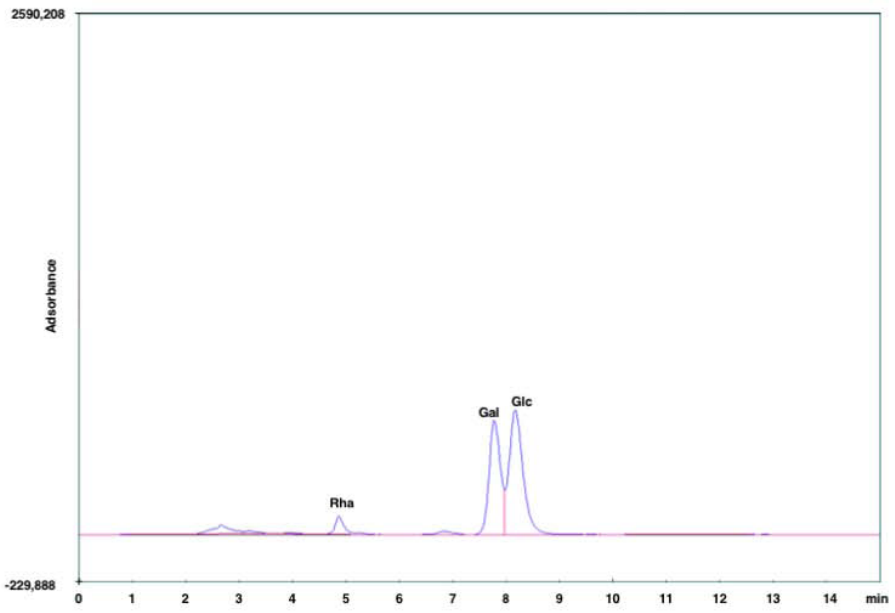

Fig. (1). HPLC chromatogram of the EPS produced by *Bifidobacterium longum* W11 on Prevail Carbohydrate ES column.

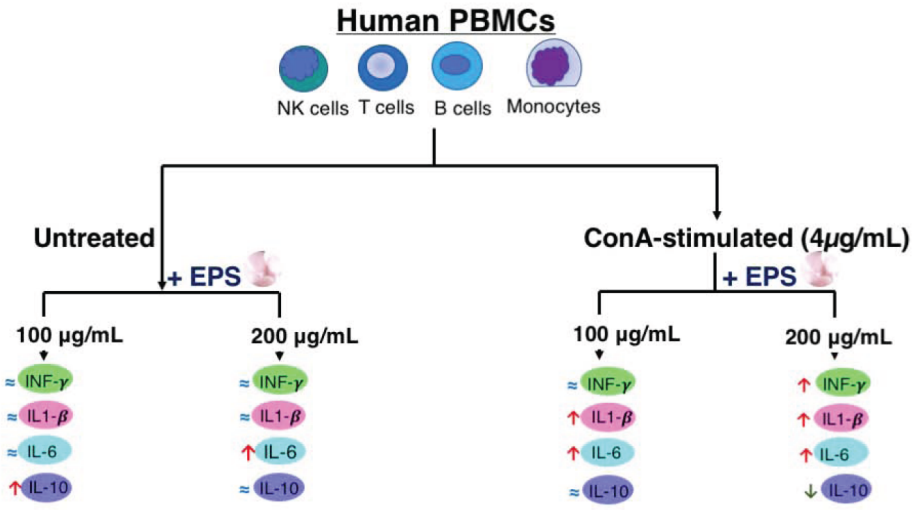

Fig. (2). Summary scheme of the cytokine pattern produced by human PBMCs with or without stimulus as immune response elicited by the EPS of *Bifidobacterium longum* W11.

tokine production by PBMCs alone or stimulated with ConA are summarized in Fig. (**2**).

Cytokines participate in many physiological processes, including the regulation of immune and inflammatory responses. Either excessive or insufficient production of cytokines may contribute significantly to the pathophysiology of a range of diseases. In this study, we measured the concentration of INF-γ, IL-1β, IL-6 and IL-10 after the addition of EPS alone at two different concentrations and after the addition of EPS in the presence of ConA in order to obtain an immune potentiation.

No statistical differences were detected for INF-γ levels (Fig. **3-A**) in the presence of the purified-EPS at both concentrations (100μg/mL and 200μg/mL), compared to the control (PMBCs with RPMI medium). By contrast, in ConA-stimulated PBMCs, the levels of INF-γ (Fig. **3-A$_1$**) were significantly increased ($p<0.05$) in the presence of purified EPS at the higher concentration (200μg/mL), compared to the positive control (PMBCs treated with ConA). Therefore, our results obtained using EPS partially confirm the results of Medina *et al.* (2007) [30] who demonstrated for live *B. longum* W11, a significantly higher ($p<0.05$) ability to induce the production of INF-γ, compared to non-stimulated and LPS-stimulated PBMCs.

No statistical differences were detected for IL1-β levels (Fig. **3-B**) in the presence of purified EPS at both concentrations, compared to the control (PMBCs with RPMI medium). In ConA-stimulated PBMCs, the levels of IL1-β (Fig. **3-B$_1$**) in the presence of both 100 and 200 μg/mL of purified EPS, were observed to be significantly higher ($p<0.05$ and $p<0.01$, respectively) compared to the positive control (PMBCs treated with ConA).

In not-stimulated-PBMCs, IL-6 levels (Fig. **3-C**) were significantly increased ($p<0.05$) in the presence of purified EPS at the higher concentration (200μg/mL) and IL-10 levels (Fig. **3-D**) were significantly increased ($p<0.05$) at the lower concentration of EPS (100μg/mL), compared to the control (PMBCs with RPMI medium). Our results contrasted with those obtained by Wu *et al.* (2010) [44] using EPS (5μg/mL) produced by *Bifidobacterium longum* BCRC 1434 on murine macrophage cell line J77A.1. Moreover, in ConA-stimulated PBMCs, the levels of IL-6 (Fig. **3-C$_1$**) were significantly increased in the presence of purified EPS at 100μg/mL ($p<0.01$) and at 200μg/mL ($p<0.001$ vs $p<0.05$), compared to the control (PMBCs treated with ConA). On the contrary, in ConA-stimulated PBMCs, the levels of IL-10 (Fig. **3-D$_1$**) significantly ($p<0.01$) decreased at the concentration of 200μg/mL, compared to the control (PMBCs treated with ConA).

Although IL-6 was originally considered a pro-inflammatory cytokine, several studies indicated that it also has anti-inflammatory properties [45, 46]. In this *in vitro* study, the modest but significant increase of IL-10 together with the increase of IL-6 suggested a potential immunomodulatory effect for EPS. Instead, when the EPS was incubated together with ConA-stimulated PBMCs, it potentiated the effects of ConA alone by increasing the IL-6 and IL-1β levels, also at the lower concentration. Nevertheless, these effects are self-limited by the fact that increase in IFN-γ and decrease in IL-10 are observed only at the higher concentration of EPS.

IFNγ plays a critical and physiologically relevant role in promoting host resistance to microbial infection. The IL-10 reduction observed in the presence of ConA could be attributed to the IFNγ increase that switches *versus* a Th1 response with a reduction of Th2.

Considering our results, the EPS produced by *B. longum* W11 could provide protection against early stages of infection via Th-1 production, in agreement with the results reported by Medina *et al.* (2007) [30], using live *B. longum* W11 cells.

This observational and preliminary study needs to be continued to better understand the immunomodulation mechanism of EPS produced by *B. longum* W11 such as identification of the immune receptor or the activated pathway. Moreover, to better understand the type of Th response

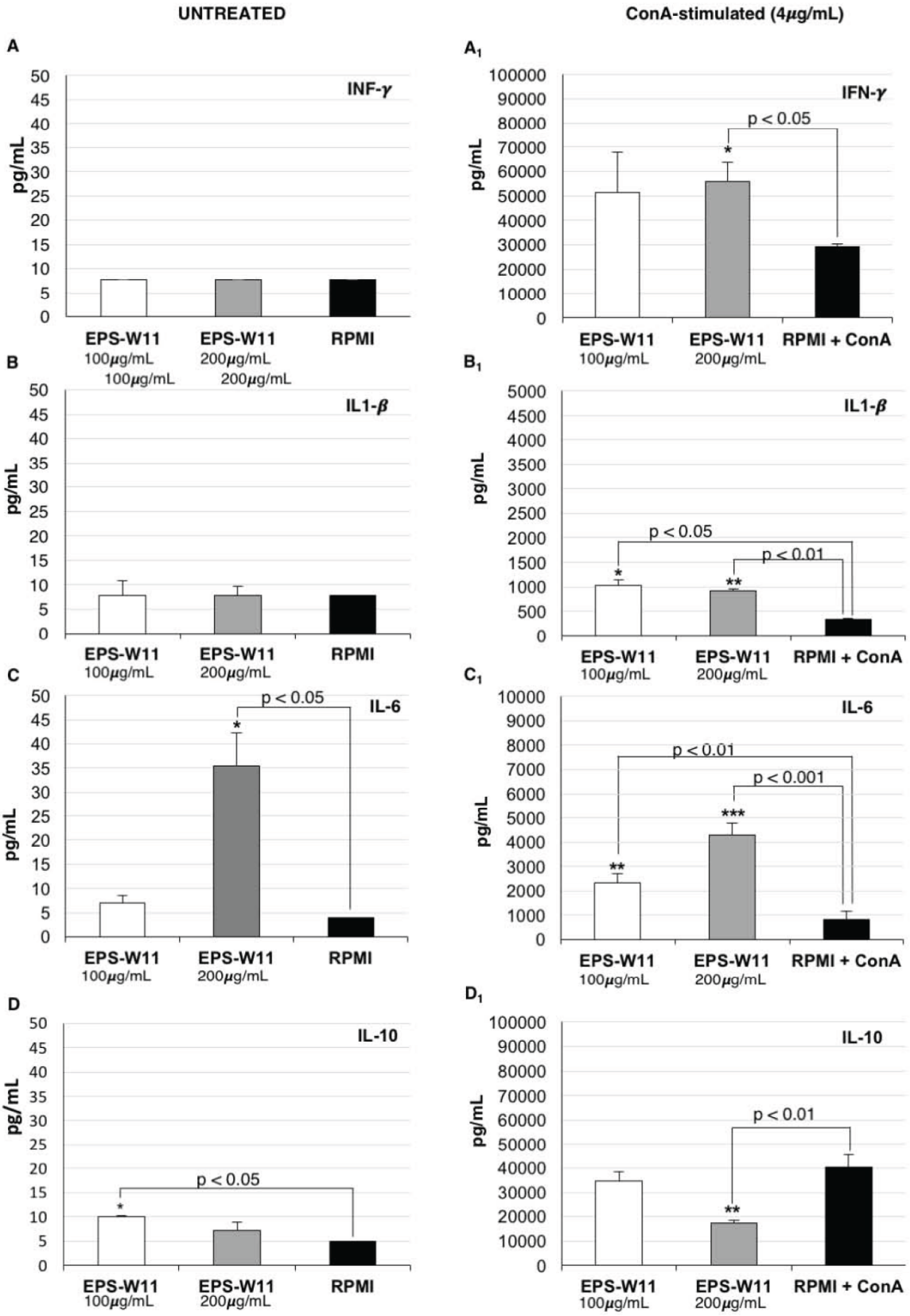

Fig. (3). Effects on cytokine production by not-stimulated PBMCs and ConA-stimulated-PBMCs cultivated for 72 h with 100 μg/mL (white bars) or 200 μg/mL (grey bars) of purified EPSs from *B. longum* W11. Controls (black bars): not-stimulated-PMBCs (RPMI) and ConA-stimulated-PBMCs (RPMI + ConA). The significant differences of the cytokine levels compared to the control are indicated by asterisks (*p<0.05, **p<0.01, ***p<0.001).

that the EPS produced by *B. longum* W11 may promote, it could be useful to study the production of other cytokines such as IL-12 and TNF-α. In fact, the ratios between some cytokines (IL-12/IL-10), (IL-10/TNF-α) and (IL-1β/IL-12) are relevant for the differentiation of Th1, Th2 and Th17 cells, respectively [2, 14].

CONCLUSION

The EPSs from the strains of *Bifidobacterium* spp. are based on basic monosaccharides such as glucose, galactose and fructose; furthermore, in the primary structure of polysaccharides, other non-carbohydrate groups could be present, linked to hydroxyl, carboxyl or amine groups [1, 12, 31].

In the present study, the methodologies used for the EPS purification [32, 33] and the HPLC analysis revealed the presence of galactose and glucose monomers as the main components of *B. longum* W11 HePS, and rhamnose to a lesser extent.

The effects of the EPS produced by *B. longum* W11 on the immune response of PBMCs with or without stimulus were investigated. In particular, in not-stimulated-PBMCs, the EPS induced the production of IL-6 at the higher concentration tested and IL-10 only at the lower concentration tested. Moreover, using ConA-stimulated PBMCs, the EPS increased the production of the tested cytokines, except IL-10.

Overall, EPS isolated from *B. longum* W11 added to human PBMCs showed an immune regulatory profile when administered alone, and an immune stimulatory profile when administered in addition to a co-stimulus such as ConA used to induce an immune potentiation.

Although there is evidence supporting this hypothesis, further research would be useful to demonstrate which are the key parameters determining the ability of EPS to modulate the immune response and also to investigate the putative mechanisms of action.

In conclusion, the results of this study could be useful to better understand the putative application of *B. longum* W11 and its EPS in different pathological conditions.

ETHICS APPROVAL AND CONSENT TO PARTICIPATE

Not applicable.

HUMAN AND ANIMAL RIGHTS

No Animals/Humans were used for studies that are base of this research.

CONSENT FOR PUBLICATION

Not applicable.

CONFLICT OF INTEREST

The authors declare no conflict of interest, financial or otherwise.

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