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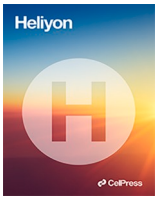

Research article

Anti-inflammatory effects in LPS-induced macrophages and antibiofilm activity of the mannose-rich exopolysaccharide produced by *Bacillus licheniformis* B3-15

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ABSTRACT

The mannose-rich exopolysaccharide EPS B3-15, produced by the thermophilic *Bacillus licheniformis* B3-15, was previously reported to possess promising potentialities as antiviral and immunomodulatory agent, and in preventing the adhesion of *Pseudomonas aeruginosa* and *Staphylococcus aureus*. In this study, EPS B3-15 was evaluated for its anti-inflammatory activity in LPS-induced macrophages and the ability to contrast the adhesion of *Klebsiella pneumoniae* and *Streptococcus pneumoniae* as pathogenic bacteria of the respiratory tract. Without affecting the macrophages viability, the EPS at low concentration (300 μg/mL) significantly downregulated the gene expression of iNOS and the consequent NO generation, and it also decreased the production of pro-inflammatory cytokines. Moreover, the EPS reduced the adhesion of *Str. pneumoniae* (47 %) more efficiently than *K. pneumoniae* (38 %), due to its ability to modify the abiotic surfaces properties and alter the charges of bacterial-cell surface of Gram-positive more than Gram-negative. As able to reduce the inflammatory responses in macrophage cells and simultaneously prevent biofilm-related to the respiratory tract infections, EPS B3-15 could have potential use as nasal spray with anti-inflammatory action and surface-coating agent for medical devices.

1. Introduction

Inflammation, characterized by redness, heat, swelling, and pain, is a physiological response of the host due to microbial infections, toxic compounds and damaged cells, to restore injured tissues and initiate a healing process [1,2]. The inflammatory response implicates a series of events, such as vascular, molecular, and cellular processes, able to modulate activation of signaling pathways that control inflammatory cytokine levels in tissue-resident macrophages and inflammatory cells recruited from the blood [3,4]. The modulation of signaling pathways contributes to the restoration of tissue homeostasis and to the resolution of acute inflammation, that if uncontrolled could lead to the onset of several chronic inflammatory diseases [5], including asthma [6], rheumatoid arthritis [7], Alzheimer's disease [8] and cancer [9].

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Bacterial infection is the consequence of an active proliferation of microorganisms in the tissues, able to induce the inflammatory reaction. Biofilms greatly increase the tolerance of bacteria to antibiotics and cause harmful changes in the host itself. The steps of inflammation are the infiltration of the primary inflammatory cells, for example macrophages, lymphocytes, and plasma cells in the tissue location, producing inflammatory cytokines, enzymes, growth factors to the progression of tissue damage and reparation. Nonsteroidal and steroidal anti-inflammatory drugs can be used to hinder inflammatory disorders; however, their excessive consumption can cause side effects, including gastrointestinal complications and reduced resistance to infections and ulcers [10,11]. In this context, in the last years several researchers have focused their attention on natural polymers, such as bacterial exopolysaccharides (EPSs), since they are considered safe, biodegradable, and biocompatible and possess various bioactivities, including antibacterial, antifungal, antiviral, antioxidant, antitumor, antidiabetic, antiulcer, anticoagulant, antiaging, cholesterol-lowering, immunomodulatory and wound healing [12,13]. The composition of bacterial exopolysaccharides can vary greatly, as they can be homo- or hetero-polysaccharides and contain different chemical groups (such as sulphate, phosphate, acetic and acetylated acid) and even small proportions of proteins and uronic acid, offering a broad spectrum of properties and therefore biological activities. EPSs from marine bacteria, greatly differing in their composition and biological activities, have been the subject of many reviews [14–16]; however, only a few of them focused on immunomodulatory activity. EPSs help bacterial colonization and biofilm formation, offering in situ protection to bacteria from stress factors, or as adaptative strategy in extreme environments like in the case of acidophilic and thermophilic bacteria. EPSs produced by the thermophilic *Bacillus licheniformis* strain B3-15 [17], *Geobacillus thermodenitrificans* strain B3-72 [18], and *B. licheniformis* strain T14 [19], isolated from the shallow hydrothermal system of Eolian Islands (Italy), were reported among the few polymers until now obtained from marine bacteria with antiviral and immunomodulatory activity [20–22]. Our previous studies reported that EPS B3-15 was able to prevent the adhesion and biofilm formation of *Pseudomonas aeruginosa* ATCC 27853 and *Staphylococcus aureus* ATCC 29213 on various abiotic surfaces (*e.g.*, polystyrene and medical devices), without exerting any antibiotic activity or interfering with quorum sensing [23,24]. Furthermore, this EPS reduced the adhesion of *P. aeruginosa* (five-log scale) and *S. aureus* (one-log scale) on human nasal epithelial cells, prospecting it as a promising agent for the prevention of biofilm-related infections [24]. These activities were related to the EPS B3-15 chemical composition, which was reported as mainly constituted by carbohydrates (67 %) (as a disaccharide repeating unit with a manno-pyranosidic configuration) and low content of proteins (5 %) (attributed to the poly-gamma glutamic acid) [25].

In the ongoing search for natural products with suitable safe properties that may inhibit inflammatory disorders and contextually prevent biofilm-related infections, in this study EPS B3-15 was evaluated for its anti-inflammatory activities on RAW 264.7 macrophages, and the ability to contrast the adhesion of *Klebsiella pneumoniae* and *Streptococcus pneumoniae* as pathogenic bacteria of the respiratory tract.

2. Materials and methods

2.1. Bacillus licheniformis strain B3-15 and EPS B3-15 production

Bacillus licheniformis strain B3-15, isolated from a shallow hydrothermal vent at the Porto di Levante of Vulcano Island, Italy) grew aerobically from 25 to 60 °C, with optimal temperature occurred at 45 °C, and it was able to produce the exopolymer EPS B3-15 [17]. To produce EPS B3-15, the strain was cultivated in a 1 L flask containing 250 mL of the medium SG17 (amended with 5 % glucose) and incubated at 45 °C for 48 h, under agitation at 250 rpm [25]. The culture was centrifuged (9318×*g* rpm for 10 min), the cell-free supernatant was treated with absolute ethanol (1:1, v/v), and the precipitated EPS was dialyzed and finally freeze-dried. Crude EPS had a high carbohydrate content (67 %), mannose being the major component, and a low protein content (5 %), mainly attributed to the poly-gamma glutamic acid constituent [25]. The EPS, dissolved in phosphate buffer saline (PBS, Thermo Fisher Scientific, Milan, Italy), was sterilized by filtration through a (0.2 μm pore size) nitrocellulose membrane (Biogenerica, Catania, Italy).

2.2. Cell cultures

The cell line used in this study, mouse leukemic monocyte-macrophage (RAW 264.7), was obtained from Sigma- Aldrich (Merk Life Science S.r.l., Milan, Italy). RAW 264.7 cells were cultured in DMEM High Glucose (Merk Life Science S.r.l., Milan, Italy) supplemented with 2 mM L-Glutamine (Euroclone, Milan, Italy), 10 % Fetal Bovine Serum (FBS) (Merk Life Science S.r.l., Milan, Italy) and penicillin/streptomycin/amphotericin (PSA) (Merk Life Science S.r.l., Milan, Italy), and maintained at 37 °C in a humidified atmosphere containing 5 % CO_2. The medium was replaced twice a week, and cells were split at about 80 % confluence.

Before starting the treatment with EPS B3-15, Raw 264.7 cells were induced to inflammation by adding LPS (1 μg/mL, O26:B6 *E. coli*) (Merk Life Science) for 24 h. The following day, LPS was removed, and the medium was replaced with fresh medium containing the EPS at different concentrations (50, 150 and 300 μg/mL). After one or 6 days, the medium was removed, and further analyses were carried out.

2.3. Cell viability assays

To evaluate the cytotoxic effect of EPS B3-15, a colorimetric assay based on MTT [3-(4,5- dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] (Merk Life Science S.r.l., Milan, Italy), was used as previously reported [26].

To induce inflammation, 5×10^3 cells/well were cultured in 96 wells and after 24 h, a fresh medium containing LPS (1 μg/mL) was added for 24 h, and then viable cells were counted.

LPS was removed and fresh medium containing EPS B3-15 at different concentrations (from 50 to 300 μg/mL) was added. After one and 6 days of incubation, the medium was removed, cells were washed and incubated with 200 μL of MTT solution (1 mg/mL in FBS-free medium) for 2 h in the presence of 5 % CO_2 at 37 °C. After incubation, MTT solution was removed, each well washed twice with cold PBS, and the formazan crystals were dissolved in DMSO (200 μL/well). Finally, the optical density (OD_{570nm}) was measured using a synergy HT plate reader (BioTek Instruments, Inc., Winooski, VT, United States).

2.4. Anti-inflammatory activity of EPS B3-15

The anti-inflammatory activity of EPS B3-15 was evaluated by measuring the production of NO• and gene expression level of specific inflammatory mediators.

To evaluate the inhibitory effect of EPS on NO• level production, we performed the Griess reagent assay (Merk Life Science), as previously described [26]. Briefly, LPS-induced RAW 264.7 cells were cultured in a medium containing EPS B3-15 at different concentrations for one and 6 days. After treatments, 100 μL of the culture medium was mixed with an equal volume of Griess reagent and incubated at room temperature for 10 min. The nitrite amount produced in culture medium was determined spectrophotometrically at 540 nm, using a synergy HT plate reader (BioTek Instruments, Inc.).

The expression of genes related to specific inflammatory mediators was achieved by qRT-PCR analyses. In Table 1 are reported the sequences of primers of each molecular endpoints examined, designed on exon/exon junction of the mRNA by using primer blast. Results were normalized to the levels of Glyceraldehyde 3-Phosphate Dehydrogenase (GAPDH).

Total RNA from LPS-induced RAW 264.7 treated with EPS B3-15, was extracted using Rneasy Mini Isolation Kit (Qiagen, Germantown, MD, USA) and quantified (in triplicate) as already reported [27]. cDNA was reverse transcribed from 1 μg of RNA by ImProm-II Reverse Transcription System (Promega, Milan, Italy). qRT-PCR reaction was carried out with the Sso Advanced universal SYBR1 Green supermix (BioRad, Laboratories, Heracles, CA, USA), using a 7500 Fast Real-Time PCR System (Applied Biosystems). Each sample was examined in triplicate and gene expression was evaluated using the $2^{-\Delta\Delta Ct}$ method.

2.5. Bacterial pathogens

Klebsiella pneumoniae (*K. pneumoniae*) ATCC 8047 and *Streptococcus pneumoniae* (*Str. pneumoniae)* ATCC 6303 were purchased from the American Type Culture Collection (LGC Promochem, Milan, Italy). *K. pneumoniae* ATCC 8047 was routinely maintained into Luria Bertani broth (LB, Sigma Aldrich) and 2 % agarized LB (LA), and *Str. pneumoniae* ATCC 6303 was grown in Brain heart infusion (BHI, Sigma Aldrich) broth. *Pseudomonas aeruginosa* (*P. aeruginosa*) ATCC 27853, maintained into LB and LA, and *Staphylococcus aureus* (*S. aureus*) ATCC 29213, grown in Tryptic Soy Broth (TSB, Sigma Aldrich) and Tryptic Soy Agar (TSA, Sigma Aldrich), were used as comparison strains. All strains were kept frozen at −80 °C in 20 % (v/v) glycerol for long term storage.

2.6. Antibiofilm activity of EPS B3-15

The effects of EPS B3-15 on biofilm formation of *K. pneumoniae* and *Str. pneumoniae* was evaluated in 96-well microtiter plates (Falcon®, Fisher Scientific, Milan, Italy) [28]. Overnight cultures (180 μL) of *K. pneumoniae* or *Str. pneumoniae* (six replicates, $OD_{600nm} = 0.1$ corresponding to 4.5×10^7 CFU/mL) were added to each well, and EPS dissolved in PBS (20 μL) was added to each well at different final concentrations (50, 100, 200 and 300 μg/mL), or PBS (20 μL) as a control. After 48 h or 24 h (for *K. pneumoniae* and *Str. pneumoniae*, respectively) at 37 °C, the microtiter plates were washed five times with distilled water to remove non-adherent bacteria. Each biofilm mass was stained with crystal violet (CV, 0.1 %, w/v) for 20 min and, after removing excess CV by aspiration, the plates were washed (5 times) and air dried (for 45 min). The biofilm mass was determined by measuring the CV content in the de-staining solution, using a microtiter plate reader (OD = 585 nm), (Multiskan GO, Thermo Scientific, Waltham, MA, USA). Biofilm formation reduction (%) was calculated as follows:

$$\text{Reduction of biofilm formation } (\%) = \frac{\text{OD585nm control} - \text{OD585nm sample}}{\text{OD585nm control}} \times 100$$

The standard deviation (±SD) of each data point was calculated by averaging six replicates. Statistical significance ($^*p \leq 0.05$ and $^{**}p \leq 0.01$) was determined by one-way ANOVA.

Table 1
qRT-PCR target genes and primer sequences.

GENE	Forward	Reverse
GAPDH	ATGGTGAAGGTCGGTGTGAA	TGGAAGATGGTGATGGGCTT
IL1β	ACTCATTGTGGCTGTGGAGA	TTGTTCATCTCGGAGCCTGT
IL6	GCCAGAGTCCTTCAGAGAGA	ATGGTCTTGGTCCTTAGCCA
iNOS	TCTTGGAGCGAGTTGTGGAT	TGACACAAGGCCTCCAATCT
COX2	CCCCTCTCTACGCATTCTGT	TGGCAGAACGACTCGGTTAT

2.7. Antibacterial activity

The antibacterial activity of EPS was evaluated to verify whether the inhibition of bacterial biofilm formation induced by EPS was due to the reduction of cell viability. The effects of EPS on bacterial growth were evaluated spectrophotometrically as reported by Zammuto et al. [23], using EPS solution in PBS (300 μg/mL) and PBS as a control.

2.8. Surface coating assay

To test the effect of EPS B3-15 on the polystyrene surface, aliquots (20 μL) of the EPS solution in PBS (final concentration of 300 μg/mL) or PBS (20 μL) used as a control, were poured into each well of a 24-well polystyrene plate (Falcon #353047). The plates were incubated at 37 °C for 30 min to allow the liquid to evaporate completely. One milliliter (mL) of diluted overnight bacterial culture (10^5 CFU/mL) in LB (for *K. pneumoniae*) or BHI (for *Str. pneumoniae*) was added to each well. Following an 18-h static incubation period at 37 °C, the wells were gently emptied, cleaned with distilled water, and stained with 1 mL of 0.1 % crystal violet solution.

2.9. Cell-surface charges and hydrophobicity properties

The effects of the EPS B3-15 on the charges and the hydrophobicity of *K. pneumoniae* and *Str. pneumoniae* cell surface were evaluated by the microbial adhesion to hydrocarbons (MATH) as reported by Bellon-Fontaine et al. [29]. Bacterial cultures (10 mL), grown overnight in LB or BHI at 37 °C, were centrifuged at (6000 × rpm for 10 min) and the pellets were recovered and suspended in sterile PBS. Each bacterial suspension (9 mL) was treated with 1 ml EPS B3-15 solution in PBS (final concentration 300 μg/mL) or PBS for 30 min. The untreated or EPS-treated bacterial suspension (3 mL) was poured into the tube, and an aliquot (0.4 mL) of each solvent

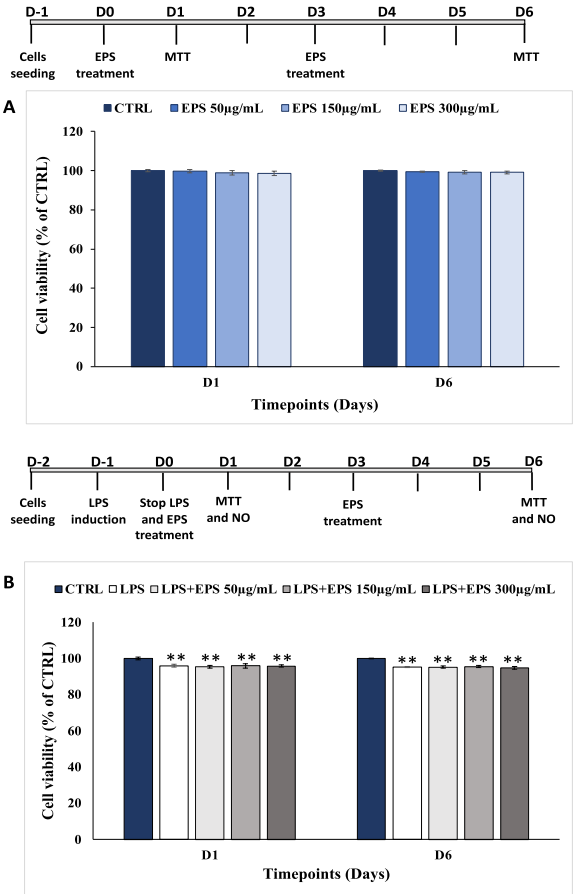

Fig. 1. Cytotoxic effect of EPS B3-15 on Raw 264.7 and LPS-induced Raw 264.7 cells. (A) MTT test performed on Raw 264.7 without LPS stimulation and treated with EPS B3-15 (50, 150 and 300 μg/mL) for 1 and 6 days. (B) MTT test performed on Raw 264.7 stimulated with LPS and treated with EPS B3-15 (50, 150 and 300 μg/mL). CTRL (untreated Raw 264.7 cells), EPS 50, 150 and 300 μg/mL (Raw264.7 cells + of EPS B3-15 at different concentrations), LPS (LPS-induced Raw 264.7 cells), LPS + EPS (LPS-induced Raw 264.7 cells + EPS B3-15 at different concentrations). Data are represented as the mean% ± SD of three independent experiments. $^{**}p \leq 0.01$ indicated significant differences between the EPS at different concentrations and the CTRL, as reported by the Holm *post hoc* test.

(ethyl acetate, chloroform, decane or hexadecane, Sigma Aldrich) was added. The tubes were vortexed for 1 min and placed in static at room temperature until phase separation. The affinity for hydrocarbons (expressed as percentage) was calculated by measuring the absorbance (OD_{400nm}) of the aqueous phase of each tube (A1):

$$\% \text{ Affinity} = (A0 - A1/A0) \times 100 \quad (1)$$

Three independent experiments were carried out.

2.10. Statistical analysis

Statistical analysis was performed by One-way and Two-way ANOVA followed by Holm multiple comparison test or Tukey test where appropriate to assess the significative differences ($^*p \leq 0.05$) between the various groups.

3. Results

3.1. Evaluation of EPS B3-15 on cell viability of Raw 264.7 with and without LPS stimulation

The cytotoxic effects of EPS B3-15 at different concentrations, evaluated using a cell viability assay on both Raw 264.7 cells and LPS-induced Raw 264.7 cells, are reported in Fig. 1.

Compared to the untreated cells used as control (CTRL), cells treated with EPS B3-15 at different concentrations did not exhibit significant cytotoxic effects on Raw 264.7 cells after 24h (D1) and 6 days (D6) from the treatment (Fig. 1A). Differently, the EPS B3-15 treated LPS-induced Raw 264.7 cells as well as the untreated LPS-induced Raw 264.7 cells (LPS) displayed a significant ($^{**}p \leq 0.01$), cell viability reduction compared to CTRL at both the tested timepoints. These data indicate that this slight reduction can be attributed to the LPS induction and not to the EPS treatment (Fig. 1B).

3.2. Evaluation of the anti-inflammatory activity of EPS B3-15 on LPS-induced Raw 264.7 cells

In LPS-stimulated Raw 264.7 (LPS) the nitrite levels increase of about 30 % in culture medium after 24 h from induction, compared to the control not stimulated (CTRL) (100 ± 0.4 % vs 69.9 ± 0.3 %), indicating the induction of the inflammatory condition (Fig. 2A).

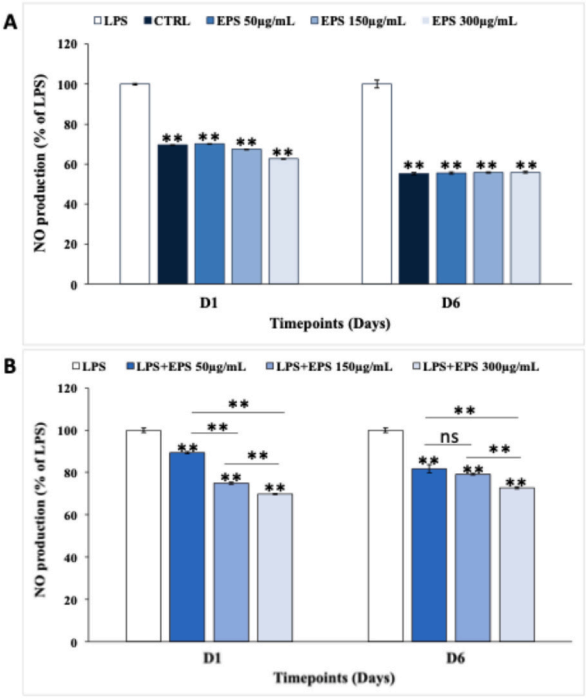

Fig. 2. Effect of EPS B3-15 on NO production in (A) Raw 264.7 cells, and (B) LPS-induced Raw 264.7 cells. NO production was measured by Griess assay performed on Raw 264.7 cells stimulated with 1 µg/mL of LPS for 24 h, and treatment with EPS B3-15 (50, 150 and 300 µg/mL) for 1 and 6 days. CTRL (untreated Raw 264.7 cells), EPS 50, 150 and 300 µg/mL (Raw264.7 cells + of EPS B3-15 at different concentrations), LPS (LPS-induced Raw 264.7 cells), LPS + EPS (LPS-induced Raw 264.7 cells treated with EPS B3-15 at different concentrations. Data are represented as the mean% ± SD of three independent experiments. $^{**}p \leq 0.01$ indicated significant differences between the EPS at different concentrations and the LPS, as reported by the Holm *post hoc* test. ns = not significant.

Furthermore, Raw 264.7 cells not stimulated with LPS and treated with EPS B3-15 at different concentrations did not show any difference in NO production compared to the control.

Contrarily, in EPS B3-15 (50, 150 and 300 μg/mL) treated LPS- stimulated cells the NO production decreases significantly, in dose-dependent manner, compared to the LPS-induced condition at both analyzed timepoints (D1 and D6) (Fig. 2B). Specifically, after 24h (D1) of EPS B3-15 (50, 150 and 300 μg/mL) treatment NO levels in culture media were 89.4 ± 0.4 %, 75 ± 0.3 % and 69.8 ± 0.4 %, respectively (Fig. 2B, left side). After 6 days (D6) of treatment with EPS 50, 150 and 300 μg/mL the nitrite levels were 81.8 ± 0.4 %, 79.1 ± 0.5 %, 72.9 ± 0.7 %, respectively (Fig. 2B, right side).

3.3. Inflammatory mediators' production in LPS-induced Raw 264.7 cells before and after EPS B3-15 treatment

The inflammatory mediator's gene expression profile of LPS- stimulated Raw 264.7 cells compared to the control cells are reported in Fig. 3 mRNA levels encoding IL6, IL1β, iNOS, and COX2 significantly increased after LPS stimulation, at both the analyzed time points (Fig. 3). In detail, the mRNA upregulation levels were the following: 15.0-fold and 6.4-fold for IL-6, 1.8-fold and 1.5-fold for IL-1β, 2.8-fold and 4.1-fold for iNOS, 1.9-fold and 1.2- fold for COX2, at D1 and D6 respectively.

Prior to evaluating the immunomodulatory effects of the EPS B3-15 on LPS-induced Raw264.7 cells, the expression of the genes IL-6, iNOS, IL-1β and COX2 was evaluated in Raw264.7 macrophages treated with EPS at different concentrations (Fig. 4A–D). As it is possible to note, at both the analyzed timepoints, IL6 and IL1β expression levels (Fig. 4A,C) did not show any significant difference compared to the untreated control (CTRL). Unlike, a significant reduction of iNOS and COX2 expression levels (Fig. 4B,D) was highlighted after 6 days of treatment with EPS B3-15, at all the tested concentrations. Differently, LPS-induced Raw 264.7 cells treated with EPS B3-15 for one and 6 days showed a significant downregulation (*$p \leq 0.05$ and **$p \leq 0.01$) of the cytokines (IL6 and IL1β) (Fig. 4A,C) and pro-inflammatory enzymes (iNOS and COX2) expression levels (Fig. 4B,D), compared to the LPS-stimulated cells (Fig. 4).

Precisely, data reported in Fig. 4(A–D) displayed that the treatment with EPS B3-15, at all the analyzed concentrations and timepoints, strongly reduced the production of inflammatory mediators to the same expression levels, or even less, than cells not stimulated with LPS (CTRL), except that IL6, even if the effect was more evident after 6 days of treatment.

3.4. EPS B3-15 antibiofilm activity

The effects of EPS B3-15 addition at different concentrations (from 50 to 300 μg/mL) on the biofilm formation of *K. pneumoniae* and *Str. pneumoniae,* in comparison with *P. aeruginosa* and *S. aureus*, as model strains, are reported in Fig. 5.

The EPS B3-15 reduced the biofilm formation of both Gram-negative and Gram-positive strains in a dose-dependent manner, being

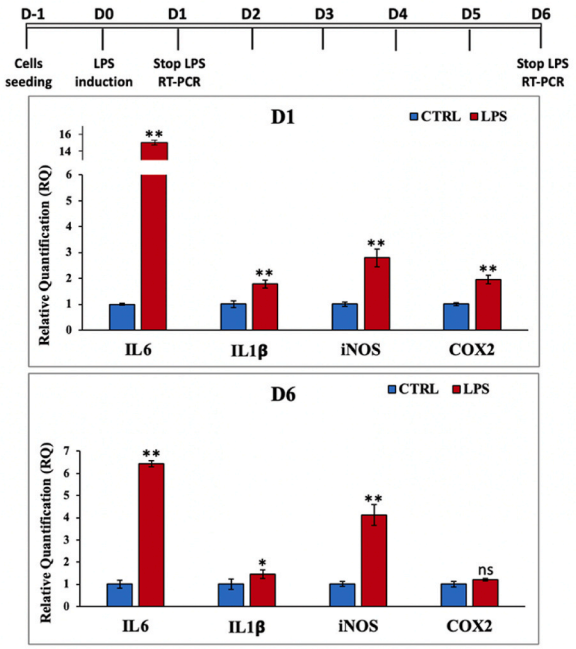

Fig. 3. Effects of LPS stimulation on inflammatory mediators' production in Raw 264.7 cells. IL6, IL1β, iNOS and COX2 mRNA levels were determined by qRT-PCR, performed on Raw 264.7 cells stimulated with 1 μg/mL of LPS for 24 h (D1), and quantified after 1- (D1) and 6 days (6D) post-induction. Data are represented as the mean ± SD of three independent experiments. **$p \leq 0.01$ and *$p \leq 0.05$ indicated significant differences between the CTRL (cells not stimulated with LPS) and LPS (LPS-induced Raw 264.7 cells) as reported by the Holm *post hoc* test. ns = not significant.

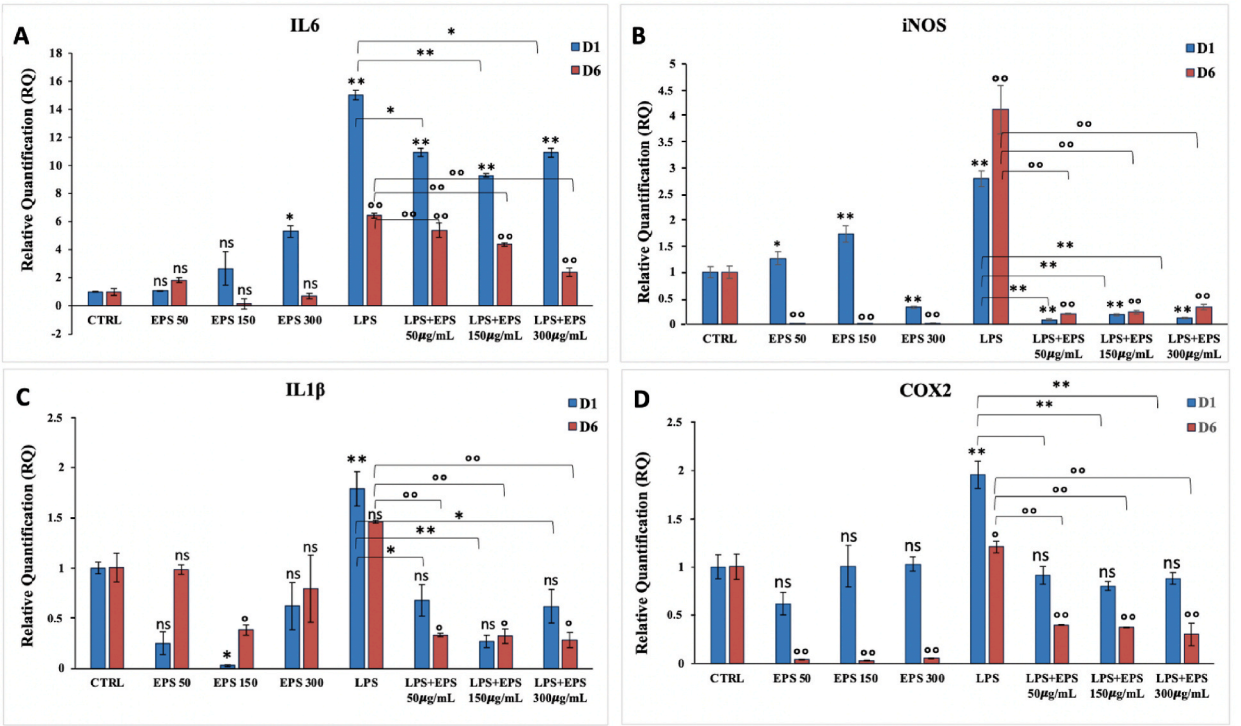

Fig. 4. Inflammatory mediator's gene expression profile of (A) IL-6, (B) iNOS, (C) IL-1β and (D) COX2 performed on EPS-treated Raw 264.7 cells, LPS-induced Raw 264.7, LPS-induced and EPS-treated Raw 264.7 cells. IL6, IL1β, iNOS, COX2 and GAPDH mRNA levels were determined by qRT-PCR performed on Raw 264.7 cells stimulated with 1 μg/mL of LPS for 24 h, and treatment with EPS B3-15 (50, 150 and 300 μg/mL) for 1 and 6 days. CTRL (cells not stimulated with LPS), EPS 50, 150 and 300 (cells not stimulated with LPS and treated with EPS at different concentrations), LPS (LPS-induced Raw 264.7 cells), LPS + EPS (LPS-induced Raw 264.7 cells treated with EPS at different concentrations). Data are represented as the mean% ± SD of three independent experiments. **, $p \leq 0.01$ and *, $p \leq 0.05$ indicated significant differences between the several groups and the CTRL, as reported by the Holm *post hoc* test.

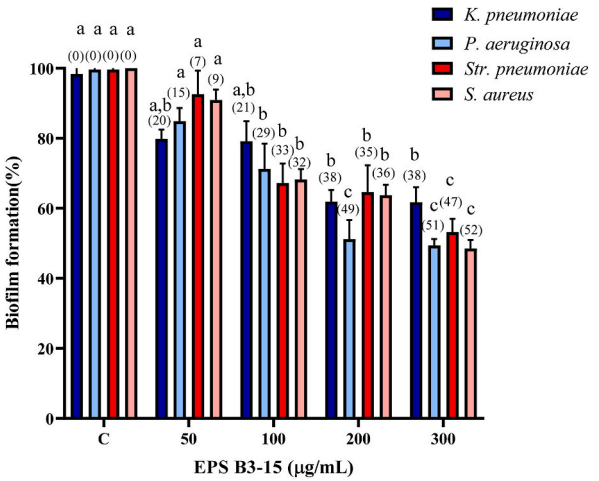

Fig. 5. Effects of EPS B3-15 on biofilm formation by *K. pneumoniae* ATCC 8047, *Str. pneumoniae* ATCC 6303, *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213. The biofilm formation is expressed as percentage compared to the biofilm produced in the absence of EPS B3-15 used as control (C). Data represent mean ± SD for six replicates (n = 6). The lowercase letters indicate different significative ($p \leq 0.01$) groups assigned by ANOVA and the Tukey *post hoc* test.

the concentration of 300 μg/mL the most active. EPS B3-15 was able to reduce the biofilm of *S. aureus* (52 ± 2.4 %), *P. aeruginosa* (51 %), *Str. pneumoniae* (47 ± 4.2 %) and *K. pneumoniae* (38 ± 2.7 %), indicating that the EPS was efficient against both Gram-positive and Gram-negative bacteria.

3.5. Antibacterial activity

The growth curves of *K. pneumoniae* and *Str. pneumoniae* in the absence or in the presence of EPS-B3-15 (300 μg/mL), compared with those of *P. aeruginosa* and *S. aureus* are reported in Fig. 6 (A and B).

The presence of EPS B3-15 did not affect the growth rates of the tested strains (Fig. 6A and B), indicating that the biopolymer has no antibacterial activity.

3.6. Microbial cell surface hydrophobicity and charges by MATH assay

The affinity to polar (ethyl acetate and chloroform) and non-polar solvents (decane and hexadecane) of the Gram-negative *K. pneumoniae* and *P. aeruginosa,* and Gram-positive *Str. pneumoniae* and *S. aureus* strains is shown in Fig. 7.

Untreated *K. pneumoniae* and *P. aeruginosa* possessed high affinity to chloroform (66 % and 62 %), and similar affinity to decane, and hexadecane. However, *K. pneumoniae* showed higher affinity to ethyl acetate (69 %) than *P. aeruginosa* (<50 %), indicating that the cellular surfaces of both Gram-negative strains were differently charged and moderately hydrophobic (Fig. 7A). Untreated *Str. pneumoniae* cells showed lower affinity to chloroform, decane and hexadecane (30 %, 31 %, 32 %, respectively) than *S. aureus* (85 %, 74 %, 69 %, respectively), whereas the affinity to ethyl acetate of *Str. pneumoniae* (47 %) was higher than *S. aureus* (14 %), suggesting that cellular surfaces of *S. aures* were more negatively charged and hydrophobic than those of *Str. pneumoniae* (Fig. 7B).

EPS B3-15 significantly modified the affinity to hydrocarbons of the *Str. pneumoniae* more efficiently than *K. pneumoniae*. Moreover, after the EPS treatment, the cell-wall hydrophobicity of *Str. pneumoniae* was reduced less than the Gram-positive *S. aureus*. The EPS altered the cell-surface properties (charges and the hydrophobicity) of Gram-positive strains more than Gram-negative.

3.7. Surface-coating assay

The effects of EPS B3-15 on the adhesion to pretreated abiotic surface of *K. pneumoniae*, *Str. pneumoniae* in comparison with *P. aeruginosa,* and *S. aureus* are reported in Table 2.

The EPS B3-15 interfered with the adhesion of *Str. pneumoniae* more than *K. pneumoniae,* but less than the model strain *S. aureus.*

4. Discussion

EPSs produced by marine bacteria possess different chemical composition, leading to highly diverse structures with different properties and biological activities, useful in different applications in many fields. However, there are relatively few studies on the anti-inflammatory activity of EPSs produced by bacteria from extreme marine environments, such as those of shallow hydrothermal vents of Eolian Islands (Italy).

The mannose-rich exopolysaccharide produced by the thermophilic *Bacillus licheniformis* B3-15, was previously reported to possess promising potentialities as antiviral and immunomodulatory agent, as stimulator of Th1-type cytokines (IFN-γ, IFN-α, TNF-α, IL-12 and IL-18), and also suppressor of anti-inflammatory cytokines (IL-4 and IL-10) in PBMC infected by HSV-2 [20]. In this study the anti-inflammatory potential of the EPS was assayed *in vitro* against murine macrophage cell line (RAW 264.7) in LPS stimulated/not stimulated cells. As previously reported, LPS induces the production of mediators and cytokines, such as nitric oxide (NO), pro-inflammatory cytokines IL1β, IL6, and tumor necrosis factor (TNF-α) or inhibits the secretion of anti-inflammatory cytokine, such

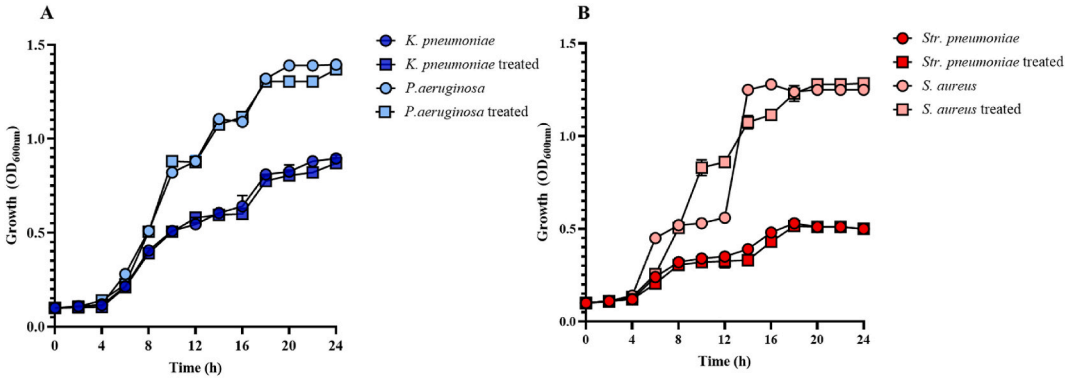

Fig. 6. Growth curves of pathogen strains (A) *K. pneumoniae* ATCC 8047 and (B) *Str. pneumoniae* ATCC 6303, untreated or treated with EPS-B3-15 (300 μg/mL), in comparison with the two model strains *P. aeruginosa* ATCC 27853 and *S. aureus* ATCC 29213, respectively [23].

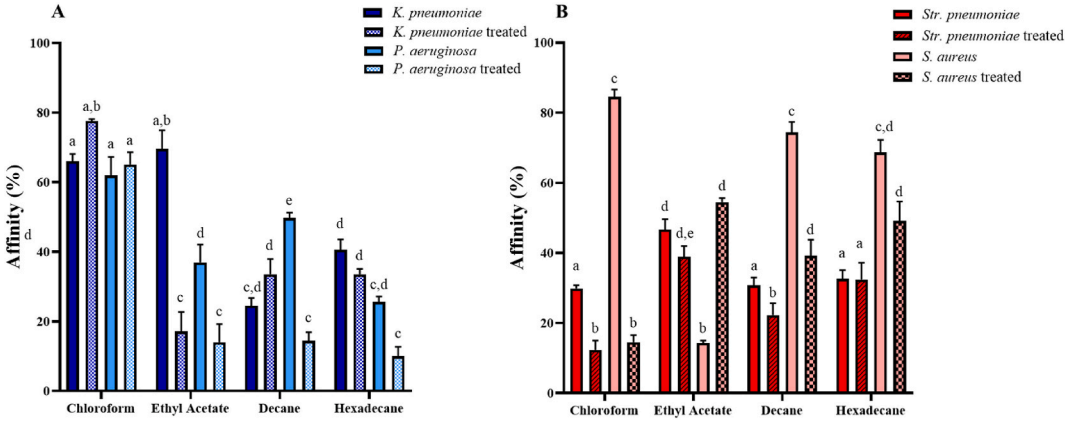

Fig. 7. Affinity (expressed as percentage) to polar (chloroform and ethyl acetate) and non-polar (decane and hexadecane) solvents of (A) Gram-negative *Klebsiella pneumoniae* and *Pseudomonas aeruginosa*, and (B) Gram-positive *Streptococcus pneumoniae* and *Staphylococcus aureus* cells in untreated (Control) or treated with EPS B3-15 (300 μg/mL) conditions. All assays are representative of three independent experiments. Significant statistical differences ($p \leq 0.01$) are indicated by different lower-case letters above the bar graph.

Table 2
Inhibition of adhesion to polystyrene surfaces by *Klebsiella pneumoniae*, *Streptococcus pneumoniae, Pseudomonas aeruginosa* and *Staphylococcus aureus,* in the absence (Control) or pre-coated with crude EPS B3-15 (300 μg/mL) after 18 h treatment.

	K. pneumoniae	Str. pneumoniae	P. aeruginosa[a]	S. aureus[a]
Control	–	–	–	–
Treated with EPS B3-15	+	++	+++	+++

(−) = negative capacity of inhibition of adhesion to polystyrene.
(+) = slight inhibition of adhesion to polystyrene.
(++) = moderate inhibition of adhesion to polystyrene.
(+++) = high inhibition of adhesion to polystyrene.
[a] data from Zammuto et al. [23].

as IL-10 in cells [30]. The addition of EPS B3-15 (300 μg/ml) to LPS- inflamed macrophages significantly reduced the NO production (Fig. 2), and down-regulated the gene expression of proinflammatory markers (IL6, IL1β, iNOS and COX2) (Fig. 4), indicating that the EPS could suppress TLR4 pathway [31]. TLR4 has been identified as an immunopharmaceutical target to control pathogenic bacteria and viruses [32]. The ability of mannose receptors (MR) of macrophages to recognize the carbohydrate structure, including mannose and fructose, can induce the intracellular signaling cascade, leading to the transcriptional expression of inflammatory mediators (*i.e.* IL1β) [33]. Moreover, the rapid down regulation of IL6, IL1β, iNOS and COX2 observed in the presence of EPS B3-15 could be related to the suppression of the mitogen-activated protein kinase (MAPK) and the nuclear factor -κB signaling pathways, considered as the core pathways in the regulation of inflammatory mediators and cytokines, similarly to that previously reported for other exopolysaccharides [34]. Therefore, the EPS B3-15 could prevent diseases caused by uncontrolled inflammatory responses. However, future molecular studies and *in vivo* experiments could confirm our hypothesis and clarify the anti-inflammatory action mechanism of EPS B3-15.

In this study, we also investigated the effects of EPS B3-15 on the adhesion and biofilm formation of two respiratory pathogens *K. pneumoniae* and *Str. pneumoniae* and its activity was compared to the model strains *P. aeruginosa* and *S. aureus* [23]. Crude EPS B3-15 impaired adhesion and thus biofilm formation in a dose-dependent manner, without any bacteriostatic or bactericidal activity. The EPS B3-15 (300 μg/mL) reduced the biofilm formation of *K. pneumonia* (38 %), *P. aeruginosa* (51 %) *Str. pneumoniae* (47 %) and *S. aureus* (52 %), although with different antiadhesive effects. It is well known that the ability of bacterial cells to adhere to an abiotic or biotic substrata greatly depends on the electric charges and the hydrophobicity of surfaces. The presence of the polysaccharide capsule of *K. pneumoniae* is involved in different stages of biofilm formation, from the initial adhesion to the maturation and dispersion of cells [35]. In detail, the presence of capsular (K) and lipopolysaccharide (O) antigens determine the hydrophobicity of the cell-surface of *Klebsiella* sp. and therefore its susceptibility to phagocytosis [36]. Moreover, the residues of glutamic acid confer negative charges to the external bacterial surfaces. These properties were confirmed by the high affinity to ethyl acetate observed by the MATH assay, whereas the moderate affinity to decane and hexadecane (<40 %) indicated a moderate hydrophobicity. The addition of EPS modified the cell surface properties of *K. pneumoniae*, reducing the affinity to ethyl acetate and therefore the surface negative charges, whereas it moderately affected its hydrophobicity, with a similar action to that previously reported for *P. aeruginosa* [23]. These observations could justify the moderate antibiofilm activity (<30 % of biofilm reduction) of the EPS B3-15 against *K. pneumoniae*.

As resulted by the affinity to ethyl-acetate assay, cell surfaces of *Str. pneumoniae* possess higher negative charges than *S. aureus*. The main effect of EPS B3-15 on the *Str. pneumoniae* cell surface was the reduction of negative charges (as observed by the low affinity to

chloroform), that could motivate its antiadhesion activity, according to previous works [37–39]. Moreover, the presence of the EPS inhibited the adhesion of *Str. pneumoniae* on pretreated polystyrene surfaces, suggesting a pivotal role of the EPS in altering both cellular and substratum surfaces properties. Compared with the antibiofilm activity of EPS1-T14 (56 % of biofilm inhibition), produced by thermophilic *Bacillus licheniformis* T14 [40] against *K. pneumoniae*, EPS B3-15 resulted less effective (30 %). The different activities among the two EPSs could be ascribed to their different carbohydrate composition and consequently to their surfactant properties, since EPS1-T14, mainly constituted by fructose, possessed higher emulsifying activity (E_{24} = 58.4 %) than EPS B3-15 (E_{24} = 34 %).

5. Conclusions

Without affecting vitality of Raw 264.7 cells, EPS B3-15 at low concentration (300 μg/mL) significantly reduced the NO production, and down-regulated the gene expression of proinflammatory markers (IL6, IL1β, iNOS and COX2) of LPS induced macrophages. The mannose receptors of macrophages could recognize the carbohydrate structure of EPS B3-15, mannose-rich exopolysaccharide, inducing the intracellular signaling cascade, leading to transcriptional expression of inflammatory mediators. However, it is essential to confirm the effects of EPS on *in vivo* experiments.

As able to reduce the inflammatory responses and simultaneously prevent biofilm-related to the respiratory tract infections, EPS B3-15 could have potential use as nasal spray with anti-inflammatory action or surface-coating agent for medical devices (*i.e.*, endotracheal and respiratory devices) or as plasters for wounds.

Data availability

Data included in article/supplementary material is referenced in the article.

CRediT authorship contribution statement

Maria Giovanna Rizzo: Writing – original draft, Visualization, Methodology, Investigation, Data curation, Conceptualization. **Vincenzo Zammuto:** Writing – original draft, Visualization, Software, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Antonio Spanò:** Resources, Methodology. **Concetta Gugliandolo:** Writing – review & editing, Visualization, Supervision, Software, Conceptualization. **Giovanna Calabrese:** Writing – review & editing, Writing – original draft, Validation, Investigation, Data curation, Conceptualization. **Salvatore Guglielmino:** Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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