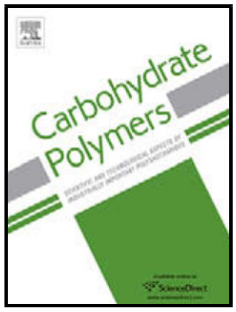

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Rheology and bioactivity of high molecular weight dextrans synthesised by lactic acid bacteria

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Abbreviations: C*, critical concentration for interaction between dextran molecules; CDMS, CDM define medium lacking glucose and supplemented with 0.8% sucrose; cfu, colony forming unit; CM9, CM30, SM34 and RTF10, *Leuconostoc mesenteroides* strains; DMEM, Dulbecco's Modified Eagle medium; EDTA, ethylenediaminetetraacetic acid; EPS, exopolysaccharide; $\dot{\gamma}$, shear rate; HePS, heteropolysaccharides; HoPS, homopolysaccharides; HPLC, High-performance liquid chromatography; LAB, lactic acid bacteria; LPS, lipopolysaccharide from *Escherichia coli* O111:B4; MRSG, Man Rogosa Sharpe broth containing 2% glucose; MRSS, MRS medium containing 2% sucrose instead of glucose; MSE, Mayeux Sandine Elliker medium; η_0, viscosity at near-zero shear rate**;** η, apparent viscosity**;** PBS, phosphate-buffered saline; PDA, photodiode array; PMA, phorbol 12-myristate 13-acetate; SEC-MALLS, size exclusion chromatography coupled to multiangle laser light scattering detection; PMA-THP-1, THP-1 monocytes differentiated to macrophages with PMA; TEM, transmission electron microscopy.

Abstract

Dextrans synthesised by three *Leuconostoc mesenteroides* strains, isolated from mammalian milks, were studied and compared with dextrans produced by *Lc. mesenteroides* and *Lactobacillus sakei* strains isolated from meat products. Size exclusion chromatography coupled with multiangle laser light scattering detection analysis demonstrated that the dextrans have molecular masses between $1.74x10^8$ Da and $4.41x10^8$ Da. Rheological analysis of aqueous solutions of the polymer revealed that all had a pseudoplastic behaviour under shear conditions and a random, and flexible, coil structure. The dextrans showed at shear zero a difference in viscosity, which increased as the concentration increased. Also, the purified dextrans were able to immunomodulate *in vitro* human macrophages, partially counteracting the inflammatory effect of *Escherichia coli* O111:B4 lipopolysaccharide.

During prolonged incubation on a solid medium containing sucrose, dextran-producing bacteria showed two distinct phenotypes not related to the genus or species to which they belonged. Colonies of *Lc. mesenteroides* CM9 from milk and *Lb. sakei* MN1 from meat formed stable and compact mucoid colonies, whereas the colonies of the other three *Leuconostoc* strains became diffuse after 72 h. This differential behaviour was also observed in the ability of the corresponding strains to bind to Caco-2 cells. Strains forming compact mucoid colonies showed a high level of adhesion when grown in the presence of glucose, which decreased in the presence of sucrose (the condition required for dextran synthesis). However no influence of the carbon source was detected for the adhesion ability of the other *Lc. mesenteroides* strains, which showed variable levels of binding to the enterocytes.

Key words: exopolysaccharides, dextran, lactic acid bacteria, *Leuconostoc mesenteroides*, rheological properties, immunomodulation, adhesion

Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of gram-positive bacteria, traditionally used in food fermentation and preservation (Gaspar, Carvalho, Vinga, Santos, & Neves, 2013). Currently, their usage has been expanded to the manufacture of functional food, as efficient microbial cell factories for production of industrially relevant metabolites and as probiotics that impact general health and well-being (Mozzi et al., 2015). The positive contribution of LAB to functional food manufacture is assured by the production of beneficial compounds such as lactic acid, carbon dioxide, aroma compounds, vitamins, antimicrobial agents, polyols and exopolysaccharides (EPS) (Nuraida, 2015). The latter bio-products are synthesised by some LAB and remain associated with the cell surface constituting a capsule, or released as a free polymer into the environment (Nwodo, Green, & Okoh, 2012). The LAB EPS can differ according to their chemical structure, molecular mass and linkage types, which could modulate their biological functionality (Surayot et al., 2014). According to monomeric composition, EPS are classified into two groups: homopolysaccharides (HoPS) contain a single type of monosaccharide and heteropolysaccharides (HePS), which are composed of repeated units of at least two types of monosaccharides and various types of linkages (Patel, Majumder, & Goyal, 2012).

The HoPS can be α-D-glucans, β-D-glucans or β-D-fructans and they are respectively synthesized by glucansucrases, glycosyltransferases or fructansucrases (Werning et al. 2012; Pérez-Ramos et al. 2015). HoPS are able to modulate rheological properties of materials (Welman & Maddox, 2003), and several studies have shown that they can replace or reduce the use of more expensive hydrocolloids and could find applications such as texturizing agents (Bounaix et al., 2009). Dextran is a HoPS composed of a main linear chain of α-1,6 glycosidic linkages with a few branches of α-glucopyranose at positions *O*-2, *O*-3 or *O*-4. This biodegradable polymer is synthesised by LAB mainly belonging to the *Leuconostoc*,

Lactobacillus and *Streptococcus* genera (Ciszek-Lenda, 2011, Patel et al., 2012). Its yield is affected by environmental conditions, biosynthetic pathways and rate of microbial growth (Sarwat, Ahmed, Aman, & Qader, 2013). Dextrans have various uses in the food, pharmaceutical and oil drilling industries (Aman, Siddiqui, & Qader, 2012). This wide spectrum of applications is due to physicochemical differences such as their molecular mass, which influences the viscosity and rheological properties of the polymer solution. Dextrans of high molecular mass are used for petroleum recovery; those of average molecular masses are employed in the chemical industry; whereas low-molecular mass dextrans find applications in the pharmaceutical and photographic industries (Vettori, Blanco, Cortezi, de Lima, & Contiero, 2012). In cosmetics, they are used as moisturizers and thickeners (Vu, Chen, Crawford, & Ivanova, 2009), and in the food industry, they are added to bakery products and confectionery to improve softness or moisture retention, to prevent crystallisation, and to increase viscosity, rheology, texture and volume (Pérez-Ramos et al., 2015). These HoPS also play an important role in stabilisation of frozen foods and can be used as films, to preserve the surface of fish, meat, vegetables or cheese from oxidation and other chemical changes (Harutoshi, 2013). In the medical field, dextrans are broadly used as blood plasma replacers/expanders and as heparin substitutes, for anticoagulant therapy. They can also produce iron dextrans, which are used in solution for treatment of human and veterinary anaemic deficiency. In addition, dextran sulphate has also shown an antiviral effect against the human immunodeficiency virus (Piret et al., 2000), and we have recently demonstrated that dextrans synthesized by LAB have potential as antivirals and immunomodulatory agents in trout (Nácher-Vázquez et al., 2015). Thus, dextrans can be used in the future for the manufacture of functional food and feed.

In the present work, we have performed a physicochemical characterization, and analysed the rheological properties, of dextrans produced by several strains of *Leuconostoc* and *Lactobacillus* isolated from food. Two physiological aspects were then tested *in vitro* for (i) the ability of the

purified dextrans to immunomodulate human macrophages and, (ii) the influence of the dextrans on the ability of the producing LAB to interact with human enterocytes.

2. Materials and methods

2.1. Bacterial strains and growth conditions. Five LAB strains were used in this work. Three of them were isolated in Algeria: CM9 and CM30 from camel milk and SM34 from sheep milk. They were isolated using agar plates containing the MSE medium (Mayeux, Sandine, & Elliker, 1962) supplemented with 10% sucrose and vancomycin (30 μg mL^{-1}) at 30 ºC for 72 h. These bacteria were identified as *Leuconostoc mesenteroides* (accession number in GenBank: KY083048 (CM9), KY082929 (CM30) and KY083047 (SM34)) by sequencing their 16S rRNA coding genes at Secugen (Madrid, Spain). The other two LAB were *Lactobacillus sakei* MN1, (CECT 8329) and *Lc. mesenteroides* RTF10, both isolated from meat products (Chenoll, Macian, Elizaquivel & Aznar, 2007). LAB were grown at 30 ºC in MRS (De Man, Rogosa, & Sharpe, 1960) containing 2% glucose (MRSG) or in MRS supplemented with 2% sucrose (MRSS) instead of glucose. For long-term storage at -80 ºC, MRSG supplemented with 20% (v/v) glycerol was used. For EPS production, LAB were grown in defined CDM medium (Sánchez et al., 2008) supplemented with 0.8% sucrose (CDMS).

2.2. Production, purification, quantification and characterization of EPS. For production, LAB were precultured at 30 ºC in MRSS to A_{600} = 2.0. Then, the bacteria were sedimented by centrifugation (12,000 x *g*, 10 min, 4 ºC), resuspended in the same volume of fresh MRSS, diluted 1:100 in fresh CDMS medium and, incubated at 30 ºC until the end of the exponential phase of growth. For EPS isolation and purification, cells were removed by centrifugation (10,651 × *g*, 30 min, 4 ºC). The EPS was precipitated by addition of cold absolute ethanol to the supernatant (v/v) and storage at 4 ºC for 24 h. After centrifugation (10,651 × *g*, 60 min, 4 ºC), the

sediment was air dried, dissolved in distilled water and dialysed for 3 days against ultrapure water, using a 12–14 kDa cut-off membrane. Then, the EPS was frozen at -80 ºC and lyophilised until dry. Fractionation by size exclusion chromatography (SEC) was performed as previously described (Notararigo et al., 2013), as the final step of purification.

The concentration of EPS at each step of purification was determined by by the phenol-sulphuric acid method, using a glucose calibration curve, (Dubois, Gilles, Hamilton, Rebers, & Smith, 1956). The amount of EPS present in the cultures supernatants was determined after their precipitation with three volumes of absolute ethanol and two washes with 80% (v/v) ethanol. During purification and after chromatographic fractionation, the EPS concentrations were determined in aqueous solutions at 2 mg mL^{-1}. In parallel, tests for detection of potential contaminants (DNA, RNA and proteins) were carried out using specific fluorescent staining kits and the Qubit® 2.0 fluorometric detection methods (ThermoFhiser Scientific) in the same solutions. This technique allows the detection of more than 0.5 µg mL^{-1} of DNA, 20 ng mL^{-1} of RNA, and 1 µg mL^{-1} of proteins.

The chemical characterisation of EPS was performed by: (i) determination of monosaccharide composition and phosphate content, (ii) methylation analysis and (iii) infrared (IR) spectroscopy as previously described (Notararigo et al., 2013).

2.3. Molar mass distribution of dextrans by SEC-MALLS. The molar mass distribution of the purified dextrans was analysed by SEC-MALLS as previously reported (Nikolic et al., 2012). In short, each lyophilised dextran sample was resuspended (5 mg mL^{-1}) in 0.1 M $NaNO_3$, kept overnight under gentle stirring and centrifuged (10,000 × *g*, 10 min) before analysis. The HPLC system (Waters, Milford, MA, USA) consisted of a separation module Alliance 2695 connected with three detectors: a photodiode array (PDA 996, Waters) checked at 280 nm for protein detection, a refractive index (RI 2414, Waters) to determine the amount of dextran using calibration curves obtained from standards of different molar mass and the MALLS Dawn

Heleos II (Wyatt Europe GmbH, Dembach, Germany). Dextrans were quantified using Empower software (Waters) and the molar mass distribution analysis using Astra 3.5 software (Wyatt Europe GmbH). Two SEC columns placed in series were used: TSK-Gel G3000 PW_{XL}+TSK-Gel G5000 PW_{XL} protected with a TSK-Gel guard column (Supelco-Sigma St. Louis, MO, USA) and the separation was carried out at 40 ºC with a flow rate of 0.45 mL min^{-1} using 0.1 M $NaNO_3$ as mobile phase.

2.4. Rheological Analysis. For determination of the rheological behaviours of dextran solutions the lyophilised EPS were dissolved in ultrapure water at different concentrations. The solutions were prepared at room temperature to avoid denaturation and were kept in the rest state before each analysis. Rheological measurements were made in a Termo-Haake Rheostress I viscoelastometer (ThermoFisher Scientific), equipped with a cone-plate (60 mm diameter, 2° cone angle). All measurements were performed using two steps after sample loading (2 mL): first the sample was maintained at the desired temperature for 3 min without shear and, secondly, the shear-rate was increased from 1 to 500 s^{-1} within 3 min. Each experiment was repeated at least three times. Continuous steady-state flow behaviour was determined from the apparent viscosity and shear rate relationship data using Haake Rheowin Data Manager. Viscosity at shear near to zero (η_0) was extrapolated and regressed using the Cross model (Cross, 1965):

$$\eta=\eta_0/(1+(\lambda.\dot{\gamma})^{1-n}) \qquad \text{Eq. (1)}$$

where 'η' is the apparent viscosity, 'λ' is a time constant related to the relaxation times of the polymer in solution, '$\dot{\gamma}$' is shear rate and 'n' is the power law flow behaviour index.

Variation of viscosity (η_0) as a function of dextran concentration (C) was determined using the following power law equation:

$$\eta_0=k.c^b \qquad \text{Eq. (2)}$$

Where '*b*' and '*K*' are the constants to be determined from the log-log plot of viscosity versus dextran concentration.

2.5. THP-1 cell line culture and immunomodulation assay. The human monocytic leukemia cell line THP-1, obtained from the cell bank at Centro de Investigaciones Biológicas (CIB, Madrid, Spain) was used. The cells were grown in RPMI 1640 medium (Gibco) supplemented with 10% heat-inactivated fetal bovine serum (Gibco), penicillin (100 U mL^{-1}) and streptomycin (100 µg mL^{-1}) at 37 ºC in an atmosphere containing 5% CO_2. Cells were sub-cultured every 2 days. The mature macrophage-like state was induced by treating the THP-1 monocytes (10^6 cells mL^{-1}) with 40 nM of phorbol 12-myristate 13-acetate (PMA, Sigma-Aldrich) for 72 h in 12-well cell culture plates (Falcon-Software, Canada) with 1 mL cell suspension in each well, using the above RPMI 1640 supplemented medium. Differentiated, plastic-adherent cells were washed twice with the same medium without PMA and incubated for 24 h at 37 ºC. Then, the PMA-THP-1 cells were co-stimulated with 700 ng mL^{-1} of the *Escherichia coli* O111:B4 lipopolysaccharides (LPS, Sigma) and with the purified dextrans (100 µg mL^{-1}) for 18 h in the same conditions of incubation (Chanput, Mes, Vreeburg, Savelkoul, & Wichers, 2010). Cell-free culture supernatants were collected and stored at -80 ºC to analyse the concentration of the secreted pro-inflammatory TNF-α and the anti-inflammatory IL10 cytokines using commercially available ELISA kits (BD OptEIA™; BD biosciences, USA) as indicated by the manufacturers. The THP-1 monocytes (un-differentiated cells) and the supplemented medium were used as controls. In this experiment, also, three commercial dextrans (T10, T40 and T2000; Pharmacia Biotechnology AB, Sweden) were analysed. The experiments were performed in triplicate.

2.6. Location of EPS production at cellular level. For phenotypic determination at cellular level, LAB cultures were grown in MRSG liquid medium to A_{600} = 1. Then, 100 µL of appropriate dilutions were streaked on MRSS and MRSG agar plates and incubated for the time indicated in the results section. To detect EPS location by transmission electron microscopy

(TEM), one colony of each strain from MRSS-agar was carefully suspended in 50 µL of sterile distilled water to form a suspension, that was subjected to negative staining as described previously (Maeyama, Mizunoe, Anderson, Tanaka, & Matsuda, 2004). To visualise EPS preparations, aqueous solutions at 30 mg mL^{-1} were subjected to negative staining. Glow-discharged carbon-coated Formvar grids were used and, immediately prior to use, the grids were subjected to ionic discharge for one minute. Then, they were placed facedown over a droplet of each suspension. After 30 s, each grid was removed, washed, blotted briefly with filter paper, negatively stained with 2% uranyl acetate, blotted quickly and air-dried. Samples were examined using a JEOL 1230 microscope operated at 100 kV at the Electron Microscopy service of Centro de Investigaciones Biológicas (CIB, Madrid, Spain).

2.7. Caco-2 cell culture and adhesion assays. The Caco-2 human enterocyte cell line, obtained from the cell bank at CIB, were seeded in 96-well tissue culture plates (Falcon Microtest™, Becton Dickinson, Franklin Lakes, NJ, USA) at a final concentration of 1.25 x 10^5 cells mL^{-1} and grown as monolayers of differentiated and polarised cells for 21 days as previously described (Nácher-Vázquez et al., 2017). Cell concentrations were determined as previously described (Garai-Ibabe et al., 2010).

For the adhesion assays, exponential-phase LAB cultures grown in either MRSG or MRSS were sedimented by centrifugation (12,000 × *g*, 10 min, 4 ºC), resuspended in the appropriate volume of either Dulbecco's modified Eagle medium (DMEM, Invitrogen) containing 0.5% glucose (G) or DMEM supplemented with 0.5% sucrose (S) to give a final concentration of 1.25 x 10^6 cfu mL^{-1}. 0.1 mL of bacterial suspension was added per well (ratio 10:1, bacteria:Caco-2 cells) and the plates were incubated for 1 h at 37 ºC. Then, un-adhered bacteria were removed and, the cell-associated bacteria quantified after platting onto MRSG plates, as previously described (Nácher-Vázquez et al., 2017). All adhesion assays were conducted in triplicate.

2.8 Statistical analysis

In the adhesion analysis, data are expressed as a mean ± standard deviation calculated from three independent replications performed in triplicate. Data were subjected to one-way analysis of variance (ANOVA) by using the SAS software. Tukey's test was employed to determine the significant differences between the variables at $p \leq 0.05$. In the immunomodulation experiments, data are expressed as a mean ± standard deviation calculated from one replication in triplicate. Data were subjected to ANOVA by using the SAS software. Dunnett's test was employed to determine the significant differences between the control group and the various treatment groups. Comparisons significant at the 0.05 level are indicated by ***.

3. Results

3.1. Characterization of dextrans produced by LAB. Three *Lc. mesenteroides* strains (CM9, SM34, CM30) isolated from Algerian milks and selected for abundant production of EPS in the presence of sucrose but not with glucose (Table 1S) were compared with two previously characterised dextran-producing bacteria, *Lc. mesenteroides* RTF10 and *Lb. sakei* MN1 isolated from Spanish fermented meat (Notararigo et al., 2013; Nácher-Vázquez et al., 2015; Nácher-Vázquez et al., 2017). LAB were grown in CDM defined medium instead of a rich medium in order to avoid, or diminish, the co-precipitation of interfering compounds during EPS quantification and further purification (Montersino, Prieto, Muñoz, & Rivas, 2008; Notararigo et al., 2013). The medium was supplemented with 0.8% sucrose. This relatively low concentration of the predicted substrate for EPS synthesis was chosen to avoid viscosity problems during purification. Quantification of the EPS present in the culture supernatants by the phenol sulphuric acid method, revealed the concentrations of the polymers, namely: for *Lc. mesenteroides* CM9, CM30, SM34 and RTF10 strains, respectively, 1.47 $\pm$ 0.08 g L^{-1}, 2.15 $\pm$ 0.15 g L^{-1}, 3.14 $\pm$ 0.06 g L^{-1} and 1.25 $\pm$ 0.11 g L^{-1} and for *Lb. sakei* MN1 1.72 $\pm$ 0.12 g L^{-1}. The

estimated yield of the production varied among the different bacteria, with the highest being SM34 EPS and the lowest being RTF10 EPS (Table 1).

Bacteria were grown in CDMS to the end of the exponential phase (A_{600} of 3.25, 2.84, 2.34, 3.16 for *Lc. mesenteroides* CM9, CM30, SM34 and RTF10 strains respectively, and to A_{600} = 4.15 for *Lb.sakei* MN1) and, the concentration of the EPS and contaminants (proteins, DNA and RNA) present in the culture supernatants and after the steps of purification was determined.

[a]The concentration of proteins, DNA and RNA was measured directly in the supernatants. EPS concentration was determined after precipitation from the supernatants with three volumes of ethanol.
[b]The concentrations of the biomolecules were determined from preparations of the freeze-dried compounds at a concentration of 2 mg mL^{-1} dissolved in water.
[c]The yield of EPS was calculated as the ratio of the total amount of the polymer (indicated in the text) and the bacterial biomass estimated from the final A_{600} of the cultures.
[d]The recovery (RC) of EPS is expressed as percentage of the EPS present in the culture supernatants.
ND indicates levels below the limit of detection corresponding to 0.5 μg mL^{-1} of DNA, 20 ng mL^{-1} of RNA and 1 μg mL^{-1} of proteins.

In all cases a purity higher than 95% was detected when the concentration of the potential contaminants DNA, RNA and proteins was measured (Table 1). Ethanol precipitation (v/v) was used for the recovery of high molecular weight EPS and their initial purification from the supernatants of the cultures, followed by dialysis of EPS solutions as an intermediate purification step. This treatment allowed a recovery of the EPS (68%-87%), eliminated the contaminant DNA and RNA, and only residual levels of proteins (0.1%-0.25%) were detected (Table 1). This degree of purity was deemed sufficient for further physicochemical studies but for immunomodulation experiments (see below) the EPS were further purified by SEC to remove the protein contaminants (Table 1). Depending on the strain considered, *Lc. mesenteroides* has been reported to produce three types of extracellular HoPS using sucrose as substrate: dextran and alternan (α-glucans) or levan (β-fructan) (Harutoshi, 2013; Sanlibaba & Çakmak, 2016). Thus, to determine, which type of HoPS was produced by *Lc. mesenteroides* CM30, SM34 and CM9 strains, monosaccharide composition analysis was carried out. The results (data not shown)

demonstrate that all EPS produced were glucan, composed strictly of glucose units. In addition, IR spectra of the three HoPS preparations presented the same profile (Fig. 1A) and they were typical of α-linked polymers, with absorption bands between 847-849 cm^{-1} and 913-917 cm^{-1} which correspond to α-anomers (Notararigo et al., 2013; Park, Ahn, Kim, & Chung, 2013; Vettori, Franchetti, & Contiero, 2012b).

Methylation analysis showed that the three HoPS had a main chain of glucopyranose units with α-(1,6) linkages (between 86.7% and 89.4%) and partially branched in the *O*-3 position by a single α-glucopyranose unit (between 8.5% and 10.3%) (Fig. 1B). Therefore, the overall data revealed that the polymers from the three *Lc. mesenteroides* strains are dextran with the structure depicted in Fig. 1C, very similar to that of the dextrans produced by MN1 and RTF10, with approximately 6% *O*-3 substitutions (Nácher-Vázquez et al., 2015; Notararigo et al., 2013) and dextrans produced by other *Lc. mesenteroides* strains (Han et al., 2014; Siddiqui, Aman, Silipo, Qader, & Molinaro, 2014; Werning et al., 2012; Yang et al., 2015).

Previous analysis of the dextran from MN1 and RTF1 by 2D-DOSY revealed that they possess a molecular mass higher than that of the commercial T2000 dextran which has a theoretical molecular mass of 2.0 x 10^6 Da (Nácher-Vázquez et al., 2015). Thus, to get a more precise estimation of the molecular mass of the five dextrans they were subjected to a SEC-MALLS analysis. The results revealed that they have a high molecular mass within the range of 1-5 x 10^8 Da (Table 1 and Fig. 1S).

3.2. Rheological properties of dextran produced by LAB. Aqueous solutions of dextrans from the five LAB were analysed, and the results revealed that the viscosity of the five polymers varied depending on the concentration and the shear rate (Fig. 2). At low concentrations, they showed a shear rate-independent Newtonian viscosity, as already observed for some commercial dextrans (Tirtaatmadja, Dunstan, & Boger, 2001). The upper concentration limits for this Newtonian or linear plateau were 1.0%, 0.8%, 0.4%, 0.6% and 0.8%, respectively, for the MN1,

CM30, SM34, RTF10 and CM9 EPS. At higher concentrations, dextrans show a different behaviour with decreased viscosity when the shear rate was increased. This corresponded to a non-Newtonian or pseudoplastic behaviour and it could be explained by the hydrodynamic forces generated during the shear, which could break structural entanglements and physical networks between α-glucan chains present in the solution.

The rheological experiments also predicted further properties of the dextran molecules by examining the concentration dependence of 'zero shear' specific viscosities. This relationship can demonstrate the presence of interactions between polymer chains by defining the critical concentration (*C**), i.e. the concentration of polymer at which the equivalent sphere of a given polymer molecule just touches the equivalent spheres of all of its nearest neighbour molecules (Kulicke & Clasen, 2013). *C** was calculated by performing a double logarithmic plot of Newtonian viscosity *versus* the different dextran concentrations. Using the Cross model (see Eq. (1) in Materials and Methods), the Newtonian viscosity (state of rest of zero shear) of the five dextrans was determined by extrapolation. Two straight lines were obtained, which corresponded to the diluted and more concentrated domains (Fig. 3).

The results suggested that the Newtonian viscosity increases with dextran concentration, and the critical concentration of the five dextrans varied between 0.5% and 1%. It had been shown, that the critical concentration is inversely proportional to the molecular mass of polysaccharides (Dong et al., 2001; Dong, Wang, & Qing, 2000), which is consistent with most of the dextrans used in this study. For example MN1, a dextran of 1.74×10^8 Da showed a *C** of 1% (Fig. 3A), whereas RTF10 of 4.41×10^8 presented a *C** of 0.65% Da (Fig. 3D). Values of slope *b* between 5.62 and 9.36 were obtained, when the equation 2 (see Eq. (2) in Materials and Methods) was applied to the second straight line of each of the five dextrans. Previous works showed that random coil (flexible) polymers have *b* exponent values in the range of ≥ 5, and that stiff polymers displayed lower values (Bohdanecky & Kovar, 1982;

Velasco et al., 2009). Thus, our results revealed that the LAB dextrans under investigation can be considered as random coil with flexible chains.

In addition, the results obtained (Fig. 3F) showed that the viscosity of 3% aqueous solutions (representative viscosity of the concentrated domain) varies between 158 Pas and 13.44 Pas of which dextran MN1 has the highest viscosity and dextran CM9 possesses the lowest viscosity. By comparing these results with the molecular masses of these polymers, it can be inferred that the viscosity is not only influenced by the molecular mass, but by the same parameters determining the critical conductivity of the polymer (cited above) (Glöckner, 2008; Graessley, 1977; Mezger, 2014). No significant variation in viscosity in individual solutions of dextrans was observed with low shear (10 s^{-1}) (data not shown) and strong shear rate (206 s^{-1}) (Fig. 3F).

3.3. Immunomodulatory activity of dextrans from LAB. These type of polymers are considered non-toxic compounds (Jean, 2008) and used for medical purposes including, among others, their utilisation as plasma substitutes (reviewed in Pérez-Ramos et al., 2015). Moreover, we have previously shown that dextran from meat LAB are not toxic for salmonid cell lines and provided *in vivo* results supporting that MN1 dextran stimulates innate and acquired immunity of trout (Nácher-Vázquez et al., 2015). Therefore, the five dextrans from LAB were tested for their potential ability to immunomodulate PMA-THP-1 monocyte-derived macrophages. Influence of these EPS on the secretion of the proinflamatory TNF-α and antiinflamatory IL-10 by macrophages stimulated with *E. coli* LPS was investigated (Fig. 4). It has been observed that immunomodulatory activities of EPS synthesised by LAB are based on many parameters such as the physicochemical properties of the polymers and their average molecular mass (Shao et al., 2014). Thus, to test a wider range of polysaccharide molecular masses, the effect of the commercial dextrans T10, T40 and T2000 was also assayed (Fig. 4). These dextrans are produced by a *Leuconostoc* strain, purified and hydrolysed, and like the EPS studied in this work, they had a main chain of glucopyranose units with α-(1,6) linkages and partially branched

in the *O*-3 position. Moreover, analysis of these polymers by SEC-MALLS confirmed the molecular masses of T10 ($1.22x10^4$ Da) and T40 ($3.98x10^4$ Da) which are lower than the mass of T2000, which was characterized as $1.33x10^6$ Da (results not shown).

Evaluation of the cytokines levels in the PMA-THP-1 cell supernatants showed that the treatment with LPS alone was able to induce the production of both TNF-α and IL10 (Figs. 4A and 4B).

Moreover, as expected from the inflammatory role of the *E. coli* LPS (Hambleton, Weinstein, Lem, & DeFranco, 1996; Seow et al., 2013) the ratio TNF-α/IL10 increased considerably. This activation of the macrophages was partially counteracted by co-treatment with all the dextrans tested by decreasing the levels of TNF-α and increasing the levels of IL10, effect that resulted in a significant decrease of the TNF-α/IL10 inflammatory ratio (Fig. 4C).

3.4. Macroscopic and microscopic detection of dextran production. Detection of mucous colonies has been used as a phenotypical test to select EPS-producing bacteria (Patel, Kothari, Shukla, Das, & Goyal, 2011). In addition, we have previously detected production of dextran by the meat LAB in solid medium containing sucrose (Nácher-Váquez et al., 2017). Thus, the ability of the five LAB to produce EPS was analysed in MRS solid media, containing either sucrose or glucose. Our results revealed that all bacteria presented viscous and mucous colonies, when grown for 48 h on MRSS (Figs. 5A and 2S) but not on MRSG even when incubated for 72 h or longer (Fig. 5C and results not shown). In the presence of sucrose, two types of morphologies were observed, *Lc. mesenteroides* CM9 and *Lb. sakei* MN1 presented convex colonies firmly adhered to the agar with persisting consistent gel gum texture even after 120 h of incubation (Fig. 2S). By contrast, the other three *Lc. mesenteroides* strains showed flat colonies with low adherence to the agar. After 72 h of growth, the colonies appeared surrounded by a semi-liquid gel (Fig. 5B), and they expanded all over the plate at longer incubation times (Fig. 2S). Previous TEM analysis of meat LAB colonies, allowed us to detect the dextran surrounding

cells grown in MRSS and not in MRSG (Nácher-Vázquez et al., 2017). Thus, in order to determine the dextran location at cellular level, Algerian LAB colonies were analysed by TEM. The analysis revealed that in all samples of cultures grown in MRSS (Fig 5D and results not shown), and not in MRSG (Fig 5D and results not shown), bacteria were surrounded by some molecules, and these had the same appearance as dextran preparations (Fig. 5F and results not shown).

3.5. Analysis of the adhesion ability of LAB. The ability to adhere to enterocytes is a potentially important characteristic to select probiotic strains, and previous results indicated that the adhesion ability of *Lb. sakei* MN1 but not of *Lc. mesenteroides* RTF10 to Caco-2 cells was affected by the presence of dextran (Nácher-Vázquez et al., 2017). Therefore, the influence of dextran synthesis on the binding ability of the milk *Lc. mesenteroides* strains to Caco-2 cells was tested in comparison to that of the meat LAB (Fig. 6).

The results revealed that MN1 and CM9 were able to adhere to the enterocytes with a level of 4.9 $\pm$ 0.43% and 4.12 $\pm$ 0.41%, respectively, in the presence of DMEM medium containing glucose. These levels are similar to those (around 5%), which we previously reported for the probiotic *Lactobacillus acidophilus* LA-5 (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010; Nácher-Vazquez, 2017).

Moreover, the binding of MN1 and CM9 strains to the Caco-2 cells decreased to 1.09 $\pm$ 0.25% and 2.55 $\pm$ 0.21%, respectively, when sucrose was present during bacterial growth in MRSS and during the assay in DMEM medium. By contrast, adhesion of *Lc. mesenteroides* CM30 (3.52 $\pm$ 0.17%), SM34 (3.68 $\pm$ 0.36%) and RTF10 (0.46 $\pm$ 0.01%) strains was not significantly affected by the presence of sucrose in the medium.

Discussion

The rheological study performed here revealed that the high molecular weight (ranging from $1.74x10^8$ Da to $4.41x10^8$ Da) synthesized by LAB isolated from Algerian milk and Spanish fermented meat products have a pseudoplastic behaviour of viscosity. This behaviour has been also observed for other dextrans isolated from bacterial cultures or some commercial hydrolysed dextrans (Kothari, Tingirikari, & Goyal, 2015; McCurdy, Goff, Stanley, & Stone, 1994; Moosavi-Nasab, Alahdad, & Nazemi, 2010) and for others biopolymers, such as xanthan, an anionic HePS produced by Gram-negative bacteria belonging to the genus *Xantomonas* which are used as a food additives and rheology modifiers (Petri, 2015) or immunomodulatory β-glucans synthesised by LAB (Velasco et al., 2009). The pseudoplastic property contributes to better sensory qualities of food and also helps in food processing, when different shear rates may be applied during mixing, pouring and pumping (Moreno et al., 2000). Thus, the dextrans described here are deemed to have potential as food additive.

Differential biological effect of EPS in different ranges of molecular masses has been previously observed (Hidalgo-Cantabrana et al., 2012), since HePS having negative charges and/or small molecular masses were able to act as strong/mild stimulators of immune cells, whereas those EPS with a large size exhibited a weak stimulation. Thus, in the immunomodulation assays of PMA-THP-1 by dextrans performed here, it was expected that the number of polymer molecules decreased upon increase of molecular weight, since the same weight to volume concentration (100 μg mL^{-1}) was used for all dextrans. For the commercial dextrans a partial correlation of their effect with the molecular masses was observed, with T2000 producing the lowest and T10 the highest anti-inflammatory effect and resulting, respectively, in the highest and lowest TNF-α/IL10 ratios. However, a high unexpected immunomodulatory effect of the high molecular mass (in the 10^8 power range) EPS produced by our strains was detected. The five dextrans purified and tested in this work had a more pronounced global anti-inflammatory effect (lower

TNF-α/IL10 ratio) than T2000, and the four HoPS from *Lc. mesenteroides* strains have a beneficial influence similar to that of T10. Nevertheless, the overall results, and the knowledge of the THP-1 response to pathogens (Murthy, Dennis, Lasater, & Philipp, 2000), indicate that dextrans from *Lb. sakei* MN1 and from *Lc. mesenteroides* strains have the potential to contribute to down-regulate inflammatory and microbicidal mechanisms of the innate immune response against bacterial pathogens.

We have previously shown that dextran production by *Lb. sakei* MN1but not by *Lc. mesenteroides* RTF10 coincides with reduced autoagglutination, biofilm formation and epithelial cell adhesion (Nácher-Vázquez et al., 2017). Here, the test of the LAB ability to bind to Caco-2 cells revealed two types of bacterial behaviour upon inclusion of sucrose in the assay independent of the genus or species, and correlating with the two patterns observed for the phenotype of the colonies in the presence of the disaccharide. These differences could be due to variations among the EPS production by different bacteria. However, according to the results presented in Table 1, this does not seem to be the case, because differences in EPS yield, high (1342 and 757) for SM34 and CM30 and medium (452, 396 and 414) for CM9, RTF10 and MN1 did not correlate with the strains grouping (CM9 and MN1 *versus* SM34, CM30 and RTF10) for phenotypical and adhesion patterns. Rather, the two phenotypes could be connected to the properties or fate of the dextran. The specific chemical structure and composition of the EPS molecule could affect adhesion. Thus, we have shown that the *0*-2 substituted (1-3)-β-D-glucan HoPS synthesised by *P. parvulus* LAB strains contributes to the attachment to colon epithelial cells (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010). However, this is not a general feature for EPS from LAB, since it has been shown in *Lactobacilli* that their adhesion to enterocytes increased, when various EPS (i.e. rich in either galactose or rhamnose or HePS) production was abolished (Lebeer et al., 2009; Horn et al., 2013). In agreement, our results showed that in the presence of dextran synthesis adhesion decreased or it was not affected. In

addition, the phenotype observed for CM30, RTF10 and SM34 colonies could be related to synthesis or generation of low molecular weight EPS (< 10^4 D), lost during purification. In this line, it is feasible that these bacteria could be able to synthesise dextranases, since some *Lc. mesenteroides* subsp. *dextranicum* strains possess this enzymatic activity (Mahmoud et al., 2014), which has not been yet ascribed to any sequenced gene. Consequently, if this is the case these LAB could not only synthesise dextran, but also degrade it later to use it not only as a carbon source, when required, but also to expand its niche by planktonic growth. This hypothesis deserves to be subjected to further investigation in the future.

Conclusions

Results of the present study expand our knowledge on LAB EPS production and its biological role. Regarding the molecular mass of LAB dextrans, a fine determination was obtained by the use of SEC-MALLS, which led to disclose that high EPS mass correlate with a pattern of immunomodulation. The physicochemical and rheological studies showed that LAB EPS analysed here have a low percentage of branching and a flexible configuration that can be at least partially responsible for their beneficial behaviour in immunomodulation assays.

In addition, our results indicate that dextran-production does not have a beneficial effect on initial interaction with enterocytes, but it can contribute to the further colonisation of the ecological niche.

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Figure 1. Physicochemical characterization of dextrans produced by *Lc. mesenteroides* CM30, SM34 and CM9 strains. (A) Infrared spectrum of the three LAB. (B) Types of bonds deduced by the methylation analysis. (C) Structure of the three dextrans.

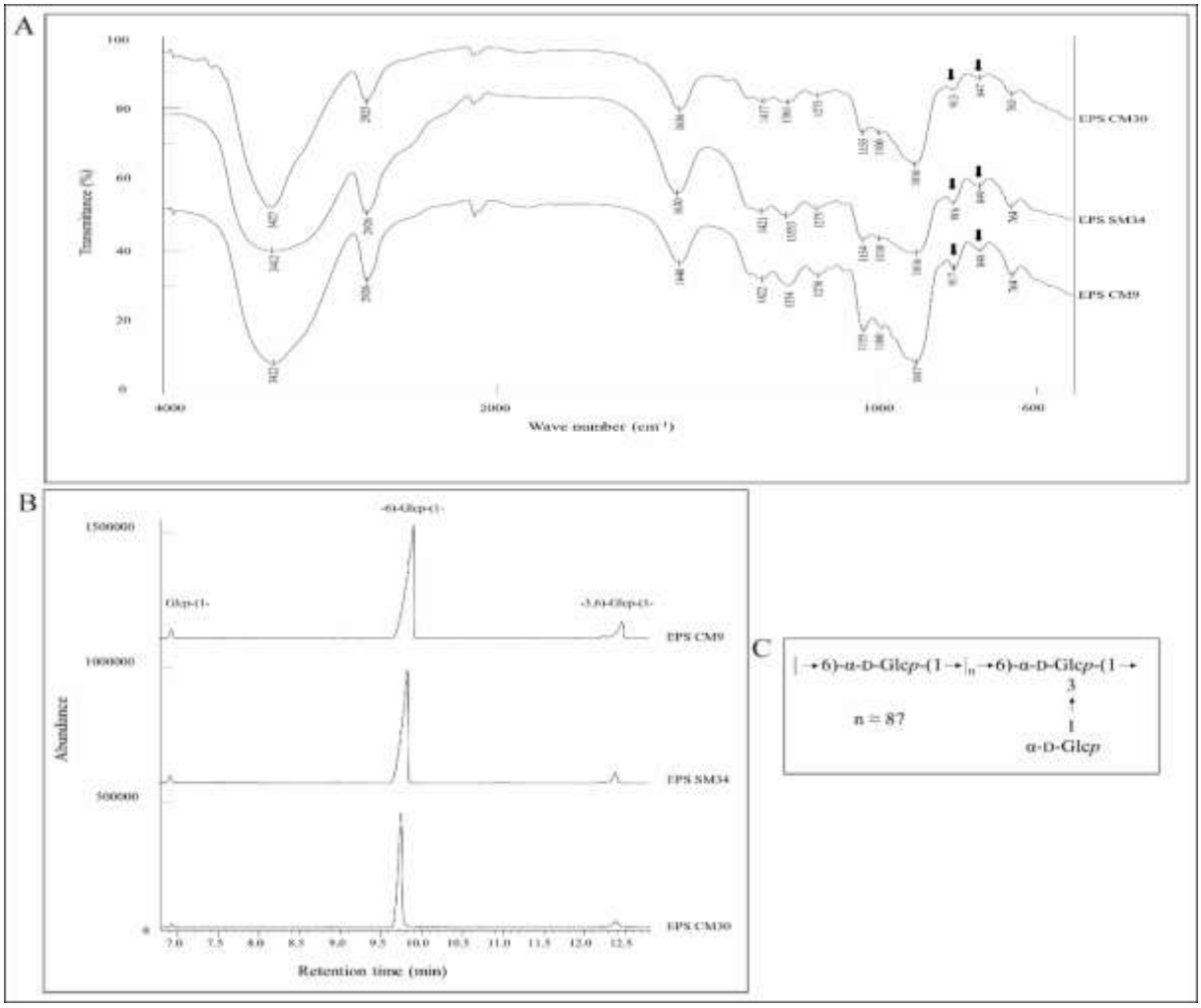

Figure 2. Apparent viscosity of the LAB dextrans *versus* the shear rate. The name of the strains from which the dextrans were purified is indicated. The experiments were performed at 20 ºC and dextran concentrations used were: 0.2% (■), 0.4% (●), 0.6% (▲), 0.8% (♦), 1% (□), 1.25% (□), 1.5% (○), 2% (Δ), 2.5% (∇), 3% (◊), 4% ( ), 5% (☒).

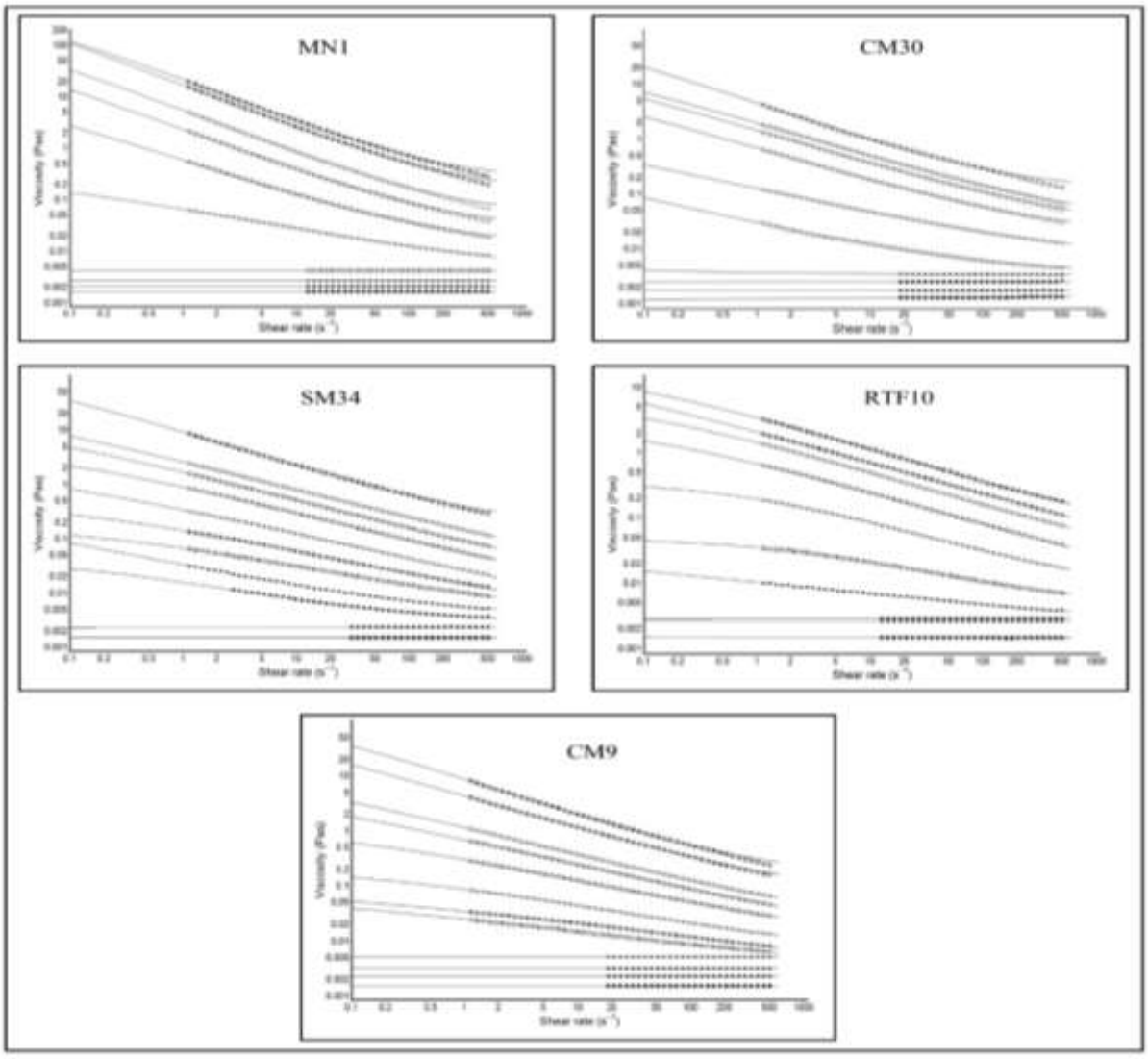

Figure 3. Influence of dextrans molecular masses in viscosity. Double-logarithmic plot of the Newtonian viscosity *versus* dextran concentrations (A-E), and (F) viscosity of the aqueous solutions (3% w/v) at zero (η_0, black) and 206 s^{-1} (η, white) shear rates.

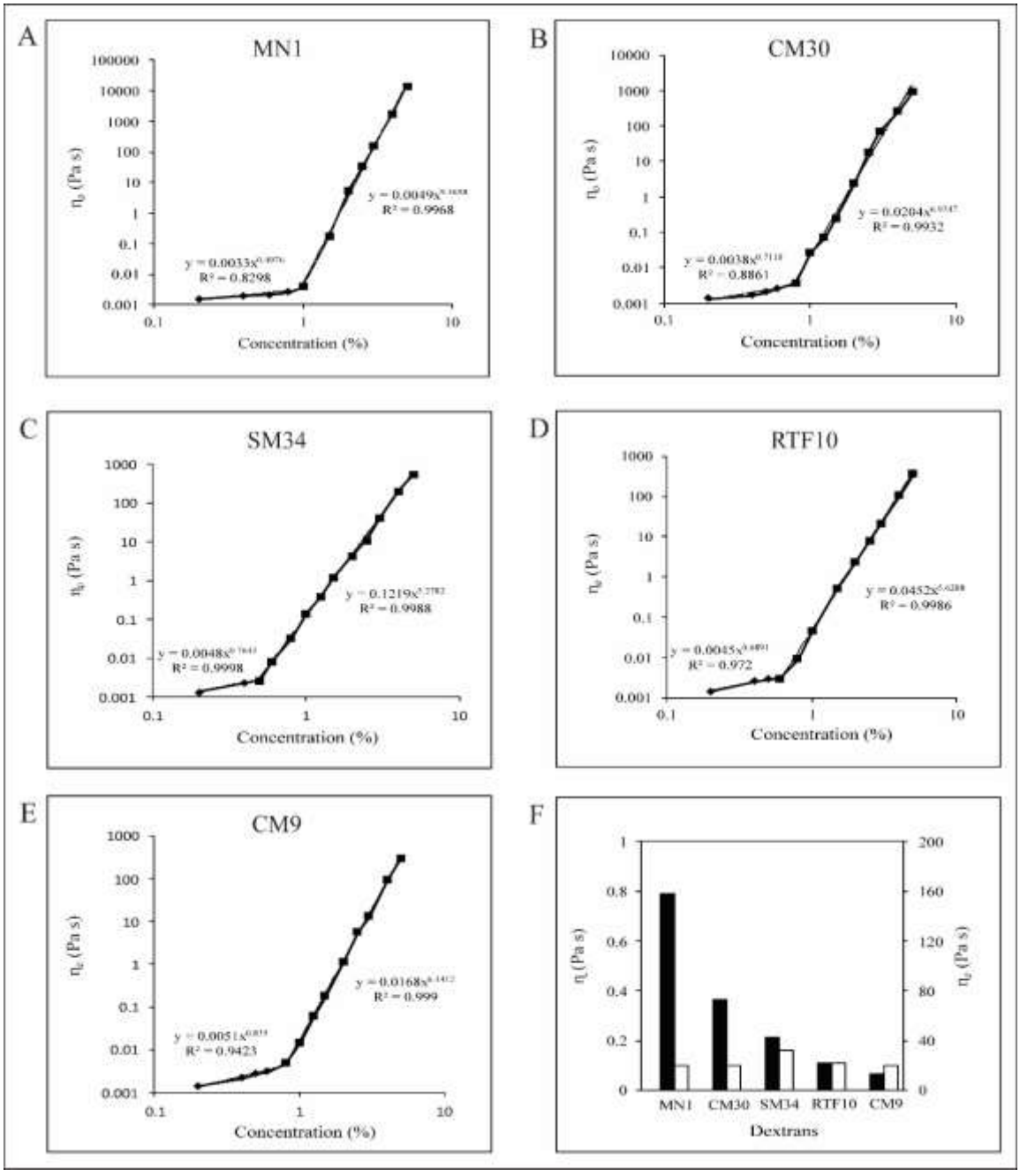

Figure 4. Influence of dextrans on cytokine production by PMA-THP-1 macrophages stimulated with LPS (*E. coli* O111:B4). (A) TNF-α, (B) IL-10 and (C) ratio TNF-α/IL-10. Dextran produced by *Lb. sakei* MN1 (MN1) and *Lc. mesenteroides* (CM9, CM30, SM34 and RTF10) strains as well as commercial T10, T40 and T2000 dextrans were assayed. Data were analysed using ANOVA. The Dunnett's test was employed to test the statistically significant differences between control and samples. Comparisons significant at the 0.05 level are indicated by ***.

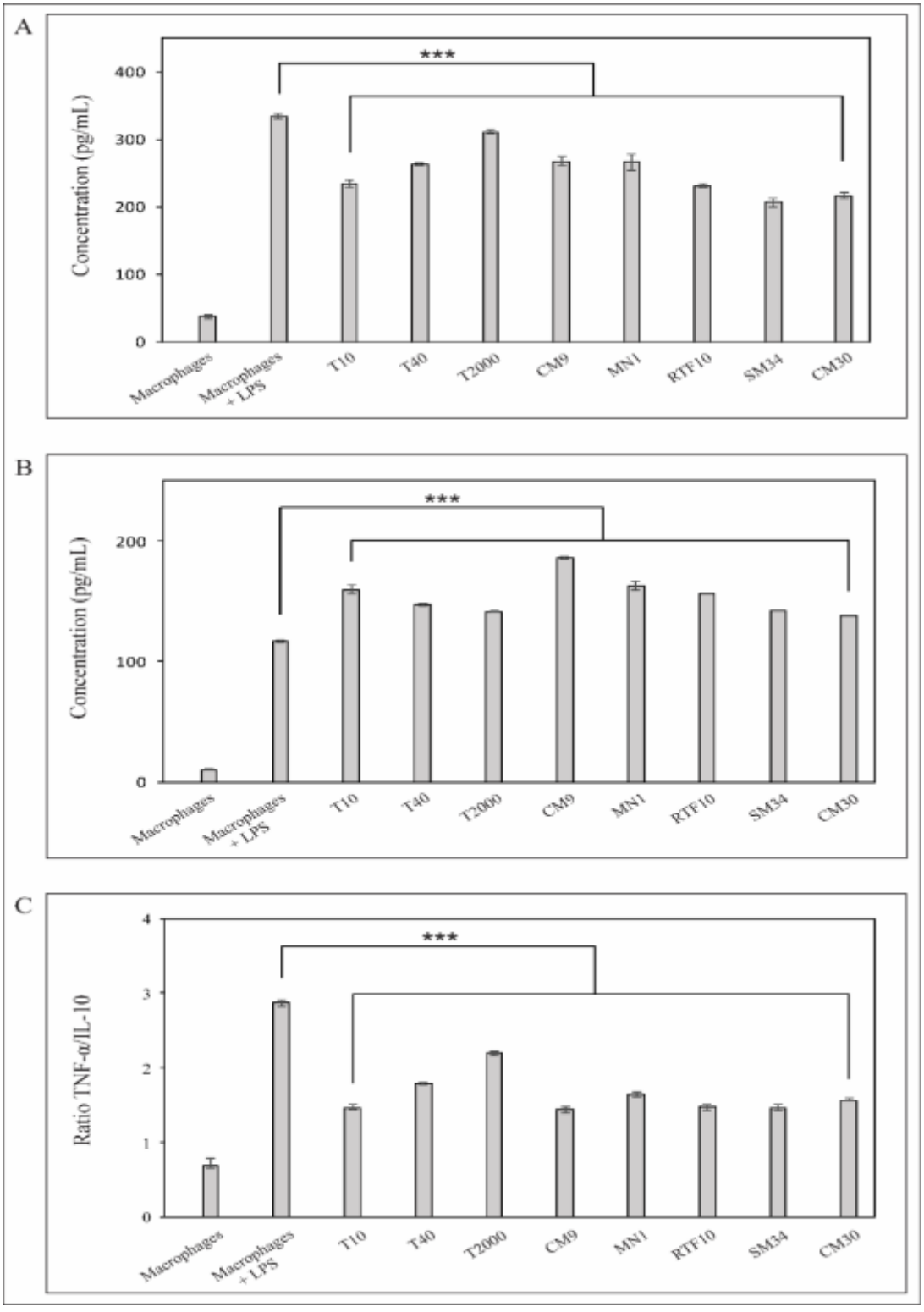

Fig. 5. Detection of EPS production by LAB on solid media. Bacterial colonies in MRSS after 48 h (A) or 72 h (B) incubation, and after 72 h in MRSG (C). Analysis of bacterial colonies of *Lc. mesenteroides* CM9 grown in MRSS (D) or MRSG (E) or its EPS preparation (F) by TEM. Arrows indicate EPS molecules.

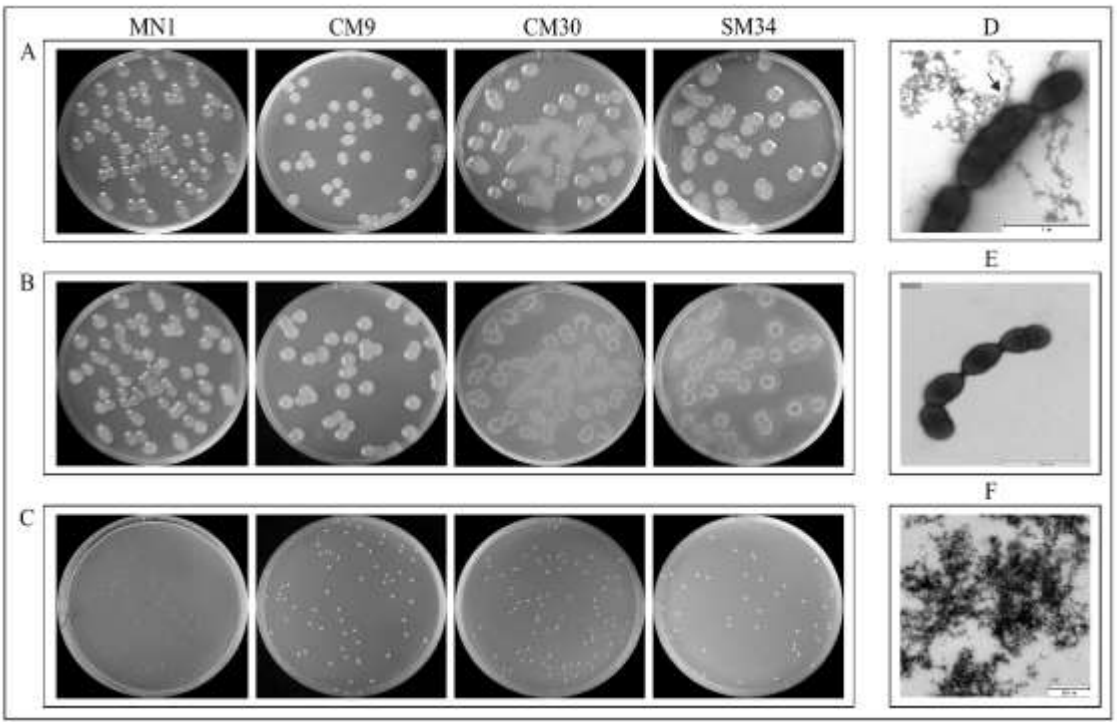

Fig. 6. Adhesion of LAB strains to Caco-2 cells. The assays were performed in DMEM (G) and DMEM supplemented with 0.5% sucrose (S) during 1 h. Adhesion levels of the indicated LAB strains are expressed as the percentage of cfu. 100% corresponds to the number of bacteria added to the Caco-2 cells. Data were analysed by ANOVA. The Tukey's test was employed to test the statistically significant differences between samples. Differences (A-D) were significant with a * $p \leq 0.05$.

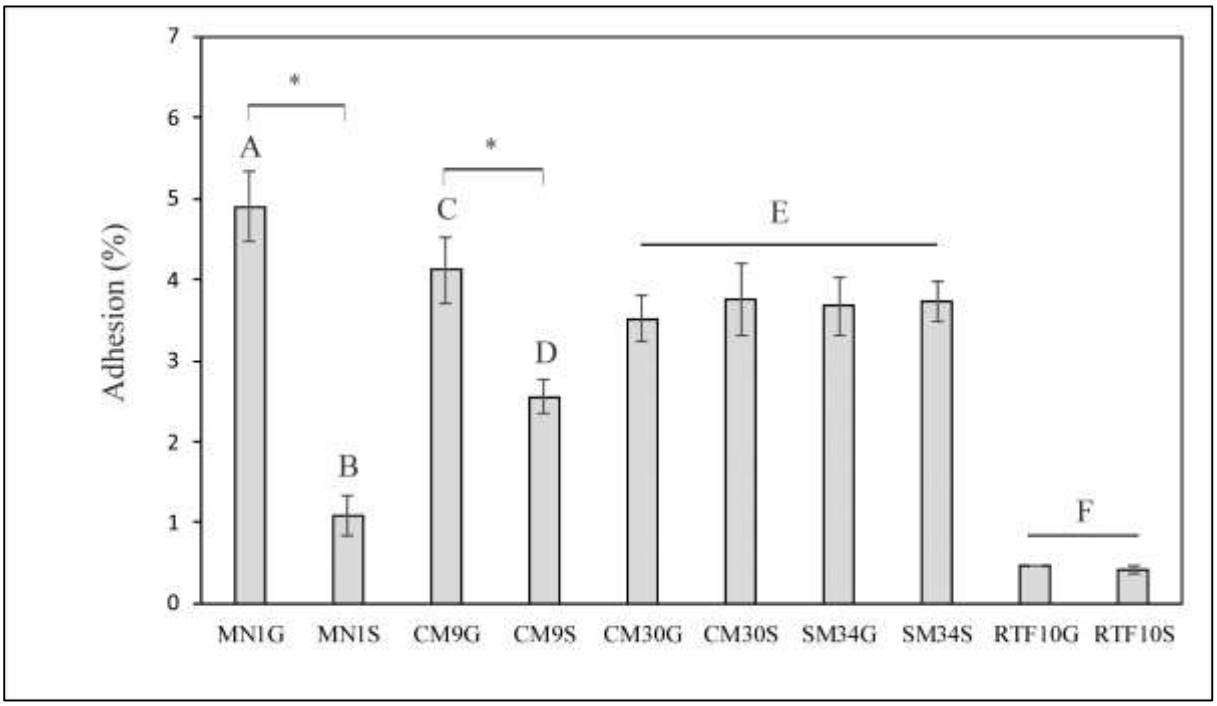

Table 1. Analysis of the EPS yield during purification and determination of their molecular masses

Samples	[a]Supernatant				[b]After precipitation and dialysis				[b]After chromatographic fractionation				Molecular mass (Da)
Molecules / Strains	[c]EPS yield ($mg\ L^{-1}/A_{600}$)	Protein ($mg\ L^{-1}$)	DNA ($mg\ L^{-1}$)	RNA ($mg\ L^{-1}$)	[d]EPS RC (%)	Protein ($mg\ L^{-1}$)	DNA ($mg\ L^{-1}$)	RNA ($mg\ L^{-1}$)	[d]EPS RC (%)	Protein ($mg\ L^{-1}$)	DNA ($mg\ L^{-1}$)	RNA ($mg\ L^{-1}$)	
CM9	452.30	48.87	3.60	0.40	75.17	2.13	ND	ND	56.84	ND	ND	ND	$2.3x10^8$
CM30	757.04	106.93	0.71	0.27	70.46	7.46	ND	ND	46.74	ND	ND	ND	$3.9x10^8$

SM34	1341.88	63.80	0.62	ND	67.67	5.86	ND	ND	46.36	ND	ND	ND	$2.1x10^8$
RTF10	395.57	52.53	0.23	ND	86.80	2.53	ND	ND	60.89	ND	ND	ND	$4.4x10^8$
MN1	414.45	66.13	0.37	ND	83.43	6.00	ND	ND	61.40	ND	ND	ND	$1.7x10^8$