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A novel rhamnose-rich hetero-exopolysaccharide isolated from *Lactobacillus paracasei* DG activates THP-1Human Monocytic cells

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Running title: *Lactobacillus paracasei* DG exopolysaccharide

Key words**:** Capsular EPS, Probiotic, Enterolactis, L. casei DG®, Immunostimulatory, THP-1, MAMPs

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Abstract

Lactobacillus paracasei DG is a bacterial strain with recognized probiotic properties and is used in commercial probiotic products. However, the mechanisms underlying its probiotic properties are mainly unknown. In this study, we tested the hypothesis that the capability of strain DG to interact with the host is, at least partly, associated with its ability to synthesize a surface-associated exopolysaccharide (EPS). Comparative genomics revealed the presence of putative EPS gene clusters in DG genome; accordingly, EPS was isolated from the surface of the bacterium. A sample of the pure EPS from strain DG (DG-EPS), upon NMR and chemical analyses, was shown to be a novel branched hetero-EPS with a repeat unit composed of L-rhamnose, D-galactose, and *N*-acetyl-D-galactosamine in a ratio of 4:1:1. Subsequently, we demonstrated that the DG-EPS displays immunostimulating properties by enhancing the gene expression of the pro-inflammatory cytokines TNF-α and IL-6, and, particularly, the chemokines IL-8 and CCL20 in the THP-1 human monocytic cell line. In contrast, the expression of the cyclooxygenase enzyme COX-2 was not affected. In conclusion, the DG-EPS is a bacterial macromolecule with the potential ability to boost the immune system as either a secreted molecule released from the bacterium or as a capsular envelope on the bacterial cell wall. This study provides additional information about the mechanisms supporting the cross-talk between *L. paracasei* DG and the host.

Importance

The consumption of food products and supplements named probiotics, i.e. containing live microbial cells, to potentially prevent or treat specific diseases, is constantly gaining popularity. The lack of knowledge on the precise mechanisms supporting their potential health-promoting properties, however, greatly limits a more appropriate use of each single probiotic strain. In this context, we studied a well-known probiotic, *Lactobacillus paracasei* DG, in order to identify the constitutive molecules that can explain the documented health-promoting properties of this bacterium. We found a novel polysaccharide molecule, named DG-EPS, that is secreted by and covers the bacterium. We demonstrated that this molecule, which has a chemical structure never identified before, has immunostimulatory properties and, therefore, may contribute to the ability of the probiotic *L. paracasei* DG to interact with the immune system.

Introduction

Strains of *Lactobacillus paracasei* are Gram-positive, non-spore-forming bacteria that are common inhabitants of the human intestinal tract. Specific strains of *L. paracasei* are found naturally in a number of fermented food products, and they have traditionally been used in the production of fermented milks and cheeses. More recently, specific strains of *L. paracasei* have been used in probiotic dietary supplements, including the strain *L. paracasei* DG (commercially known as L. casei DG, Enterolactis®). A range of health-promoting properties have been assigned to *L. paracasei* DG including the improvement of ulcerative colitis (1) and a reduction in the side effects associated with therapies for eradication of *Helicobacter pylori* (2), and treatment of small intestinal bacterial overgrowth (3). It has also been shown that *L. paracasei* DG is able to modulate fecal Clostridiales bacteria and butyrate levels in healthy adults (4). Despite the significant clinical evidence for the health benefits associated with the consumption of *L. paracasei* DG, the molecular mechanisms underlying these health effects are still unknown.

A number of mechanisms have been proposed for the probiotic effect. One of the most studied mechanisms relates to the ability of probiotic bacteria to antagonize pathogenic organisms by either excretion of antimicrobial agents (5) or the displacement of pathogenic organisms through the competitive occupancy of adhesion sites (6). In addition, there are a number of reports suggesting that health benefits result from stimulation of the immune system by components presented at the surface of probiotic strains (7, 8). Both *in vivo* and *in vitro* experiments have demonstrated that the polysaccharides present at the surface of the bacteria, referred to as either capsule or as exopolysaccharides (EPSs), can play a role in both the displacement of pathogenic organisms and the stimulation of the immune system (9, 10).

In an attempt to understand the molecular mechanisms underlying the probiotic activity *of L. paracasei* DG, we undertook a study to identify EPS molecules in *L. paracasei* DG. We isolated, purified, and characterized a novel EPS molecule (that we named DG-EPS), and studied its ability to mediate DG's probiotic properties by monitoring adhesion on a Caco-2 cell layer and the immunostimulatory activity on Caco-2 enterocyte-like and THP-1 macrophage-like cell models.

Materials and Methods

Unless otherwise stated, all reagents were purchased from Sigma-Aldrich Company Ltd. (Poole, Dorset, UK) and were used as supplied.

Identification of the putative EPS gene cluster

The draft genome sequence of *L. paracasei* DG was obtained through Ion Torrent PGM (Life Technologies, Germany) as previously described (11). The raw sequence data were assembled using MIRA v.3.9 (http://www.chevreux.org/projects_mira.html), applying default parameters recommended for Ion Torrent data processing. Initial automated annotation of the genome was performed using RAST, combined with BLASTX. Results of the gene-finder program were combined manually with data from BLASTP analysis against a non-redundant protein database provided by the National Center for Biotechnology Information (NCBI). The DG draft genome sequence was compared with other *L. paracasei* genome sequences by means of BLAST Ring Image Generator (BRIG; (12)). The functional annotation of the *EPS-b* region was carried out by combining the results of BLASTN, BLASTP and the "CD-search" of the Conserved Domain Database (CDD) available at the NCBI website (http://www.ncbi.nlm.nih.gov/Structure/cdd/wrpsb.cgi).

EPS isolation and purification

Lactobacillus paracasei strain DG (deposited at the National Collection of Microorganisms Cultures of the Pasteur Institute under the code CNCM I-1572) was grown at 37°C in de Man-Rogosa-Sharpe (MRS) broth (Difco Laboratories Inc., Detroit, MI) for 24 h. This culture was used to inoculate the chemically defined medium (CDM, Table 1). The multistep extraction and purification of EPS was performed from about 1 l of CDM supplemented with 2% glucose. After growth at 37°C for 48 h, cells were collected by centrifugation at 12,000 × g for 15 min at 4°C (Avant J-26 XPI, Beckman Coulter Ltd, High Wycombe, UK) and separated from the exhausted medium. The two fractions were then treated separately. The exhausted medium was added with an equal volume of absolute ethanol and stored at 4°C for 48 h. After storage, it was centrifuged at 25,000 × g for 35 min at 4°C. The obtained pellet (fraction S1) was dissolved in deionized water (about 20-50 ml), whereas the supernatant was added to a second volume of ethanol and stored again at 4°C for 48 h. Subsequently, the centrifugation step was repeated, and the pellet (fraction S2) was dissolved in deionized water as above. Concerning cell fractions, the pellet was washed with phosphate-buffered saline (PBS) to remove polysaccharide impurities and then treated with 1 M sodium hydroxide and stirred overnight at 4°C. Afterwards, it was centrifuged again at 12,000 × g 4°C for 15 min in order to remove sodium hydroxide. Crude EPS was precipitated by the addition of an equal volume of chilled absolute ethanol; this was stored 48 h at 4°C and then centrifuged at 25,000 × g 4°C for 35 min. The recovered pellet (fraction C1) was re-dissolved in deionized water (about 20 ml). The resulting supernatant was then added to a second volume of absolute ethanol and again incubated 48 h at 4°C. Another centrifugation, as described above, a second

precipitated fraction (C2) was recovered, which was dissolved in deionized water. Small neutral sugars and proteins were then removed by dialysis (with 100 kDa cut-off cellulose acetate membranes) of the extracted fractions for 72 h at 4°C, against three changes of deionized water per day. After three days, the contents of the dialysis membrane were collected and lyophilized in a freeze-dryer (Northern Scientific, York, UK). The dry mass of EPS was then determined. The presence of contaminating bacterial DNA in the EPS preparations was tested through real-time quantitative PCR (qPCR) with two primer pairs: universal primers targeting 16S rRNA gene (EUB), and DG strain specific primers targeting *welF* gene (13). This analysis revealed the presence of 10-63 ng ml^{-1} in the 1 mg ml^{-1} stock solutions of EPS, corresponding to an overall maximum concentration of 0.6 ng ml^{-1} of DNA incubated with THP-1 cells when the highest concentration of EPS (10 µg ml^{-1}) was used in immunological experiments.**Table 1**. Chemically defined medium (CDM) used to cultivate *L. paracasei* DG for the extraction of the exopolysaccharide (EPS).

Component	**Concentration (g l^{-1})**
Sol. 1	
$(NH_4)_2SO_4$	2
$MgSO_4 \times 7\ H_2O$	0.15
$MnSO_4 \times 4\ H_2O$	0.02
Sol. 2	
Adenine	0.005
Pyridoxal	0.002
Nicotinic acid	0.001
Ca^{2+}-D-pantothenate	0.001
Riboflavin	0.001
Thiamine	0.001
Vitamin B12	0.000001
Biotin	0.00001
p-aminobenzoic acid	0.000005
Folic acid	0.00001
Sol. 4 *	
Guanine	0.005
Xanthine	0.005
Uracil	0.005
Sol. 5	
K_2HPO_4	4.56
Sol. 6	
Sodium acetate	0.05
Sodium citrate	0.02
KH_2PO_4	0.01
NaCl	0.002
$CaCl_2$	0.002
Sol. 7	
Tween 80	1
Tween 20	1
Glycerol	1
Glucose	20
Casaminoacids	10

* Dissolved in 1 M NaOH. An equal volume of 1 M HCl

Determination of monomer composition and linkage analysis

To determine the monomer composition of the polysaccharide, the EPS (3 mg) was hydrolyzed by treatment with 2 M trifluoroacetic acid (TFA, 120°C for 2 h); the released monosaccharides were subsequently derivatized to form alditol acetates which were analyzed by gas chromatography–mass spectrometry (GC-MS). To derivatize the monomers, the mix resuspended in 1 ml Milli-Q water was added with 10 mg $NaBH_4$ and incubated at 40°C for 2 h. After evaporation of the solution, 1 ml glacial acetic acid was added to the residue, and again evaporated to dryness. Subsequently, 3 ml methanol were added and then evaporated in order to remove the borate complex and to give the methylated sugar alditols. They were then added with 2 ml pyridine and 2 ml acetic anhydride; acetylation reaction ran at 100°C for 2 h. At the end of the reaction the solution was evaporated and the acetylated monomers resuspended in water. Extraction with chloroform was performed to collect the organic phase, containing the alditol acetate sugars. Any trace of water was removed by adding anhydrous sodium sulphate and storing the sample 30 min at 4°C. Sodium sulphate was removed by filtration on filter paper and chloroform by evaporation. The resulting residue was resuspended in acetone. The GC-MS analysis was performed on an Agilent 7890A GC system (Santa Clara, CA, USA) coupled to an Agilent 5675c quadrupole MS. The samples were eluted from a HP-5 column (30 m x 0.25 mm id, 0.25 µm film) using helium as carrier (9 psi, flow rate 1 ml min^{-1}) and using a temperature program (start temperature 150°C, hold time 4 min, and a final column temperature of 250°C reached via a rising gradient of 4°C min^{-1}). The ratios of the different sugars were determined by examination of the relative responses of the different alditol acetates with reference to the relative responses determined for a standard mixture of alditol acetates. As previously reported (14), the integral area for amino sugars was low, and this is a result of their having undergone thermal decomposition during analysis. The final monomer ratio, for the amino sugar, was taken from integration of the nuclear magnetic resonance (NMR) peak integrals for the respective anomeric and H2 protons. The absolute configurations of monosaccharides were determined by conversion to their 2-butyl glycosides using the procedure described by Gerwig *et al*. (15). To determine the linkage pattern of the EPS, the sample was permethylated using the procedures described by Ciucanu and Kerek (16). The permethylated polysaccharide was then hydrolyzed (2 M TFA, 120°C for 2 h) and the methylated monosaccharides converted to methylated alditol acetates. The identity of the methylated alditol acetates was determined by analysis of their individual mass spectrum fragmentation patterns generated during GC-MS analysis. The GC-MS analyses were performed on the same instrumentation as the monomer analysis but using

the following temperature program: start temperature 155°C, hold time 1 min, and a final column temperature of 195°C reached via a rising gradient of 0.75°C min^{1}.

NMR analysis of the EPS from *L. paracasei*

Nuclear magnetic resonance (NMR) spectra were recorded for EPS samples that were dissolved (10-20 mg ml^{-1}) directly in D_2O (Goss Scientific Instruments Ltd., Essex, UK). NMR spectra were recorded at a probe temperature of 70°C. NMR spectra were recorded on a Bruker Avance 500.13 MHz ^{1}H (125.75 MHz ^{13}C) spectrometer (Bruker-Biospin, Coventry, UK) operating with Z-field gradients where appropriate and using Bruker's pulse programs. Chemical shifts are expressed in ppm relative to internal acetone (δ 2.225 for ^{1}H and δ 31.55 for ^{13}C). Spectra recorded included: a 2D gradient-selected double quantum filtered correlation spectrum (gs-DQF-COSY) recorded in magnitude mode at 70°C; total correlation spectroscopy (TOCSY) experiments recorded with variable mixing times (60, 90, 120 ms); ^{1}H-^{13}C heteronuclear single quantum coherence (HSQC) spectra (decoupled and coupled); a heteronuclear multiple bond correlation (HMBC) spectrum; and finally, a rotating frame nuclear Overhauser effect spectrum (ROESY). The 2D spectra were recorded with 256 experiments of 1024 data points. The ROESY spectrum was recorded using a Bruker pulse sequence and 256 experiments of 1024 data points using a mixing time of 200 ms. For the majority of spectra, time-domain data were multiplied by phase-shifted (squared-) sine-bell functions. After applying zero-filling and Fourier transformation, data sets of 1024-1024 points were obtained.

Bacterial adhesion to Caco-2 cell line

The adhesion of *L. paracasei* strains to Caco-2 (ATCC HTB-37) cell layer was assessed as previously described (13). In brief, for adhesion experiments, fully differentiated Caco-2 cells were used (i.e., 15 days after confluence). 100 µg ml^{-1} EPS was incubated with a monolayer of Caco-2 cells for 1 h at 37°C. Finally, monolayers were examined microscopically (magnification, 400×) under oil immersion after Giemsa staining (17). All experiments were performed in duplicate.

Nuclear factor κB (NF-κB) activation by exopolysaccharides

The activation of nuclear factor κB (NF-κB) was studied by means of a recombinant Caco-2 cell line stably transfected with vector pNiFty2-Luc (InvivoGen, Labogen, Rho, Italy) as described in detail elsewhere (18, 19). In brief, recombinant Caco-2 monolayers (approximately 3×10^{5} cells/well), cultivated in the presence of 50 µg ml^{-1} zeocin, were washed with 0.1 M Tris-HCl buffer (pH 8.0) and then suspended in fresh Dulbecco's Modified Eagle Medium (DMEM) containing 100 mM HEPES (pH

7.4) and with 0.1 ml of *L. paracasei* DG-EPS, corresponding to a final concentration of 100 µg ml^{-1}. The stimulation was conducted by adding 10 ng ml^{-1} of interleukin (IL)-1β. After incubation at 37°C for 4 h, the samples were treated, and the bioluminescence was measured as described by Stuknyte et al. (19). Two independent experiments were conducted in triplicate for each condition.

Activation of THP-1 human macrophage cell line: cell culture, growth conditions, and stimulation protocol

The monocytic THP-1 cell line was purchased from the American Type Culture Collection (Manassas, VA, USA). THP-1 cells were originally cultured from the peripheral blood of 1-year child with acute monocytic leukemia (20). They are non-adherent cells, which can be differentiated into macrophage-like cells through a protein kinase C-mediated reactive oxygen species (ROS)-dependent signaling pathway (21) by treatment with phorbol myristate acetate (PMA). The normal growth medium for THP-1 cells consisted of RPMI 1640 medium (Lonza, Basel, Switzerland) supplemented with 10% (v/v) fetal bovine serum (FBS) (Gibco-BRL, Life Technologies, Milan. Italy), 2 mM L-glutamine, 100 units ml^{-1} penicillin and 100 µg ml^{-1} streptomycin (Sigma-Aldrich). Cells were seeded at a density of 5×10^5 cells/well in 24-well plates and incubated at 37°C in a humidified atmosphere of 95% air and 5% CO_2. Differentiation was induced by the addition of PMA (Sigma-Aldrich) into the cellular medium at a final concentration of 100 nM and was allowed to proceed for 24 h. Afterwards, cells were washed once with sterile PBS buffer to remove all non-adherent cells, and fresh complete medium was added. Bacteria were used at the multiplicity of infection (MOI) of 50, EPS at final concentrations of 0.1, 1, and 10 µg ml^{-1}; lipopolysaccharide (LPS) from *Salmonella enterica* (Sigma-Aldrich) was used at a final concentration of 1 µg ml^{-1}. An untreated sample, i.e., only RPMI 1640 medium with 10% (v/v) FBS, was used as control.

Preparation of RNA and Real-time Quantitative PCR (qRT-PCR)

After incubating THP-1 cells at 37°C for 4 h, the supernatant was carefully removed from each well, and the total cellular RNA was isolated from the adhered cells with the Total RNA Blood and Cultured Cells Kit (GeneAid, New Taipei City, Taiwan). Afterwards, traces of DNA were removed by treatment with DNAse enzyme (Sigma-Aldrich), following the manufacturer's instructions. RNA concentration and purity was determined with a Take3 Multivolume Plate Reader (Biotek, Luzern, Switzerland), and reverse transcription to cDNA was performed with the iScript™ Select cDNA Synthesis Kit (Bio-Rad Laboratories, Hercules, CA), using the following thermal cycle: 5 min at 25°C, 30 min at 42°C, and 5 min at 85°C. Real-time Quantitative PCR (qRT-PCR) was carried out in order to measure the mRNA

expression levels of cytokines by means of the SYBR Green technology using the SsoFast EvaGreen Supermix (Bio-Rad) on a Bio-Rad CFX96 system according to the manufacturer's instructions. The primers were as follows (5'→3'): GAPDH forward, 5'-GGGAAGGTGAAGGTCGGAGT-3'; GAPDH reverse, 5'-TCAGCCTTGACGGTGCCATG-3'; IL-6 forward, 5'-CGGTACATCCTCGACGGCAT; IL-6 reverse, 5'-TCACCAGGCAAGTCTCCTCAT-3'; IL-8 forward, 5'- TGTGGTATCCAAGAATCAGTGAA-3'; IL-8 reverse, 5'-TATGTTCTGGATATTTCATGGTACA-3'; CCL20 forward, 5'-CTGCTTGATGTCAGTGCTG; CCL20 reverse, 5'-CACCCAAGTCTGTTTTGG-3'; TNF-α forward, 5'-TCAGCTCCACGCCATT-3'; TNF-α reverse, 5'-CCCAGGCAGTCAGATCAT-3'; COX-2 forward, 5'-CCCTTGGGTGTCAAAGGTAA; COX-2 reverse, 5'-TGAAAAGGCGCAGTTTACG-3'. All primers were designed previously, and their specificity was assessed with melting curves during amplification and by 1% agarose gels (22). Quantitative PCR was carried out according to the following cycle: initial hold at 95°C for 30 s and then 39 cycles at 95°C for 2 s and 60°C (for TNF-α and cyclooxygenase COX-2) or 58.2°C (for IL-6, IL-8 and CCL20) for 5 s. Gene expression was normalized to the reference glyceraldehyde-3-phosphate dehydrogenase (*gapdh*) gene. The amount of template cDNA used for each sample was 15 ng. All results regarding cytokine mRNA expression levels are reported as the fold of induction (FOI) relative to the control (namely unstimulated THP-1), to which we attributed a FOI of 1. Statistically significant differences have been determined through unpaired Student's t test with a two-tailed distribution.

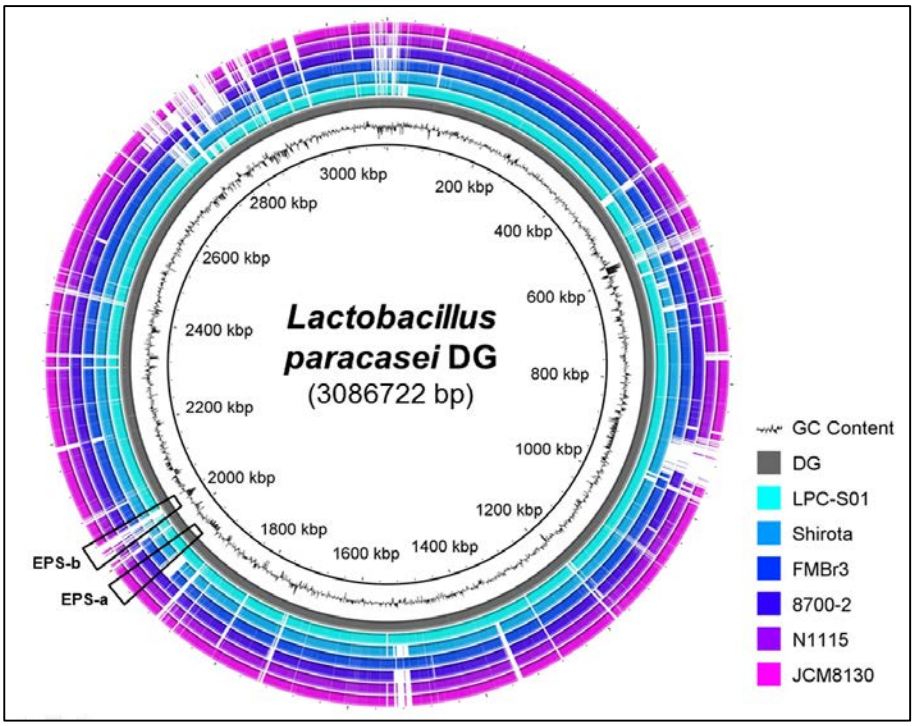
Results

Identification of putative genetic clusters for exopolysaccharide biosynthesis

In light of the potential importance of EPS molecules in the cross-talk between probiotic bacteria and host (9, 10, 23), we performed *in silico* analyses to identify putative EPS operons in the draft genome of the probiotic strain *L. paracasei* DG. Specifically, comparative analysis with other genomes of the same species led to the identification in the genome of strain DG of two different regions encoding open reading frames (ORFs) putatively involved in the biosynthesis of EPS molecules (regions *EPS-a* and *EPS-b* in **Fig. 1A**). Notably, whereas *EPS-a* region is common to all *L. paracasei* genomes investigated, *EPS-b* is a 13 kb region coding for several putative glycosyltransferases that includes a region of about 7 kb in the center of the cluster which did not find any match with other sequences in GenBank according to BLASTN search (**Fig. 1B**). The %GC of the 7 kb region is much lower (36%) than the average GC content of DG's whole genome (approximately 46%) supporting the idea of the acquisition of these genes by horizontal gene transfer from a phylogenetically unrelated host.

The identification of putative EPS operons in the genome of *L. paracasei* DG, along with the formation of a much weaker pellet after centrifugation compared to other *L. paracasei* strains (24) were considered as an indication of the probable ability of strain DG to produce EPS molecules. Consequently, we undertook experiments aimed at the extraction and purification of the EPS fraction from DG's broth cultures.

Fig 1A



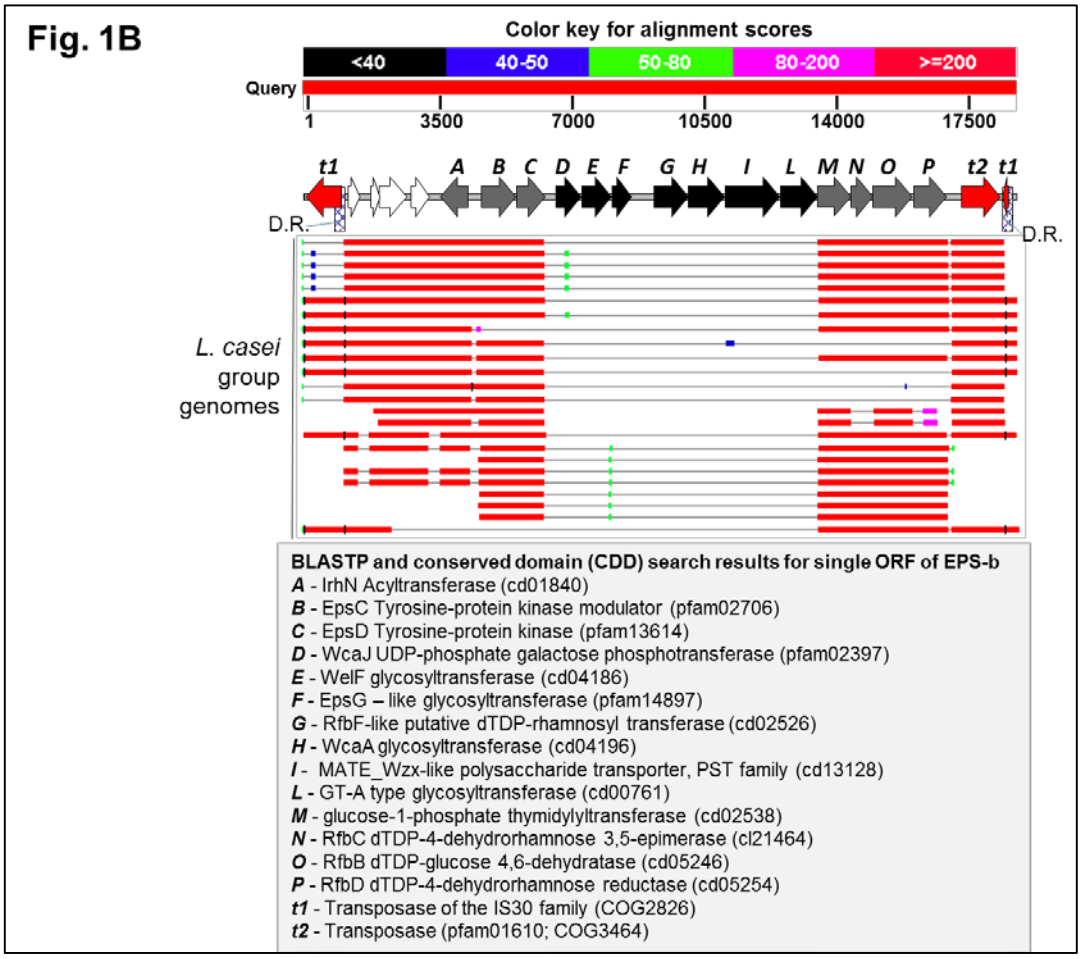


Fig. 1. Comparative genomic analysis of *Lactobacillus paracasei* DG with other complete genome sequences of *L. paracasei* strains. (**A**) Circular genome atlas of *L. paracasei* (reference genome) and six other publicly available *L. paracasei* genomes; highlighted in the atlas are the two putative exopolysaccharide (EPS) regions of strain DG. (**B**) In silico predicted functional organization of the *EPS-b* region of *L. paracasei* DG; the figure shows the BLASTN search results for the region *EPS-b* and has been obtained by adding a picture of the putative EPS gene cluster over the graphic representation of the BLASTN output. In white are indicated open reading frames (ORFs) outside the putative EPS operon; in black are ORFs that do not share significant homology with other sequences in GenBank. D.R., direct repeat sequences.

Production and isolation of the EPS from *L. paracasei* DG

A culture of *L. paracasei* DG was grown in a CDM. Although growth was slower than that observed in more conventional media such as MRS broth, CDM was chosen as it does not contain contaminating polysaccharides which interfere with the characterization of bacterial polysaccharides by NMR (25). In order to isolate a sample of polysaccharide suitable for characterization, it was necessary to grow *L. paracasei* DG for three days, at which point the cell biomass was separated from the fermentation liquors by centrifugation. High purity EPS was isolated from the supernatant by fractional precipitation of material. Adding one volume of ethanol released small amounts of an EPS material contaminated with proteins (typically 20-25 mg from a 500 ml batch fermentation). The addition of a second volume of ethanol also precipitated a relatively small amount of EPS (20-25 mg) but with much greater purity

and of a purity that was suitable for characterization by NMR. As the yields of EPS were low, and in order to determine if additional material was being retained with the biomass, various different methods were attempted in order to recover capsular material bound to the cells. Stirring a suspension of the cells overnight in an aqueous solution of sodium hydroxide (1 M) and then precipitating crude polysaccharide by adding two volumes of ethanol yielded a significant amount of material which included both polysaccharide and protein. The same approach was adopted for the isolation of extracellular polysaccharide molecules from strain *L. paracasei* LPC-S01, whose genome was reported to harbor two distinct putative EPS operons (13), one of which was identical to *EPS-a* of strain DG. However, we failed to obtain significant amounts of EPS from LPC-S01.

The purity of the EPSs released into the supernatant was established by examination of a ^{1}H-NMR spectrum of the sample (**Fig. 2A**). The low-field region of the spectrum contained six anomeric signals. Within the error of the experiments, the peak area integrals for each of the anomeric signals were the same, implying that a single EPS having a repeating unit containing six monosaccharides had been isolated. In addition to the anomeric signals, a single resonance with an integral height of three was visible at a chemical shift of 2.05 ppm which is indicative of the presence of an *N*-acetylhexosamine and a further two sets of overlapping doublets, each integrating to six protons, were present at 1.25 &1.32 ppm; these signals indicated that the repeat unit contained four 6-deoxyhexoses.

Fig. 2A

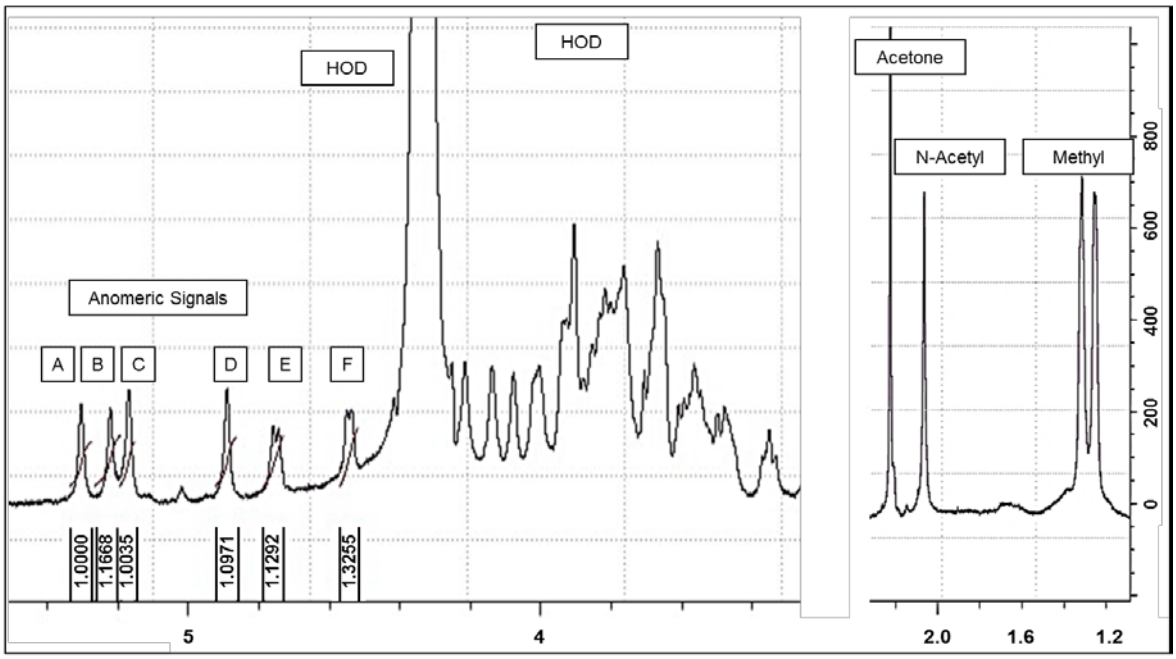
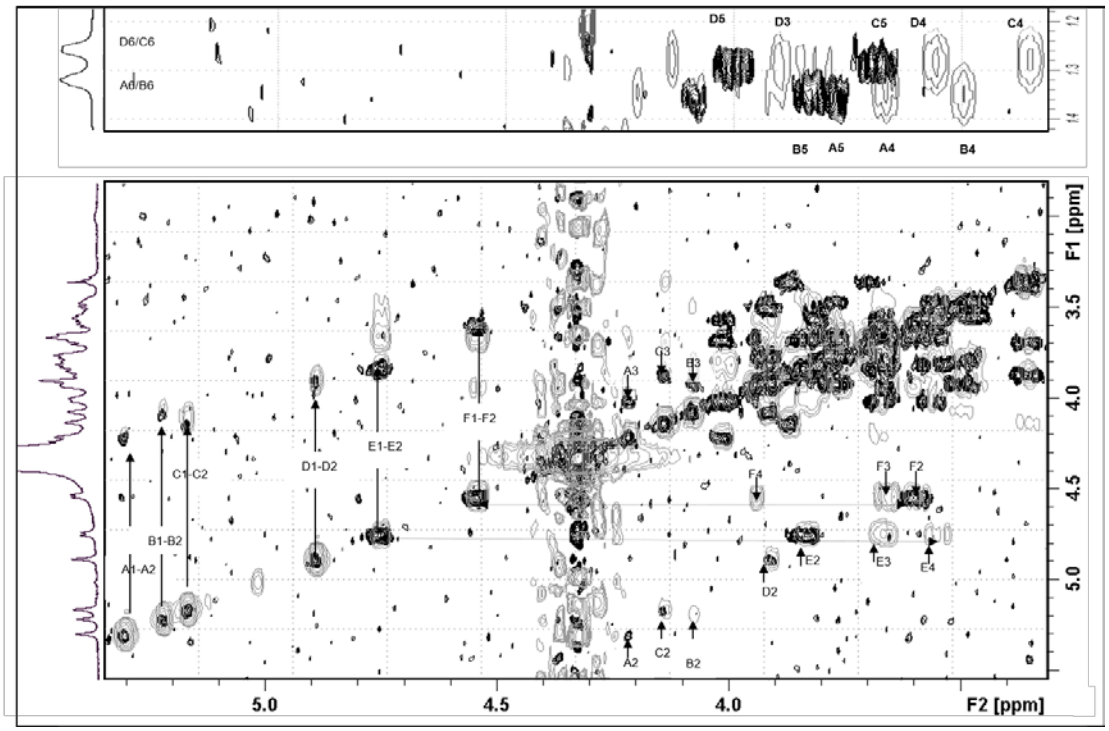


Fig. 2B


D6/C6
A6/B6
D5
D3
C5
D4
C4
B5
A5
A4
B4
F1 [ppm]
A1-A2
B1-B2
C1-C2
D1-D2
E1-E2
F1-F2
A3
B3
C3
F4
F3
F2
E2
E3
E4
D2
A2
C2
B2
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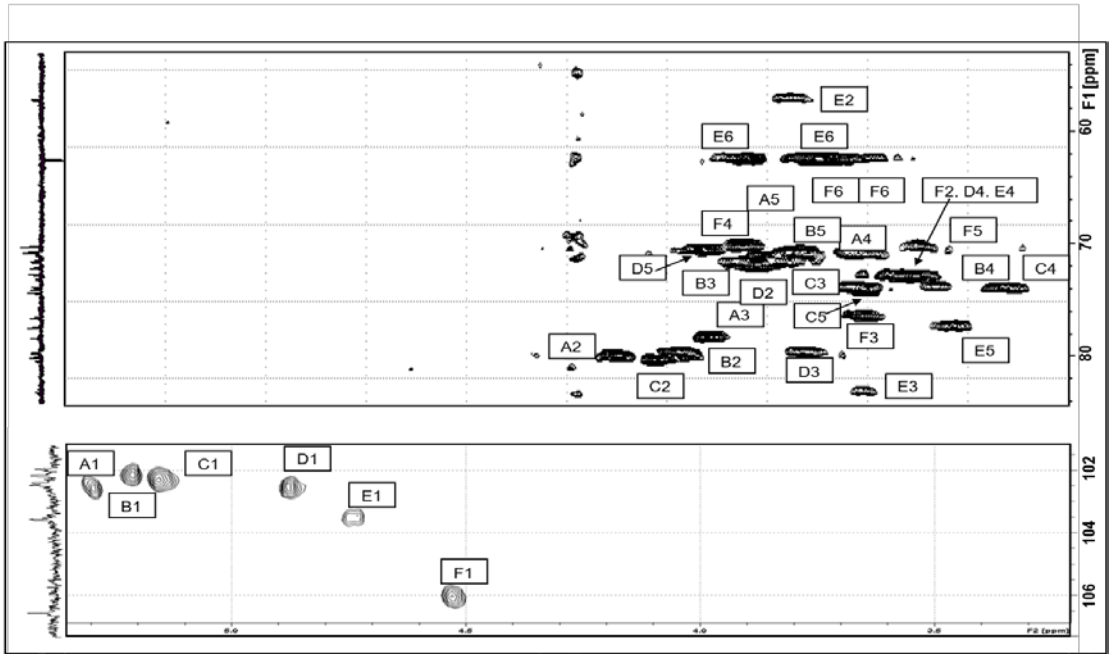
4.5
4.0
F2 [ppm]

Fig. 2C


F1 [ppm]
E2
E6
E6
A5
F6
F6
F2. D4. E4
F4
B5
A4
F5
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D2
C3
B4
C4
A3
C5
F3
A2
B2
D3
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80
102
104
106

Fig. 2D

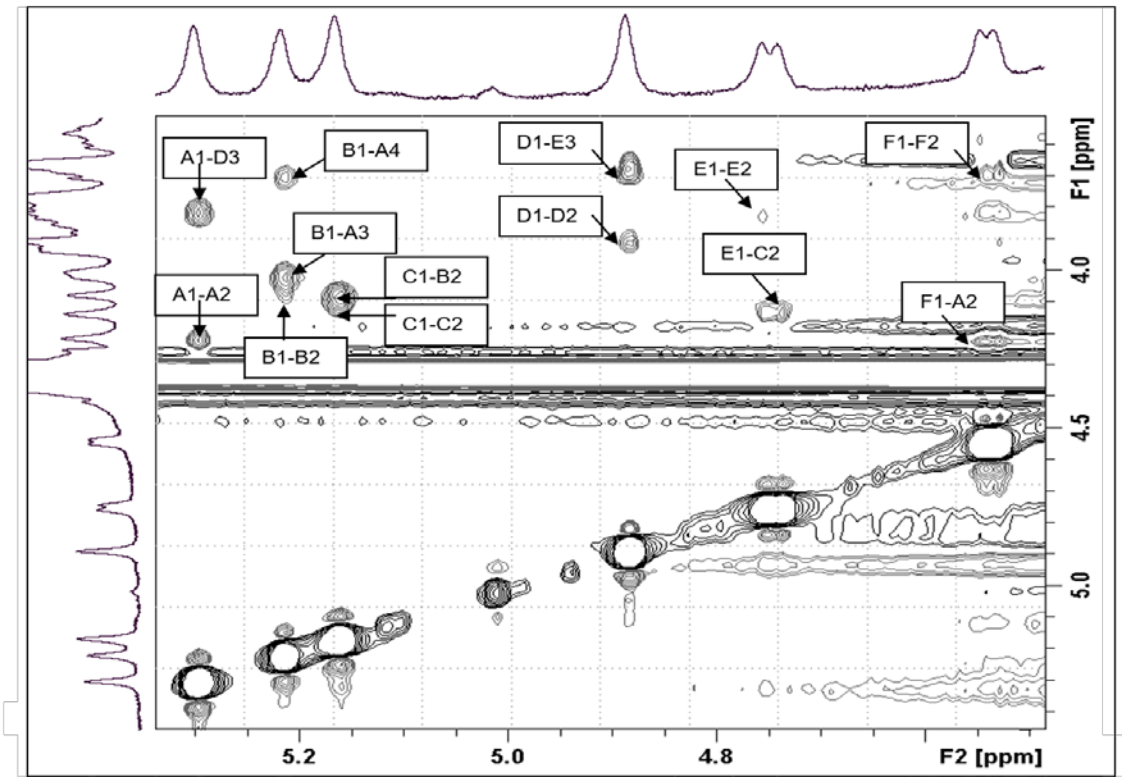


Fig. 2. NMR analysis of the exopolysaccharide (EPS) isolated from *Lactobacillus paracasei* DG. (**A**) Selected regions of the ^{1}H-NMR of the DG-EPS recorded at 70°C in D_2O and using acetone as an internal standard, anomeric (H-1) resonances are labelled A-F in order of decreasing chemical shift. **(B)** Selected regions of overlaid COSY (black contours) and TOCSY (grey contours) spectra for the DG-EPS recorded at 70°C; symbols A-F identify individual sugars and numbers (1-6) identify the C/H ring position. **(C)** Selected regions of the HSQC spectrum of DG-EPS; the location of the individual ring and H6-protons and carbons are identified on the top frame, and the location of the anomeric protons and carbons are identified on the bottom frame. The spectrum was recorded in D_2O at 70°C. **(D)** Anomeric region of a ROESY spectrum recorded for the DG-EPS; inter- and intra-residue NOEs from the anomeric hydrogens to ring protons are individually labelled.

Monomer, linkage analysis, and determination of the absolute configuration of the monosaccharides in the repeating unit structure

GC-MS analysis of the alditol acetates generated during monomer analysis of the EPS identified the presence of 1,2,3,4,5-penta-*O*-acetyl-L-rhamnitol, 1,2,3,4,5,6-hexa-*O*-acetyl-D-galactitol, and 2-acetamido-1,3,4,5,6-penta-*O*-acetyl-2-deoxy-D-galactitol in a ratio of 4:1:1. The results of the monomer analysis identified the presence of rhamnose, galactose, and *N*-acetylgalactosamine in the repeating unit.

The methylated alditol acetates generated during linkage analysis included: a 1,5-di-*O*-acetyl-2,3,4,6-tetra-*O*-methylhexitol which confirms that the galactose is present in its pyranose form as a terminal sugar; a 1,3,5-tri-*O*-acetyl-2-(acetylmethylamino)-2-deoxy-4,6-di-*O*-methylgalacitol, which confirms that the *N*-acetylgalactosamine is present in its pyranose form as a 1,3-linked

monosaccharide; two 1,2,5-tri-*O*-acetyl-3,4-di-*O*-methyl-6-deoxyhexitols, which indicates that two of the rhamnose monomers are 1,2-linked; a 1,3,5-tri-*O*-acetyl-6-deoxy-2,4-di-*O*-methylhexitol, which indicates that one of the rhamnose monomers is 1,3-linked; and finally a 1,2,3,5-tetra-*O*-acetyl-6-deoxy-4-*O*-methylhexitol suggesting that the final rhamnose is a 1,2,3-linked rhamnose present as a bridging point in the repeating unit.

Conversion of the monomers to mixtures of their epimeric 2-butyl-glycosides confirmed that all the rhamnose monomers were of L-absolute configuration whilst both the galactose and *N*-acetylgalactosamine were of D-absolute configuration.

Determination of the sequence of the monomers in the repeat unit using 1D & 2D NMR

The chemical shifts of each of the protons and carbons in the repeat unit were determined through the inspection of a series of 1D & 2D NMR spectra, and these are listed in **Table 2**. Analysis of the ^{1}H-^{1}H COSY spectrum (**Fig. 2B**, black contours) in combination with the ^{1}H-^{1}H TOCSY spectrum (**Fig. 2B**, grey contours) allowed the scalar coupling within the individual sugars to be tracked from the anomeric protons (labelled **A to F** in order of decreasing chemical shift- see **Fig. 2A**) from H1 to H6 in the rhamnose sugars (**A, B, C, and D**) and from H1 to H4 in the galactose and *N*-acetylgalactosamine monomers (**Fig. 2B**).

Table 2. The chemical shifts of protons and carbons in the repeat unit of *L. paracasei* DG exopolysaccharide (DG-EPS) determined through the inspection of a series of 1D & 2D NMR spectra.

Position	**A**	**B**	**C**	**D**	**E**	**F**
H1 (C1)	5.30 (102.6)	5.21 (102.2)	5.15 (102.4)	4.87 (102.5)	4.72 (103.5)	4.53 (106.0)
H2 (C2)	4.21 (79.8)	4.07 (79.6)	4.13 (80.3)	3.90 (71.6)	3.82 (57.1)	3.59 (72.5)
H3 (C3)	4.01 (78.2)	3.91 (72.0)	3.86 (71.3)	3.79 (79.5)	3.65 (83.1)	3.63 (76.5)
H4 (C4)	3.67 (70.4)	3.49 (73.6)	3.35 (73.7)	3.55 (72.9)	3.54 (72.6)	3.93 (70.1)
H5 (C5)	3.82 (70.6)	3.77 (70.6)	3.66 (73.8)	4.02 (70.5)	3.45 (77.3)	3.53 (70.0)
H6 (C6)	1.32 (18.6)	1.32 (18.1)	1.25 (18.0)	1.25 (17.9)	3.91/3.75 (62.4)	3.75 (62.3)
Acetyl (CH_3CO)				CH_3 δ 2.05 CH_3 δ 23.5 CO δ 175.6		

A HSQC spectrum was used to correlate ring carbons with their attached protons (**Fig. 2C**) and the distinctive position of C2 and identification of H2 of the *N*-acetylgalactosamine confirmed, by observation of scalar coupling to H1 resonance at 4.72 on the COSY spectrum, that the anomeric resonance at 4.72 ppm (**E** in **Fig. 2A**) was that of the *N*-acetylgalactosamine. The remaining anomeric resonance at 4.53 ppm must therefore belong to the terminal galactose.

Through inspection of the carbon chemical shifts of the rhamnose ring carbons, it was possible to identify points of linkages by locating those carbons whose chemical shifts had moved towards low-field positions (above 78 ppm) compared to the values normally associated with unsubstituted ring positions (less than 74 ppm for rhamnose sugars). This identified **A** as a 2,3-linked rhamnose (C2, δ 79.8 ppm; C3, δ 78.2 ppm), **B** as a 2-linked rhamnose (C2, δ 79.6 ppm), **C** as a 2-linked rhamnose (C2, δ 80.3 ppm), and **D** as a 3-linked rhamnose (C3, δ 79.5 ppm). The results of the linkage analysis already identified that the *N*-acetylgalactosamine (**E**) is 1,3-linked, and this was confirmed by the high chemical shift of C-3 in **E** (δ 83.1 ppm). Finally, the chemical shifts of the carbons in residue **F** are in agreement with this being a terminal galactose monomer.

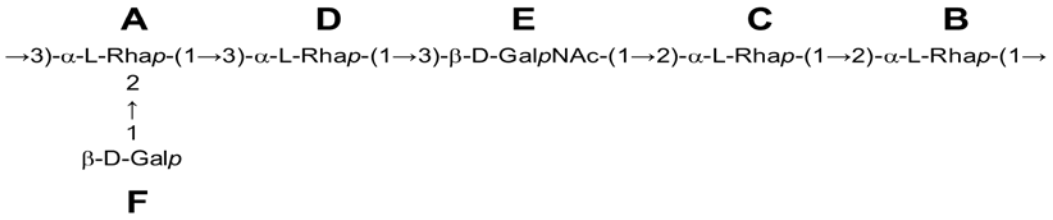
The anomeric configuration of the monosaccharides was determined by measuring the $^1J_{C1\text{-}H1}$ coupling constants which were visible on a coupled HSQC spectrum (not shown). Residues **A** to **D** had $^1J_{C1\text{-}H1}$ coupling constants **A** (177 Hz), **B** (172Hz), **C** (175Hz), and **D** (174 Hz) which are more

than 170 Hz, which indicates that the rhamnose residues are alpha-linked, whilst the size of the $^{1}J_{C1\text{-}H1}$ coupling constants in **E** (164 Hz) and **F** (157 Hz) identifies these two resonances as beta-linked monomers.

Finally, the sequence of the sugars in the repeating unit was established through inspection of both a ^{1}H-^{13}C-HMBC spectrum (not shown) and a ^{1}H-^{1}H-ROESY spectrum (**Fig. 2D**). Inter-residue scalar coupling observed on the HMBC spectrum included: coupling between **A**-H1 & **D**-C3, indicating **A** is linked to the 3-position of **D**; coupling between **C**-H1 & **B**-C2, indicating **C** is linked to the 2-position of **B**; coupling between **D**-H1 & **E**-C3, indicating **D** is linked to the 3-position of **E**; coupling between **F**-H1 & **A**-C2, indicating **F** is linked to the 2-position of **A**. On the ROESY spectrum, inter-residue NOEs were observed: between **A**-H1 & **D**-H3, confirming the **A**(1-3)**D** linkage; between **B**-H1 and **A**-H3 (strong) and **A**-H4 (moderate), identifying that **B** is linked to the 3-position of A; between **C**-H1 & **B**-H2, confirming the **C**(1-2)**B** linkage; between **D**-H1 & **E**-H3, identifying a **D**(1-3)**E** linkage; between **E**-H1 & **C**-H2, identifying the **E**(1-2)**C** linkage; and also between **F**-H1 & **A**-H2, confirming the **F**(1-2)**A** linkage.

The combined results of the chemical and NMR analysis of the EPS isolated from *L. paracasei* DG indicates that the DG-EPS is a novel heteropolysaccharide having the repeating unit structure reported in **Fig. 3**.

Fig. 3



In the following set of experiments, we studied the possible involvement of the characterized EPS molecule in mediating the cross-talk between *L. paracasei* DG and the host.

Experiments on enterocyte-like Caco-2 cell model

In order to investigate the potential ability of the DG-EPS to mediate the bacterium's interaction with the host, we first used the Caco-2 cell line, which is considered a valuable in vitro tool for studying the mechanisms underlying the interaction between bacterial cells and the human gut (7, 17, 26).

In a previous publication, *L. paracasei* DG was shown to be moderately adhesive on Caco-2 cell layer (13). In this study, the potential involvement of DG-EPS was assessed by testing also the adhesion ability of strain DG after removal of the EPS molecule ("naked" DG cells, nDG, prepared through PBS washes and mild sonication) and upon pre-incubation of Caco-2 cells with purified EPS. Several studies demonstrated that bacterial EPS molecules may both promote adhesion (27) or, on the opposite, reduce adherence by masking cell-wall adhesins (28). Nonetheless, in our experiments, purified EPS was unable to affect the adhesion properties of strain DG. In fact, the adhesion of *L. paracasei* DG was not significantly different from that of nDG and was quite modest (about 300 bacteria per 100 Caco-2 cells), as previously reported (13); in addition, the adhesion ability was unaffected by the co-incubation of the bacterial cells with purified EPS (data not shown).

Afterwards, Caco-2 cells were employed to investigate the e fDG-EPS on the activation of the transcription factor NF-κB. We recently showed that *L. paracasei* DG possesses an evident ability to reduce NF-κB activation in Caco-2 cells at baseline and upon stimulation with the pro-inflammatory cytokine IL-1β (13), as determined through a reporter system obtained by transfecting Caco-2 cells with a luciferase reporter vector (6, 7). Here, we used the same immunological model to test the EPS macromolecule isolated from strain DG. We found that the purified EPS molecule, differently from the whole bacterial cells and their exhausted broth (13), was unable to affect NF-κB activation both at baseline and in the presence of the pro-inflammatory stimulus IL-1β (data not shown). Other *L. paracasei* strains under study (namely, strains LPC-S01 and Shirota) displayed the same ability of strain DG in reducing NF-κB activation in Caco-2 cells (13), further suggesting that DG-EPS does not contribute to this specific immunomodulatory effect.

DG-EPS molecule triggers the expression of chemokines and cytokines by THP-1 cells

We exploited the THP-1 macrophage model to test the immunomodulatory properties of *L. paracasei* DG and its isolated EPS. We quantified through qRT-PCR the gene expression of the tumor necrosis factor (TNF)-α, the interleukin (IL)-6, the chemokine (C-C motif) ligand 20 (CCL20), the chemokine IL-8, and the cyclooxygenase (COX)-2. We tested three concentrations of the purified DG-EPS molecule (0.1, 1, and 10 μg ml^{-1}). We also performed the same experiments in the presence of 1 μg ml^{-1} of the pro-inflammatory stimulus LPS. We found that the purified DG-EPS can stimulate the expression of all

genes under study in a concentration-dependent manner, with the exception of COX-2 (Fig. 4). In particular, the chemokines IL-8 and CCL20 were induced approximately 70-fold by 10 µg ml^{-1} DG-EPS, whereas the pro-inflammatory cytokines TNF-α and IL-6 26- and 39-fold, respectively. A similar stimulatory profile was observed with bacterial cells of strain DG (MOI 50), even if to a lower extent (Fig. S1). In addition, when EPS was removed by PBS washes and mild sonication, the bacterial cells of strain DG lost their ability to stimulate the gene expression of TNF-α, IL-8 and CCL20; the addition of 1 µg ml^{-1} purified EPS partially reconstituted the stimulatory capacity of the cells (Fig. S1).

As expected, the stimulation of THP-1 cells with LPS determined a marked overexpression of all tested genes, particularly IL-6, IL-8, and CCL20. However, the addition of DG-EPS did not significantly affect the LPS-associated inductions of all tested genes (Fig. 4).

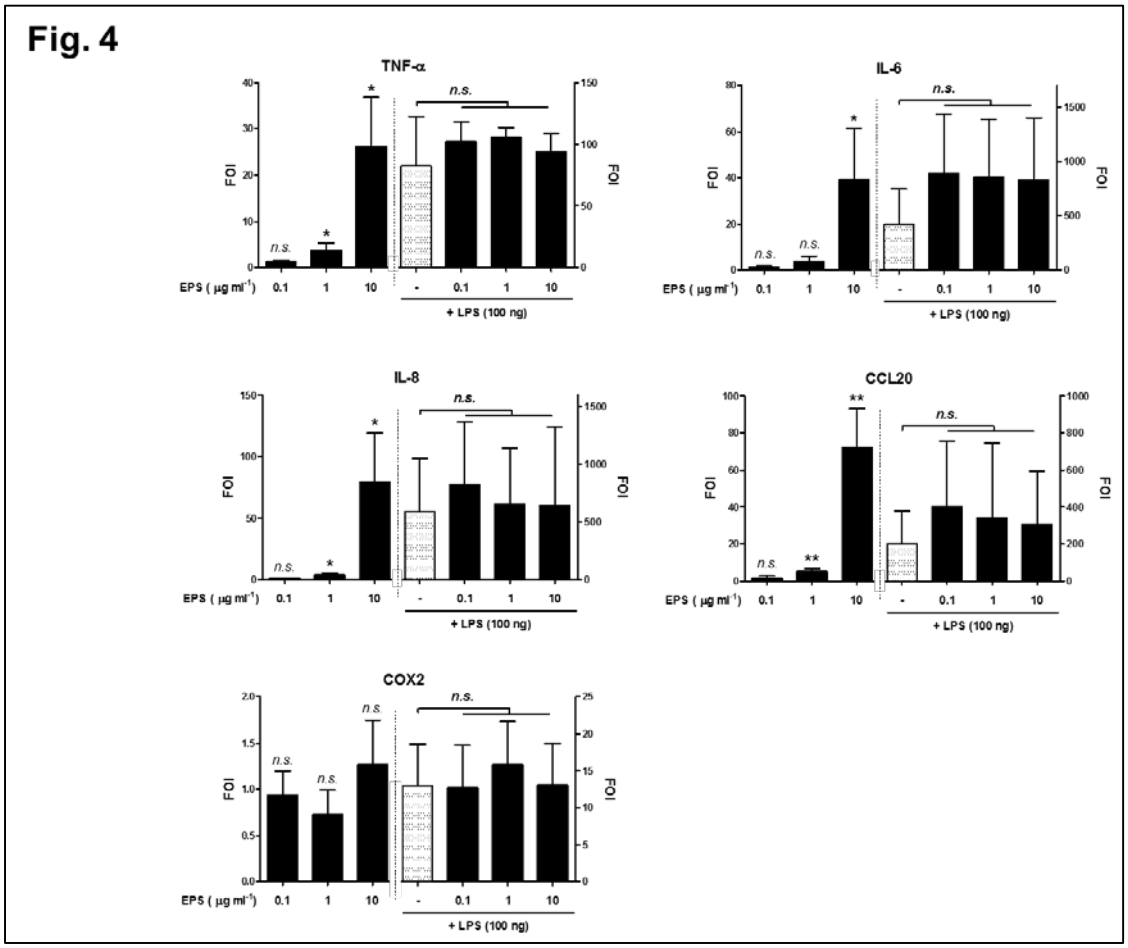


Fig. 4. Gene expression analysis by qRT-PCR in human macrophages THP-1 after 4 h stimulation with purified DG-EPS molecule (0.1, 1, and 10 µg ml^{-1}), with or without the addition of LPS (1 µg ml^{-1}). Expression levels of TNF-α, IL-6, IL-8, CCL20, and COX-2 are shown as the fold change of induction (FOI) relative to the control (unstimulated macrophages), which was set at a value of 1 (red line). Data are presented as mean of three independent experiments ± standard deviation. Asterisks indicate statistically significant differences compared to unstimulated (samples under the left Y axis) or LPS-stimulated (samples under the right Y axis) THP-1 cells: *, $P<0.05$; **, $P<0.01$.

Discussion

The use of probiotic and, in general, food-grade bacteria as modulators of host immune responses appears a promising strategy for the prevention and management of the inflammatory conditions at the gut mucosa level. To this aim, the identification of microbial cell components mediating the interaction with host cells is of pivotal importance. The enormous biodiversity of the microbial world provides a virtually inexhaustible source of molecular stimuli for the immune system, which has been to date only marginally explored. Particularly, bacterial cell wall components and secreted molecules are key ligands that can interact with the host receptors and activate various signaling pathways, thus triggering the final probiotic effect. Among the most studied probiotics, lactic acid bacteria (LAB) possess a cell wall that is typically composed of a thick peptidoglycan layer decorated with proteins, teichoic acids, and polysaccharides (29), which can act as microbe-associated molecular patterns (MAMPs). EPSs, apart from their technological properties and industrial applications, have been found to contribute to several health-promoting features of probiotics, such as antitumor, antiulcer, immunomodulatory, antiviral, and cholesterol-lowering activities (30, 31), but their mechanisms of action have not been clearly established and are probably diverse and complex.

Thanks to the genomic analysis on *L. paracasei* strain DG, we could identify two gene clusters putatively coding for EPS biosynthesis. Interestingly, GenBank search revealed that one of these regions, *EPS-b*, is unprecedented. Since we failed to obtain knock-out mutants for *EPS-b*, the link between this gene cluster and the production of the DG-EPS, although probable, is not definitely demonstrated. Nonetheless, the identification of EPS gene clusters in DG's genome inspired us to investigate biologically the potential presence of EPS molecules on the outer surface of the bacterium.

After purification of the exopolysaccharidic cell fraction of strain DG, we characterized the EPS repeating unit by means of chromatographic methods and NMR spectroscopy, establishing that it possesses a novel structure. Effectively, the repeating unit structures of a number of different strains of the *L. casei* group of species (i.e., *L. casei*, *L. paracasei*, and *L. rhamnosus*) have previously been published (32-38), and the one reported here is different. Moreover, it is also different to those of the published structures of the EPSs characterized for a range of LAB (30, 39). A feature of the repeating unit structure of the EPS reported here and those of a number of probiotic bacteria is the presence of rhamnose in the polymeric backbone (30); nonetheless, the very high content of rhamnose

(accounting for two third of the sugar moieties in molarity) in the DG-EPS is, to the best of our knowledge, unique in LAB.

The ability of EPSs isolated from LAB to trigger immunomodulatory responses has been observed in several studies (23, 40-43); nonetheless, the definition of a common immunological outcome for LAB's EPSs is a hard (and potentially impracticable) task, because their broad structural diversity may influence the recognition by immune system receptors (40). Therefore, any EPS molecule with a different monosaccharide sequence may represent a potential novel MAMP. In this context, we undertook an in vitro immunological characterization of DG-EPS. The initial experiments clearly demonstrated that the previously reported ability of strain DG to modulate NF-κB activation in Caco-2 enterocytes (13) is independent from the EPS.

After this preliminary immunological investigation on epithelial cells, we then performed the subsequent experiments with macrophages, which are cells more properly belonging to the immune system. Macrophages, just like monocytes and dendritic cells, are antigen-presenting cells (APCs) responsible for the detection of microorganisms and involved in their clearance through phagocytosis and production of pro-inflammatory cytokines. Specifically, in our study, we used the human leukemia monocytic cell line THP-1 that, upon differentiation with PMA, expresses a phenotype with similarities to human peripheral blood mononuclear cell (PBMC) monocyte-derived macrophages (44). With this model, we found that DG-EPS displays immunostimulating properties by enhancing the gene expression of the pro-inflammatory cytokines TNF-α and IL-6 and, particularly, the chemokines IL-8 and CCL20. On the contrary, the expression level of the cyclooxygenase enzyme COX-2 was not affected. TNF-α is a cytokine that enhances the expression of accessory molecules involved in macrophages to guide memory cells to better access the site of an infection (45). Similar to TNF-α, IL-6 is among the first cytokines to be produced by APCs during the innate immune response toward bacteria. TNF-α and IL-6 have pleiotropic effects such as induction of the acute phase response and activation of macrophages and, also together with IL-8, have a crucial role as chemoattractants for neutrophils, immature dendritic cells, natural killer cells, and activated T cells (46). Furthermore, CCL20 is a chemokine that attracts immature DCs (47), whose induction has been shown to be regulated by TNF-α (48) and found to be also elicited by probiotics such as *L. rhamnosus* GG (49).

In accordance with the results of our study, in a previous study, the EPS isolated from *L. rhamnosus* KL37 was shown to display a pro-inflammatory profile by inducing the production of TNF-α, IL-6, and IL-12 in mouse peritoneal macrophages (40). Similarly, in a different study, the EPS from

L. rhamnosus RW-9595M, which is a rhamnose-rich heteropolysaccharide (rhamnose:glucose:galactose 4:2:1 in molar ratio) like the DG-EPS, elicited TNF-α, IL-6, and IL-12p40 production by both the murine RAW 264.7 macrophage-like cell line and the PBMCs (50). The ability to stimulate the production of pro-inflammatory cytokines by APCs, therefore, could potentially be a common property of the (rhamnose-rich) hetero-exopolysaccharides produced by the lactobacilli of the *L. casei* group.

Notably, in our experiments, the induction of pro-inflammatory cytokines and chemokines was much lower upon stimulation with DG-EPS than LPS, even when DG-EPS was used at 10-times higher concentration. In addition, when we used EPS or DG cells in combination with LPS, we did not observe any additive effect over the induction levels of cytokines triggered by LPS. Accordingly, it was proposed that probiotics can moderately stimulate the synthesis of pro-inflammatory cytokines in the absence of an inflammatory response, but may have suppressive or no effects when an inflammatory response has been already triggered (51). In this context, probiotics, although inoffensive during infection-derived inflammations, act as mild boosters of the innate immunity that may contribute to a more efficient and quick immune response against potential infectious agents.

Several studies failed to assign to the EPSs evident immunostimulatory abilities, and it was proposed that the EPSs anchored on the bacterial outer surface are immunologically inert molecules that prevent cell-wall associated MAMPs from the direct contact with immune cell receptors (28, 52, 53). However, in our study, the purified EPS produced by *L. paracasei* DG displayed an immunostimulatory activity, particularly in terms of chemokines expression. Thus, the EPS from *L. paracasei* DG, rather than an inert molecule, can be considered a bacterial product that can boost the immune system as either a secreted molecule released from the bacterium or also, plausibly, as a capsular envelope on the bacterial cell wall. Our results concerning the immunostimulating abilities of DG-EPS derive, however, from a preliminary *in vitro* study, and further investigations are needed in order to decipher the potential role of DG-EPS in the in vivo setting, where the final response depends on the concertation of signals coming from diverse epithelial and immune cells in an environmental context populated by numerous diverse commensal microbes.

In conclusion, our study provides additional information about the well characterized probiotic strain *L. paracasei* DG by demonstrating that it produces a unique rhamnose-rich hetero-exopolysaccharide, named DG-EPS, possessing immunostimulatory properties. DG-EPS may represent a new molecule for potential nutraceutical and pharmaceutical applications.

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Supplementary Information:

A novel rhamnose-rich hetero-exopolysaccharide isolated from *Lactobacillus paracasei* DG activates host's immune cells

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Running title: *Lactobacillus paracasei* DG exopolysaccharide

Key words**:** Capsular EPS, Probiotic, Enterolactis, L. casei DG®, Immunostimulatory, THP-1, MAMPs

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Figure S1. Gene expression analysis by qRT-PCR in human macrophages THP-1 after 4 h stimulation with strain *L. paracasei* DG (MOI 50) and strain DG after removal of the exopolysaccharide (EPS) (nDG; MOI 50), with or without the addition of purified the EPS molecule (1 µg ml^{-1}). Expression levels of TNF-α, IL-6, IL-8, and CCL20 are shown as the fold change of induction (FOI) relative to the control (unstimulated macrophages), which was set at a value of 1 (red line). Data are presented as mean of two independent experiments conducted in duplicate ± standard deviation. Asterisks indicate statistically significant differences: *, P<0.05; **, P<0.01. Non-significant P values under 0.1 are specified in the figure.