Probiotic properties of the 2-substituted (1,3)-β-D-glucan producing *Pedioccus parvulus* 2.6

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Running title**:** β-D-glucan role in *Pedioccus parvulus* 2.6

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Abstract

Exopolysaccharides have prebiotic potential and contribute to the rheology and texture of fermented foods. Here, we have analyzed the *in vitro* bioavailability and immunomodulatory properties of the 2-substituted (1,3)-β-D-glucan-producing *Pediococcus parvulus* 2.6. It resists gastrointestinal stress, adheres to Caco-2 cells and induces the production of inflammation-related cytokines by polarized macrophages.

Lactic acid bacteria (LAB) are industrially important micro-organisms for fermented food production. The recent widespread application of LAB and bifidobacteria for elaboration of functional food is attributable to the accumulating scientific evidence showing their beneficial effects on human health (3, 16). Most of the commercialized probiotics are limited to a few strains of Bifidobacteria, Lactobacilli and Streptococci, most of which produce exopolysaccharides (EPS) (27, 30). This fact, together with reports on immunomodulating ability as well as anticarcinogenic and cholesterol-lowering activities of EPS-producing LAB (25), suggests that the beneficial properties of these micro-organisms for human health may be due to the biological activities of these prebiotic biopolymers (25, 26), whose producing bacteria are also frequently used to improve texture and taste of dairy products (5, 11, 25). The future development of functional foods will be aimed at the diversification of this class of food, and therefore the identification and characterization of further bacteria with probiotic potential isolated from habitats different from those of the currently used organisms (digestive tract and dairy products), will increase the biodiversity and utility of this class of microorganisms.

LAB strains belonging to the *Pediococcus, Lactobacillus* and *Oenococcus* genera, isolated from cider and wine, produce a 2-substituted (1, 3)-β-D-glucan EPS (6, 7, 17, 12, 4). One of these strains is *Pediococcus damnosus* 2.6 (ropy, 2.6R), originally isolated from cider (8) and later renamed *P. parvulus* 2.6 (32). Curing of its 35 kDa pPP2 plasmid generated the isogenic non-ropy (2.6NR) EPS non-producing strain (8)**.** The plasmidic *gtf* gene determinant for the EPS production was cloned into *E. coli* and determination of its DNA sequence revealed that it encodes a protein, named GTF glycosyltransferase, belonging to the COG1215 membrane-bound glycosyltransferase family (32). Cloning of the *gtf* gene and functional expression of its encoded glycosyltransferase in *Streptococcus pneumoniae* (32) and *Lactococcus lactis* revealed that indeed this enzyme is responsible for the synthesis of the β-D-glucan (33).

The GFT glycosyltransferase has identity (33%) only with the Tts glycosyltransferase of *Streptococcus pneumoniae* serotype 37 (19). This latter enzyme catalyzes the biosynthesis and secretion of this organism's capsule (18), which is a β-D-glucan similar to the EPS synthesized by *Pediococcus*, and anti-serotype 37 antibodies also agglutinate *Streptococcus pneumoniae* (32) and *Lactococcus lactis* strains that over-express *gtf* (33, 4) as well as LAB strains naturally carrying this gene (32, 4).

Analysis of the rheological properties of the β-D-glucan synthesized by *P. parvulus* 2.6 showed that it has potential utility as a biothickener (29). In addition, human ingestion of oat-based food elaborated with *P. parvulus* 2.6 resulted in a decrease of serum cholesterol levels, boosting the effect previously demonstrated for (1,3)-β-D-glucans in oat (21). Therefore, this LAB is a potential probiotic strain useful for elaboration of functional food.

In this work, we have performed a comparative analysis of the β-D-glucan producer *P. parvulus* 2.6 and its isogenic non-ropy strain, in *in vitro* models that simulate the conditions in the human gastrointestinal tract.

Cultures of the strains were grown to early stationary phase in MRS medium (Pronadisa, Madrid Spain) at 30ºC under anaerobic conditions. Aliquots containing 3.4 x 10^7 cells of each bacterium were independently subjected to agglutination tests with *S. pneumoniae* type 37-specific antisera (Statens Serum Institut, Copenhagen Denmark), as previously described (32) and production of EPS was examined under the microscope (Fig.1). Agglutination of the cultures, detected by phase contrast microscopy as previously described (33), showed that immunoprecipitation of strain 2.6R occurred with antibodies against pneumococcal 37 serotype (Fig.1A). As expected, these antibodies did not react with strain 2.6NR (Fig. 1B). This type of analysis, coupled with plate counting, revealed that growth of *P. parvulus* 2.6 up to the beginning of the stationary phase was an optimal condition for EPS production without lost of viability (results not shown). Therefore, the strains were grown to OD_{620}=1.2 (10^9 CFU ml^{-1}) as above and subjected to conditions of the human gut by using an *in vitro* model, which approximates exposure to saliva, the pH gradient of the stomach and the intestinal stress (Fig. 2), as previously described (9), with the following modifications. For gastric stress (G) analysis, bacteria after exposure to lysozyme were treated with pepsin at the following pHs: 5.0, 4.1 or 3.0 for 20 min. Moreover, gastrointestinal stress (GI) was mimicked by exposure of the G pH 5.0 samples to bile salts and pancreatin at pH 6.5 for 120 min. Treated bacteria (G and GI samples) were further analyzed for cell viability as previously described (9) and compared with untreated bacteria (C samples) by using the LIVE/DEAD^R BacLightTM fluorescent stain which permits the calculation of the percentage of live cells from the ratio of green

(live) and red (dead) fluorescence. As the presence of the EPS attached to the ropy strain could impair a proper staining of the cells, prior to this analysis we established that: (i) for both strains the green/red (G/R) ratio correlates with the number of viable cells as determined by plate count and (ii) the dyes were taken up by *P. parvulus* 2.6 cells, as determined by fluorescence microscopy analysis (data not shown). Figure 2 depicts the results of the analysis of *P. parvulus* 2.6 and 2.6NR subjected to the gastric or gastrointestinal stress. Both strains showed the same pattern of resistance to the stress, indicating that the presence of EPS did not confer to *P. parvulus* 2.6 an advantage for survival in the human digestive tract. After exposure to pH 3.0 approximately a 10 % of cell survival was detected in both strains. In addition, the intestinal conditions caused no marked loss of viability (GI pH 5.0 *versus* G pH 5.0 samples), indicating that live bacteria could be available for interaction with intestinal epithelial cells. This interaction was investigated by using human Caco-2 cell lines and a ratio of 10 bacteria per epithelial cell, as previously described (9). After 1 h of exposure to bacteria, the Caco-2 cells were washed 3 times with PBS pH 7.1 to remove unadhered bacteria, then the Caco-2 cells were detached by treatment with 0.5 % trypsin-EDTA (Invitrogen, Barcelona, Spain), and the number of adhered bacteria were determined by plate count. In the control experiments, after 1 h of exposure to bacteria the Caco-2 cells were detached with trypsin, as described above, but without any washing, and were plate-counted to determine the total number (i.e. adhered and unadhered) of bacteria. Results from the adhesion experiments are expressed as a percentage of the corresponding control. In further experiments, two probiotic strains were used, *Lactobacillus acidophilus* LA-5 and *Bifidobacterium animalis* sups. *lactis* BB-12 (Chr. Hansen A/S., Hørsholm, Denmark), that had previously showed high and intermediate levels of adhesion (9). All bacteria were grown to early stationary phase in

MRS medium as above, sedimented by centrifugation at 12.000 x g and used for adhesion experiments, after resuspension in PBS pH 7.1 at 1.25 x 10^6 cells mL^{-1}. In addition, for analysis of the influence of the EPS on the adhesion capability of the ropy strain, two sub-populations were used: (i) prepared as indicated above (2.6R) and (ii) composed of bacterial cells washed with PBS prior resuspension as above (2.6R*), with the aim to remove the EPS attached to bacterial before analyzing their adhesion. Prior to that, an analysis of the bacteria by electron microscopy was performed using samples prepared as follows. Glow-discharged carbon-coated formvar grids were placed face-down over a droplet of each culture concentrated five-fold in 0.1 M $AcNH_4$ pH 7. After 1 min, each grid was removed, blotted briefly with filter paper and, without drying, negatively stained with 2% uranyl acetate for 40 s, then blotted quickly and air-dried. The analysis (insets in Fig. 3) revealed that indeed EPS-bound to *P. parvulus* 2.6 was present which was partially removed by the washing treatment. Moreover, the analysis confirmed the absence of EPS in the 2.6NR strain. Figure 3 depicts the results of the adhesion experiments, *P. parvulus* 2.6 showed a high level of adherence (6.1 %) similar to that of *L. acidophilus* LA-5 (6.6 %) and considerably higher than that of the EPS-non-producing 2.6NR (0.25 %). In addition, an intermediate adherence (1.8 %) was detected for the 2.6R* sub-population of the 2.6R strain and for *B. animalis* BB-12. These results strongly supported a contribution of the EPS of *P. parvulus* 2.6 for attachment to colon epithelial cells. Therefore, the immunomodulatory properties of the 2.6R and 2.6NR strains on macrophages were investigated. To that end, pro-inflammatory M1 and anti-inflammatory M2 macrophages were generated from human peripheral blood mononuclear cells using 1000U mL^{-1} GM-CSF or M-CSF (10 ng mL^{-1}), as previously described (31), and their cytokine response after exposure to the ropy and non-ropy strains during 18 h, was determined by means of ELISA (34) using

antibodies against TNF-α, IL-8 and IL-10 (ELISA set, ImmunoTools, Friesoythe, Germany). With regard to pro-inflammatory cytokines, both bacterial strains induced high levels of TNF-α (Fig. 4A) and IL-8 (Fig. 4B) on M1 macrophages, but had a minor (TNF-α) or absent effect (IL-8) on M2 macrophages. Both strains also induced the production of the anti-inflammatory IL-10, although the extent of cytokine release was higher in M2 macrophages (Fig. 4C). However, the levels of TNF-α and IL-8 by M1 macrophages were higher in response to the 2.6NR strain (Figs. 4A and 4B), thus implying that elimination of the pPP2 plasmid, which encodes the *P. parvulus* 2.6 EPS, triggers a higher level of pro-inflammatory cytokines in M1 macrophages. Although a contribution by other, unknown, products encoded by pPP2 cannot be ruled out, these results strongly suggest that the presence of EPS in *P. parvulus* 2.6 counteracts the pro-inflammatory activation of M1 macrophages in response to the bacteria. Consequently, EPS might act by: (i) preventing recognition by M1 macrophage-expressed Toll-like receptor 2 (TLR2) of the major Gram-positive pathogen-associated molecular patterns lipoteichoic acid or peptidoglycan (14); or (ii) inhibiting the intracellular signaling cascade initiated upon TLR2 engagement by both cell wall components. If the latter explanation is true, then EPS could be considered as a *bona fide* beneficial immunomodulator.

In summary, the comparative analysis of the β-glucan producing and non-producing strains performed in this work has provided insights into the debated issues of probiotic properties of EPS-producing LAB (3) and its role in the immunomodulation of macrophages (20).

Our results indicate that *P. parvulus* 2.6 should be able to tolerate human gastrointestinal stress and thus could be metabolically active in the colon. This supports the detected changes in short-chain fatty acid formation in the caecum, distal colon and

faeces of rats which had been fed with fermented oat-based food elaborated with this bacterium (15).

The EPS produced and secreted by LAB seems to be implicated in cellular recognition and the formation of biofilms, e.g. the glucans and fructans of *S. mutants*, which play an important role on the adhesion of this bacterium to the tooth surface and the formation of dental plaque (13)**,** thus facilitating bacterial colonization and protection against hostile habitats. However, the involvement of these biopolymers in the *in vivo* bacterial adhesion to the intestinal epithelium has not been yet validated (25). The results of Dols-Lafargue *et al*. (4) show the contribution of the 2-substituted (1,3)-β-D-glucan on biofilm formation by LAB, and our results strongly support the involvement of this EPS in adhesion to human epithelial cells.

There are several reports that indicate host immune response to LAB, in which the involvement of various surface components of these bacteria are demonstrated (10, 22, 28). It has been reported that the suppressive effect on activation of macrophages exerted by *Lb. casei* strain Shirota is associated with its EPS content (34). It is also known that the (1,3)-β-D-glucans can promote antitumor and antimicrobial activity, by activating macrophages, dendritic cells or other leukocytes (1, 24). The immune response to eukaryote-derived glucans (either linear or with (1,6) branches), and to the prokaryotic linear curdlan, used for making functional foods (tofu), has been characterized, and their activity has been correlated with their chemical structure, molecular weight and conformation (2, 23). However, the immunomodulating properties of the β-D-glucans with (1, 2) branches have not been reported until now. Therefore, this is the first report that a 2-substituted (1, 3)-β-D-glucan affects activation of human macrophages. Further experiments are in progress to characterize the influence of this β-D-glucan and of *P. parvulus* 2.6 on the immune response.

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LEGEND TO THE FIGURES

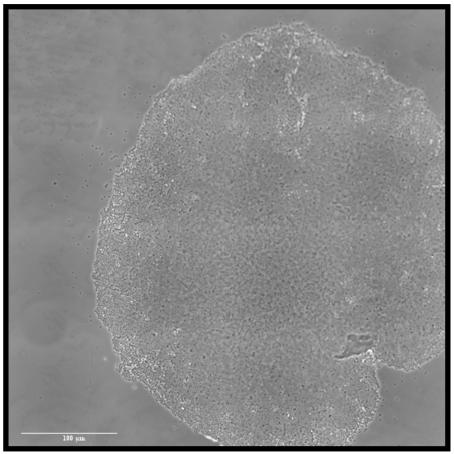
Figure 1. Detection of EPS production. The indicated strains were subjected to agglutination tests and detection by contrast phase (¿phase contrast?) microscopy. Left panels (Bar=100 μm); Right panels (Bar=10 μm).

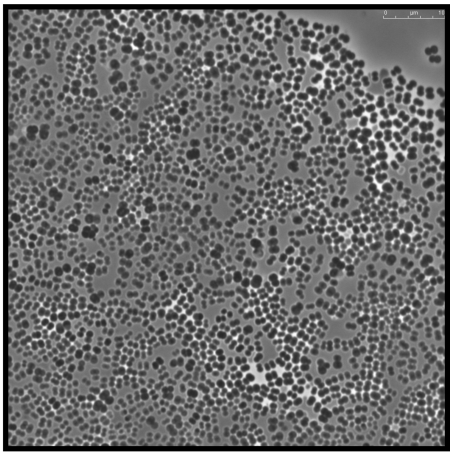
Figure 2. Analysis of cell survival after gastric (G) and gastrointestinal (GI) stresses. The indicated bacterial strains were untreated (C) or subjected to various G- or GI-stresses as described in the text. After staining, cell viability was analyzed by measurement of green and red fluorescence. The values are the mean of three independent experiments and are expressed as a percentage of the Green/Red (G/R) fluorescence ratio for untreated control samples. 100% control values for untreated 2.6R and 2.6NR were respectively 10.05 and 9.98.

Figure 3. Adhesion of bacterial strains to Caco-2 cells. Adhesion levels are expressed as percentage of the total number of bacteria (adhered plus unadhered) detected after their exposure for 1 hour to Caco-2 cells. Each adhesion assay was conducted in triplicate. The values are the mean of three independent experiments, in each of which, three independent determinations were performed. Inset. Prior to the adhesion experiments, bacteria were analyzed in a JEOL 1230 transmission electron microscope operated at 100 kV.

Figure 4. Cytokine response of macrophages to *P. parvulus* strains. M1 and M2 +macrophages were either untreated (Basal 18h) or stimulated with LPS from *Escherichia coli* 055:B5 (Sigma, Barcelona, Spain) at 10 ng mL^{-1}, *P. parvulus* 2.6 (2.6R) or its non-ropy mutant (2.6NR) and the levels of IL-10, TNFα and IL-8 released were determined. Each determination was performed in triplicate, and the mean, and standard deviations, are shown.

A *P. parvulus* 2.6R





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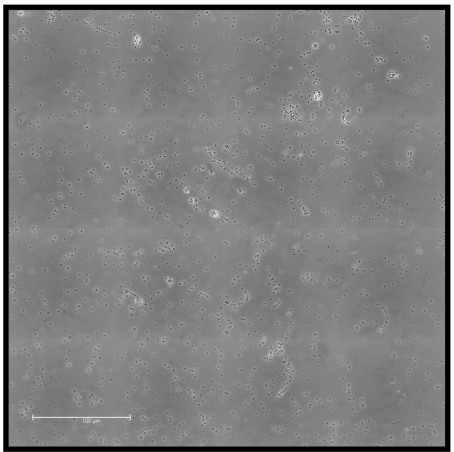

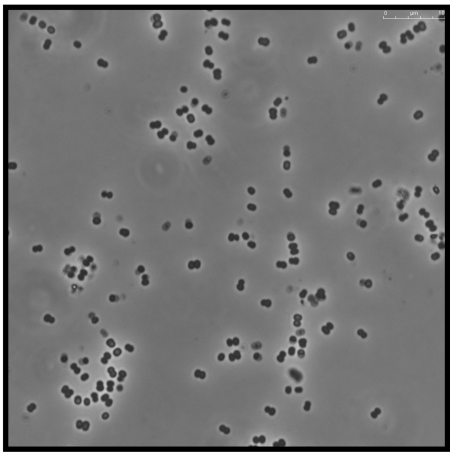

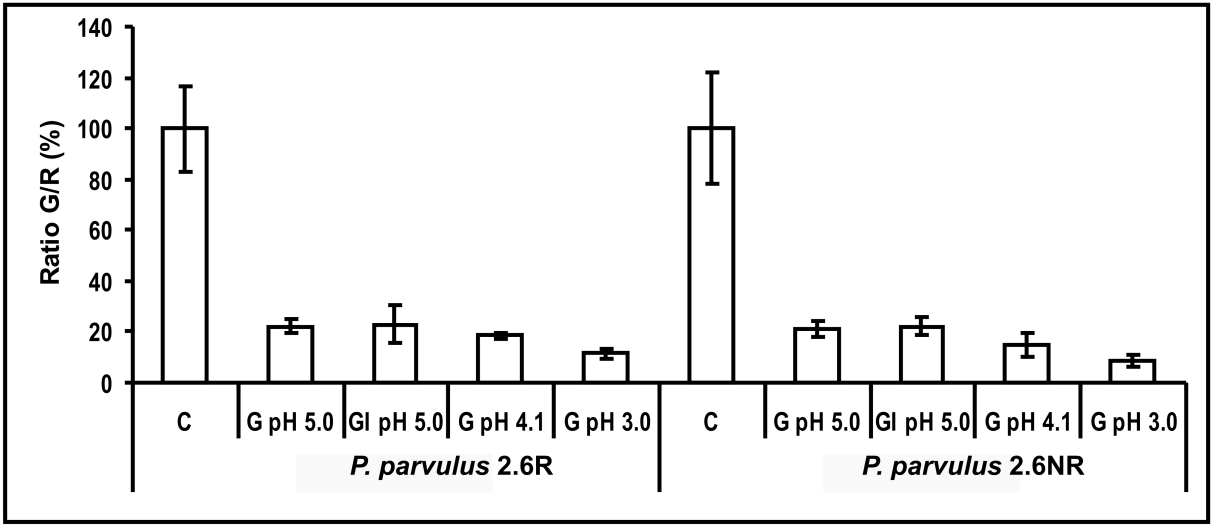
Ratio G/R (%)
140
120
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40
20
0
C
G pH 5.0
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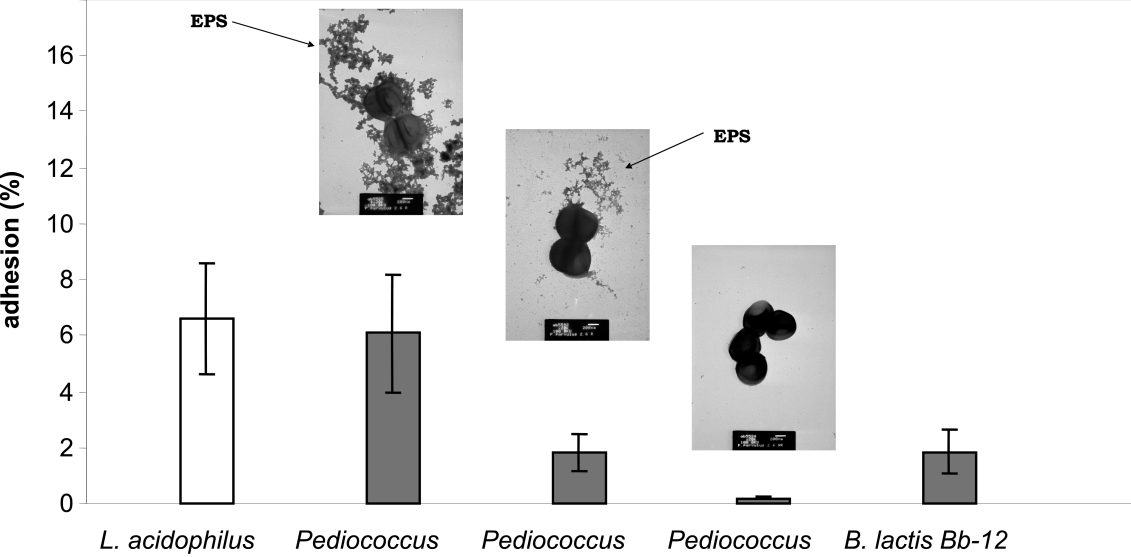
P. parvulus 2.6R
P. parvulus 2.6NR


EPS
EPS
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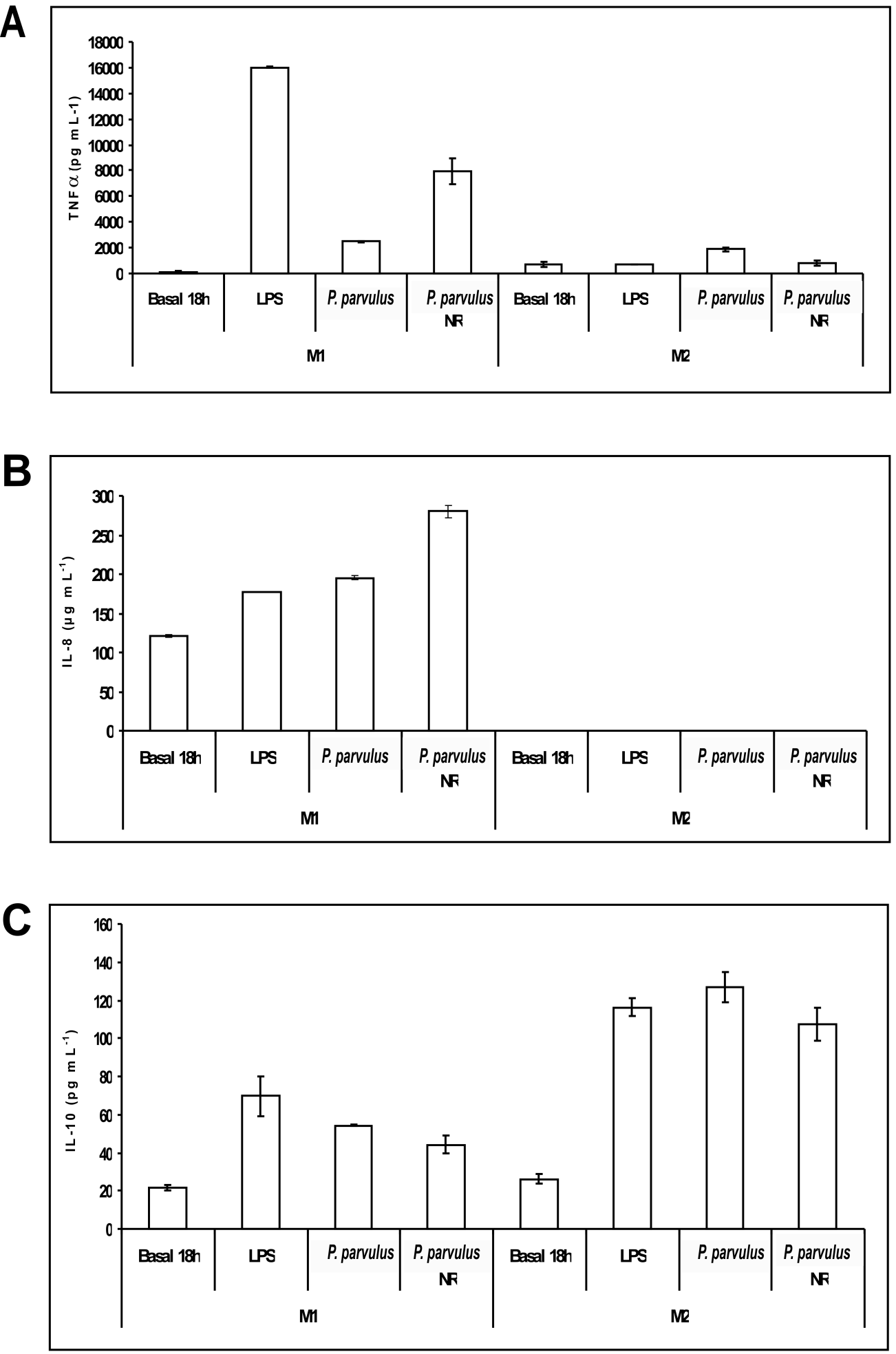
Pediococcus
Pediococcus
B. lactis Bb-12


A
TNFα (pg mL-1)
18000
16000
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12000
10000
8000
6000
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0
Basal 18h
LPS
P. parvulus
P. parvulus NR
M1
Basal 18h
LPS
P. parvulus
P. parvulus NR
M2
B
IL-8 (µg mL-1)
300
250
200
150
100
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Basal 18h
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Basal 18h
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P. parvulus NR
M2
C
IL-10 (pg mL-1)
160
140
120
100
80
60
40
20
0
Basal 18h
LPS
P. parvulus
P. parvulus NR
M1
Basal 18h
LPS
P. parvulus
P. parvulus NR
M2