Dextran production by *Lactobacillus sakei* MN1 coincides with reduced autoagglutination, biofilm formation and epithelial cell adhesion

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Abbreviations: ANOVA, one-way analysis of variance; CDMS, CDMG define médium lacking glucose and supplemented with 0.8% sucrose; cfu, colony forming unit; dpf, days post fecundation; DMEM, Dulbecco's Modified Eagle medium; EPS, exopolysaccharide; GC-MS, gas chromatography–mass spectrometry; GFP, Green fluorescent protein; HoPS, homopolysaccharides; hpi, hours post bacterial infection; LAB, lactic acid bacteria; LG, *Lactobacillus sakei* culture in 0.5% glucose solution; LS, *Lactobacillus sakei* culture in 0.5% sucrose solution; LSD, Fisher's Least Significant Difference test; MN1, *Lactobacillus sakei* MN1 strain; MRSG Man Rogosa Sharpe broth containing 2% glucose; MRSS, MRS medium containing 2% sucrose instead of glucose; NaClG, (0.5% NaCl plus 0.5% glucose solution; NaClS, 0.5% NaCl plus 0.5% sucrose solution; PBS, phosphate-buffered saline; RTF10, *Leuconostoc mesenteroides* RTF10 strain; TEM, transmission electron microscopy; TSB, trypticase soy broth; VG, *Vibrio anguillarum* culture in 0.5% glucose solution; VS, *Vibrio anguillarum* culture in 0.5% sucrose solution.

Abstract

In this work we have investigated two dextran-producing lactic acid bacteria, *Lactobacillus sakei* MN1 and *Leuconostoc mesenteroides* RTF10, isolated from fermented meat products. These bacteria synthesise dextran when sucrose, but not glucose, is present in the growth medium. The influence of dextran on bacterial aggregation, adhesion and biofilm formation was investigated in cultures challenged with sucrose or glucose. For *Lb. sakei* MN1, the synthesis of the dextran drastically impaired the three processes; in contrast it had no effect on *Lc. mesenteroides* RTF10. Therefore, the influence of dextran on probiotic properties of *Lb. sakei* MN1 was tested *in vivo* using gnotobiotic zebrafish models. The bacterium efficiently colonised the fish gut and inhibited the killing activity of *Vibrio anguillarum* NB10[pOT11]. Furthermore, under conditions of dextran synthesis, the adhesion of *Lb. sakei* MN1 to the epithelial cells decreased, without greatly affecting its anti *V. anguillarum* activity.

Keywords: Lactic acid bacteria, dextran, zebrafish models, *Vibrio anguillarum*, fish probiotics, colonisation

1. Introduction

Intensive production of fish in aquaculture causes them stress, which affects their immune system, increasing susceptibility to pathogens and promoting the emergence of diseases (Tort, 2011). Control of diseases in aquaculture is based mainly on the use of antibiotics and some vaccines. The indiscriminate use of antibiotics has significant disadvantages such as the development of resistances (Kruse & Sorum, 1994) and pollution of aquatic environments generating important problems of toxicity (Cabello, 2006). Therefore alternative strategies, such as the use of probiotics, (Newaj-Fyzul, Al-Harbi & Austin, 2014) or immunostimulants, including polysaccharides such as β -glucans (Ringø, 2012), are being introduced to prevent and control not only bacterial but also viral infections. Some lactic acid bacteria (LAB) belonging to the genera *Streptococcus*, *Leuconostoc*, *Lactobacillus* and *Carnobacterium* are part of the normal microbiota of fish (Ringø & Gatesoupe, 1998). Therefore, some LAB strains have already been evaluated as potential probiotic bacteria due to their stimulation of the immune system and their potential to compete with pathogens (Gomez & Balcazar, 2008).

We have previously shown that high molecular weight dextrans, homopolysaccharides (HoPS) produced by LAB, have antiviral and immunomodulatory properties of interest in aquaculture (Nácher-Vázquez et al., 2015). Thus, these exopolysaccharides (EPS) as well as their producing bacteria *Lactobacillus sakei* MN1 and *Leuconostoc mesenteroides* RTF10 could be of interest for the preparation of fish feed. Therefore, we have analysed these bacteria for dextran production *in vitro* during sessile and planktonic growth, for abiotic and biotic interactions as well as *in vivo* interactomic studies with fish. For *in vivo* studies, zebrafish (*Danio rerio*) have emerged as a useful model due to their small size, high fecundity, rapid development, optical transparency

of the embryos, and amenability to genetic screens, (Sullivan & Kim, 2008). This model has been extensively used to study the interactions between the host and the natural gut microbiota (Milligan-Myhre et al., 2011; Toh, Goodyear, Daigneault, Allen-Vercoe & Van Raay, 2013) including probiotics (Avella et al., 2012; Carnevali, Avella & Gioacchini, 2013; Gioacchini et al., 2012; Gioacchini, Maradonna, Lombardo, Bizzaro, Olivotto & Carnevali, 2010; Gioacchini, 2011; Rendueles et al., 2012; Rieu et al., 2014). In addition, the study of host-probiotics interactions in fish models is extremely complicated due to the diversity of microorganisms colonising the host gut, often hampering the interpretation of results. These difficulties can be overcome using gnotobiotic models in which the microbiota is either known or absent (Pham, Kanther, Semova & Rawls, 2008). Thus, we have previously used gnotobiotic zebrafish larvae for evaluation of potentially probiotic LAB isolated from food (Russo et al., 2015), as well as for analysis of the mechanism of infection of opportunistic pathogens (Oyarbide, Iturria, Rainieri & Pardo, 2015). Consequently, in addition to *in vitro* evaluation of the two dextran-producing LAB, the influence of the presence of the HoPS on the activity of *Lb. sakei* MN1 in the gut has been investigated in this work using zebrafish gnotobiotic models.

2. Materials and methods

2.1. Bacterial strains, plasmid and growth media. *E. coli* DH5 α [pRCR12] strain

(Russo et al., 2015) was grown at 37 °C with shaking in LB (Luria-Bertani broth, Pronadisa, Spain) supplemented with chloramphenicol (Cm, Sigma-Aldrich, St. Louis, MO, USA) at 10 $\mu\text{g mL}^{-1}$.

LAB used in this work: *Lb. sakei* MN1 (CECT 8329) and *Lc. mesenteroides* RTF10, both isolated from meat products (Chenoll, Macian, Elizaquivel & Aznar, 2007) and *Lactobacillus acidophilus* LA-5, kindly provided by Chr. Hansen A/S (Hørsholm, Denmark). In addition, *Lb. sakei* MN1[pRCR12] strain was constructed in this work as follows. The broad host range plasmid pRCR12, which encodes constitutively the mCherry fluorescent protein (Russo et al., 2015), was obtained from *E. coli* DH5 α [pRCR12] using the "JetStar 2.0 Plasmid Purification Kit" (Genome, Löhne, Germany). pRCR12 was transferred to MN1 strain as described (Berthier, Zagorec, Champomier-Vergès, Ehrlich & Morel-Deville, 1996). Transformants were selected on MRSG (De Man, Rogosa & Sharpe, 1960) medium plates supplemented with 1.5% agar, and Cm at 5 $\mu\text{g mL}^{-1}$. Functionality of mCherry in MN1[pRCR12] was revealed by the red colour of the colonies (Fig. 3SB).

The LAB strains were grown at 30 °C in CDM medium (Sánchez et al., 2008) supplemented with 2% sucrose (CDMS) and lacking glucose or in MRSG or supplemented with 2% sucrose (MRSS) instead of 2% glucose. For growth of MN1[pRCR12], the media were supplemented with Cm at 5 $\mu\text{g mL}^{-1}$. Plate media were prepared by adding 1.5% agar (Pronadisa) to the liquid broths.

The *Vibrio anguillarum* serotype O1 strain used in this work was NB10[pOT11] (O'Toole, Von Hofsten, Rosqvist, Olsson & Wolf-Watz, 2004), kindly provided by R. O'Toole from Umeå University. This is a GFP (green fluorescent protein)-labelled

bacterium due to the expression of the inducible *tac-gfpmut2* transcriptional fusion carried by the pOT11 plasmid, The bacterium was grown at 25 °C in TSB (trypticase soy broth, Pronadisa) supplemented with chloramphenicol at 10 µg mL⁻¹ and 0.5 mM isopropyl-β-D-thiogalactopyranoside to induce expression of the GFP protein.

2.2. Animal husbandry. Zebrafish embryos were obtained from wild-type adult zebrafish (*D. rerio*, Hamilton 1822), which were bred and maintained in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) as previously described (Russo et al., 2015) following standard conditions (Sullivan & Kim, 2008). All experimental procedures were approved by the Regional Animal-Welfare Body. Production of germ-free zebrafish was performed as previously described (Oyarbide, Iturria, Rainieri & Pardo, 2015). During experimentation fish were maintained at an average density of 1.3 animals per mL in sterile petri dishes housed in an air incubator at 27 °C on a 14 h light cycle. Mortality tests were performed in 24-well culture plates.

2.3. Detection of dextran production at colony and cellular levels. Production of the HoPS at colony level was tested by growing the LAB on MRSG and MRSS plates for 48 h at 30 °C and checking the morphology of the colonies (mucoid phenotype in the case of production). For localization of the dextran at the cellular level, the ruthenium red staining method was used (Akin & Rigsby, 1990) adapted to direct analysis of HoPS on bacterial colonies. First, 2% melted agar was poured on the colony grown in the plate and portions containing a single colony were cut with a scalpel for further analysis. The bacteria present in the sample were fixed in freshly prepared 2.5% (v/v) glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) and sections were cut. Cells included in the sections were post-fixed in 1.5% (w/v) OsO₄ in 0.1 M cacodylate buffer (pH 7.4) containing 0.075% (w/v) ruthenium red. Then, they were washed three times with 0.1 M sodium cacodylate buffer (pH 7.4), dehydrated using a graded aqueous ethanol series

(30, 50, 70, 95, and 100% ethanol, 5 min each), and embedded in LR-white resin. Sections (60 nm) were made with a Diatome diamond knife, using an Ultracut Leica UC6 ultramicrotome, and examined by transmission electron microscopy (TEM) with a JEOL JEM1010 microscope after uranyl acetate staining. Images were captured and digitised using a MegaView III camera with the software “AnalySIS”.

2.4. Analysis of metabolic fluxes and dextran production. LAB were grown in MRSS to an absorbance at 600nm (A_{600nm}) of 2. Cells were sedimented by centrifugation (9,300 x g, 10 min, 4 °C), resuspended in the same volume of fresh MRSS and diluted 1:100 in CDMS. Batch fermentations without pH control were performed, in triplicate, for each bacterium and they were grown in 100 mL screw-capped flasks for 13 h at 30 °C. Cultures were sampled every hour (from 2 h to 13 h) to monitor growth by determination of A_{600nm} as well as acidification of the media by measuring pH. Also, concentration of sugars (sucrose, glucose and fructose), lactic acid and dextrans was determined in culture supernatants. To this end, bacteria were removed from the growth medium by centrifugation (9,300 x g, 10 min, 4 °C). Then, the metabolic analysis was performed by gas chromatography–mass spectrometry (GC-MS) using myo-inositol as internal standard. For this analysis, the bacterial culture supernatants were lyophilised and derivatised with hydroxylamine chloride in pyridine to 2.5% for 30 min at 70 °C, to form the oximes of sugars. Afterwards, bis-trimethylsilyl trifluoroacetamide was added for 45 min at 80 °C, to form the trimethylsilylated derivatives. Identification and quantification were performed by GC-MS on a CG EM 7980A 5975C Agilent (Santa Clara, California, USA) equipped with a column HP 5MS (30 m x 0.25 mm I.D. x 0.2 µm film thickness) with He as the carrier gas. Injector and detector were set at 275 °C. Samples (1 µL) were injected with a split ratio of 1:50 with a temperature program: 80 °C for 4 min, then 15 °C min⁻¹ to 270 °C

and finally 30 °C min⁻¹ to 310 °C (2 min). The peaks in the chromatograms corresponding to sugars and lactic acid were identified by their retention times. Quantification was performed by determination of the peak area in comparison with a calibration standard curve.

For analysis of the HoPS, the dextrans were recovered from culture supernatants by ethanol precipitation as previously described (Nácher-Vázquez et al., 2015) and total sugar content was determined by the phenol/sulphuric acid method using glucose as standard (Dubois, Gilles, Hamilton, Rebers & Smith, 1956). Three cultures of each bacterium at each incubation time were analysed and determinations were performed in duplicate.

2.5. Detection of biofilm formation on polystyrene and glass substrates. Biofilm formation was assayed as previously described (Vergara-Irigaray et al., 2009). For detection in microtiter plates, LAB were used to inoculate MRSG or MRSS (0.1% v/v) and 200 µL of these suspensions were used to inoculate sterile, 96-well polystyrene microtiter plates (BD, New Jersey, USA). After incubation for 24 h at 37 °C, in an orbital shaker (200 rpm), the wells were gently washed twice with 200 µL of distilled water, dried and stained with crystal violet (0.5% w/v) for 5 min at 21 °C. Then, the wells were washed twice with 200 µL of distilled water, dried and the dye was solubilised with 200 µL of ethanol-acetone (80:20, vol/vol). Crystal violet-stained bacteria were quantified measuring the A_{595nm}. Determination of biofilm formation on a glass surface was carried out essentially in the same way, except that glass tubes were used instead of microtiter plates and the volume was 5 mL instead of 200 µL. Three cultures of each bacterium were analysed and experiments were performed in triplicate.

2.6. Caco-2 cell culture and adhesion assays. The Caco-2 cell line from human colon adenocarcinoma was obtained from the cell bank at the Centro de Investigaciones

Biológicas (Madrid, Spain). Cells were grown in MEM-Alpha medium (Invitrogen, Barcelona, Spain), supplemented with 20% (v/v) heat-inactivated fetal bovine serum. The incubation was carried out at 37 °C in an atmosphere containing 5% CO₂. Caco-2 cells were seeded in 96-well tissue culture plates (Falcon Microtest™, Becton Dickinson, Franklin Lakes, NJ, USA) at a concentration of 1.25 x 10⁴ cells per well (1.25 x 10⁵ cells mL⁻¹) and grown for 21 days to obtain a monolayer of differentiated and polarized cells. The culture medium was changed every 2 days. Cell concentrations were determined before adhesion experiments in a counting chamber (Type Neubauer 0.100 mm Tiefe. 2. Depth Profondeur 0.0025 mm²) using a Nikon Eclipse TS 100 microscope.

For adhesion experiments, LAB late exponential-phase cultures were sedimented by centrifugation (12,000 x g, 10 min, 4 °C) 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 1.25 x 10⁶ colony forming units (cfu) mL⁻¹, and added to Caco-2 cells (ratio 10:1) in a final volume of 0.1 mL per well. After incubation for 1 h at 37 °C, unattached bacteria were removed by three washings with 0.2 mL of phosphate-buffered saline (PBS) pH 7.1 (10 mM Na₂HPO₄, 1 mM KH₂PO₄, 140 mM NaCl, 3 mM KCl, all purchased from Merck), and then the Caco-2 cells were detached from the plastic surface by incubating for 10 min at 37 °C with 0.1 mL of 0.05% trypsin–EDTA per well. The detachment reaction was stopped by adding 0.1 mL of PBS pH 7.1. To determine the number of cell-associated bacteria, appropriate dilutions were plated onto MRS plates. All adhesion assays were conducted in triplicate.

2.7. Detection of bacterial aggregation and disaggregation. To test autoaggregation, LAB were grown without shaking in MRSG or MRSS (0.1% v/v) for 24 h at 30 °C

prior to analysis. For detection in liquid medium, tubes containing the cultures were photographed with Fujifilm FinePix S5 Pro, objective AF Micro Nikkor 60 mm. For detection at cellular level, 1 mL of LAB cultures grown in Eppendorf tubes were sedimented by centrifugation (5,000 x g, 3 min, 4 °C) and the supernatants removed. Then, the bacteria were carefully resuspended without vortexing in 50 µL of PBS (pH 7.4) and directly analysed, without fixing using a Leica AF6000 LX-DMI6000B model microscope (Leica Microsystems, Mannheim, Germany). Illumination was provided with a 100× objective. For detection of mCherry BP 620/60 excitation and BP700/75 emission filters were used. Image analysis was performed using LAS AF Core Software (Leica Microsystems).

To detect bacterial disaggregation, LAB were grown without shaking in MRSG for 24 h at 30 °C. Then, the bacteria were sedimented as described above, carefully resuspended without vortexing in MRSS or MRSG and further incubated for 3 h at 30 °C. Afterwards, the samples were subjected to sedimentation as described above, the bacteria resuspended without vortexing in 50 µL of PBS (pH 7.4) and directly analysed, without fixing, by phase contrast and fluorescence microscopy.

2.8. Interactomic studies of *Lb. sakei* MN1[pRCR12] and zebrafish. LAB strain was grown at 30 °C in MRSG supplemented with Cm. Overnight cultures were washed three times with PBS pH 7.0 at room temperature and diluted in either 0.5% glucose or 0.5% sucrose solutions to reach 10^7 cfu mL⁻¹.

Three independent experiments were performed. Twenty gnotobiotic zebrafish larvae of 4 dpf (days post fecundation) were placed into each petri dish and 15 mL of the dilution containing the bacterial culture and either 0.5% glucose (LG) or 0.5% sucrose (LS) were added. Twenty gnotobiotic larvae were placed in a petri dish containing 15 mL of PBS pH 7.1 as control. Larvae were incubated at 27 °C with agitation (60 rpm) for 18 h.

Then, the solution containing the bacteria was removed and the larvae were washed three times with PBS. After 6, 24 and 48 hours post infection (hpi) larvae were euthanized with tricaine at 300 mg mL⁻¹. Then, they were washed in 3 different baths of sterile PBS and 0.1 % (v/v) Tween 20 to remove bacteria loosely attached to the skin (Rendueles et al., 2012). Larvae were manually homogenised with a Pellet Pestle Cordless Motor (Kimble Chase Life Science and Research Products LLC). Finally, serial dilutions of recovered suspension were spotted on plates and bacterial cfu were counted after incubation for 24 h at 30 °C.

2.9. *Lb. sakei* MN1[pRCR12] activity against *V. anguillarum* NB10[pOT11] in zebrafish model. NB10[pOT11] was used to test its pathogenicity on zebrafish larvae. Overnight cultures of MN1[pRCR12] and NB10[pOT11] were washed three times with NaClG (0.5% NaCl, 0.5% glucose) or NaClS (0.5% NaCl, 0.5% sucrose) solutions and finally diluted to 10⁷ cfu mL⁻¹. Larvae exposed for 18 h to MN1[pRCR12] (LG or LS) were immersed in the above described solutions containing NB10[pOT11] and either NaClG or NaClS. Different combinations were tested to assess the influence of both sugars on bacterial metabolism.

Larvae from the LG group were divided into two groups; one was immersed in a solution containing NB10[pOT11], NaClG (LG–VG). The other one was immersed in a solution containing NB10[pOT11] and NaClS (LG–VS). Larvae from the LS group were immersed in a solution containing NB10[pOT11] and NaClS (LS–VS). Larvae exposed to *V. anguillarum* but not to *Lb. sakei* were used as positive control. Larvae immersed in either NaClG or NaClS but not exposed to any bacteria were used as negative control. Mortality was determined at 96 h post-infection. Three independent experiments per each condition were performed.

2.10. Detection of bacterial colonisation of zebrafish gut by fluorescent microscopy.

The colonisation of bacteria inside the larvae was assessed visually using a Leica MZFL III stereomicroscope with a zoom magnification range of 8× to 100×. The microscope was equipped with visible light and UV light (Hg 100 W) sources. GFP fluorescence of NB10[pOT11] was detected by exposure of the larvae to UV light in the excitation range of 450-490 nm. mCherry fluorescence of MN1[pRCR12] was detected by exposure of the larvae to ultraviolet light in the excitation range of 540-580 nm. Images were captured using a Leica DFC 360FX camera and processed using ImageJ v1.47 software (National Institutes of Health, NIH).

2.11. Statistical analysis. Data are expressed as a mean ± standard deviation calculated from three independent replications in triplicate. Adhesion data were analysed by one-way analysis of variance (ANOVA) performed with the SAS 9.4 software (SAS Institute Inc., Cary, NC). Tukey's test was used to determine the significant differences between the variables at $p < 0.05$. Zebrafish experimental data were evaluated by ANOVA using Statgraphics Centurion XVI Software (Statpoint Technologies, Warrenton, VA). Fisher's Least Significant Difference test (LSD) was employed to evaluate the significant differences among mean values at $p < 0.05$.

3. Results

3.1. Dextran-production and central metabolism of *Lb. sakei* MN1 and *Lc.*

mesenteroides RTF10. In previous works, we identified the EPS produced by MN1 and RTF10 strains as α -(1-6) glucans with approximately 6% substitution, at positions O-3, by side chains composed of a single residue of glucose (Nácher-Vázquez et al., 2015; Notararigo et al., 2013). These types of HoPS (dextrans) are synthesised by dextransucrases, using sucrose as substrate and with a concomitant release of fructose (Leemhuis et al., 2013; Moulis et al., 2006). Therefore we investigated the interrelation of metabolic fluxes with dextran production by MN1 and RTF10. Since LAB dextransucrase catalysis takes place extracellularly, levels of sucrose and metabolic products were analysed in culture supernatants as well as the pH of the media. Both bacteria consumed approximately 90% of the sucrose (23 mM) added to the medium during the first 7 h of growth, when the cultures entered the stationary phase (Fig. 1). Also during this period, an increased concentration of fructose was detected in both culture supernatants, which reached a similar maximal level (7.93 ± 0.55 mM for MN1 and 7.31 ± 0.22 mM RTF10, corresponding respectively to a yield from sucrose of 34% and 32%) which decreased during further incubation, at a faster rate in *Lc. mesenteroides* culture supernatants (Fig. 1).

Figure 1. Analysis of metabolism and EPS production of LAB during planktonic growth.

Bacteria were grown in CDMS at 30 °C. Symbols: ●, A_{600nm} ; ◆, pH; ○, Dextran; □, sucrose; △, fructose; ▲, glucose and ■, lactic acid.

After 13 h of growth, the extracellular levels were respectively 2.72 ± 0.24 mM and 0.76 ± 0.01 mM (Fig. 1), and undetectable after 24 h (results not shown). In addition, a transient accumulation of glucose was detected in the RTF10 cultures supernatants, but not observed for *Lb. sakei* MN1 (Fig. 1). The maximum level of glucose (6.94 ± 0.63 mM, corresponding to a yield from sucrose of 30%) was detected in the RTF10 supernatants after 7 h of growth, and afterwards the bacteria started to internalise the sugar. This glucose accumulation is unusual for *Leuconostoc mesenteroides* and it has been suggested for *Lc. mesenteroides* NRRL B-512F to be due to a levansucrose

activity (Dols, Rемаud-Simeon & Monsan, 1997). However, this cannot apply for *Lc. mesenteroides* RTF10, since it produces only one HoPS characterized as dextran (Notararigo et al., 2013).

The pattern of exopolysaccharide and lactic acid production correlated with the growth curves for both strains (Fig. 1). With regard to the production of dextran, after 13 h of growth, the levels in culture supernatants were $2.20 \pm 0.09 \text{ g L}^{-1}$ for MN1 and $1.25 \pm 0.04 \text{ g L}^{-1}$ for RTF10, which expressed as molecules of glucose correspond to $12.21 \pm 0.52 \text{ mM}$ and $6.92 \pm 0.21 \text{ mM}$ (Fig. 1). The efficiency of dextran production by *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10 relative to the biomasses present in the cultures was estimated ($[\text{dextran}]/A_{600\text{nm}}$) (Fig. 1S), and showed that MN1 reached a maximum during exponential growth, whereas RTF10 had similar efficiencies during exponential- and stationary-phases. The synthesis of lactic acid by MN1 was faster and approximately 2-fold higher than that by RTF10, $45.02 \pm 0.94 \text{ mM}$ versus $25.47 \pm 0.18 \text{ mM}$, after 13 h of growth. This result correlated with the behaviour of the pH of the culture media decreasing from 7.0 to 4.2 and 4.8, respectively, for MN1 and RTF10. In addition, the higher yield of lactic acid by MN1 could correlate with the fact that *Lb. sakei* strains perform a homolactic metabolism of hexoses via glycolysis (yield of 2 lactate molecules from 1 molecule of glucose) (Champomier-Vergès, Chaillou, Cornet & Zagorec, 2002), whereas *Lc. mesenteroides* strains, an obligatory heterofermentative bacteria, utilised the 6-phosphogluconate pathway to ferment hexoses (yield of 1 lactate molecule from 1 molecule of glucose) (Zaunmuller, Eichert, Richter & Uden, 2006). However, the yield of dextran corrected for the biomass for RTF10 remained constant (around 0.4) during the exponential- and stationary-phases, whereas for MN1 the synthesis increased during growth from values of 1.7 to 4.0 at late stationary-phase (Fig. 1S). Therefore, the higher yield of RTF10 could be due to the ability of dextran-

producing *Lc. mesenteroides* strains, such as NRRLB_1149, to metabolise sucrose, not only *via* dextransucrase, but also *via* sucrose phosphorylase activities (Lee, Yoon, Nam, Moon, Moon & Kim, 2006). With regard to the impact on bacterial growth due to the usage of sucrose as carbon source, comparison of cultures grown in presence of sucrose or glucose (Fig. 2S) revealed that the growth rate of both bacteria was not significantly affected by the type of carbohydrate (0.76 ± 0.18 *versus* 0.68 ± 0.11 for MN1 and 0.57 ± 0.19 *versus* 0.63 ± 0.25 for RTF10), even when no synthesis of dextran was observed in cultures grown in CDMG (results not shown). However, the final biomass of both cultures at the beginning of stationary phase was higher in CDMS than in CDMG (2.4-fold for MN1 and 1.4-fold for RTF10).

The high production of dextran by the LAB strains prompted us to detect the HoPS bound to the bacteria sedimented from liquid cultures by TEM, as we have previously described for other HoPS (β -glucans) and heteropolysaccharides produced by LAB (Notararigo et al., 2013). However, we were unable to detect the dextran attached to the bacteria grown in media containing sucrose (results not shown). Therefore, production of dextran was investigated during sessile bacterial growth in plates containing agar media. Both bacteria displayed a mucous colony phenotype when grown on MRSS but not on MRSG (Fig. 2A). This mucoid appearance of colonies grown in the presence of sucrose has been previously described in the literature as characteristic of dextran-producing bacteria (Leemhuis et al., 2013). In order to determine the dextran location at cellular level, in this study we have set up a procedure to directly analyse bacterial colonies by TEM (see details in Materials and methods). Using this procedure, the reduced sample manipulation minimised the loss of dextran attached to the LAB. The analysis of mucoid colonies of MN1 and RTF10 from MRSS plates by TEM revealed that the HoPS were associated to the bacterial cell walls and surroundings (Fig. 2B).

Figure 2. Detection of dextran production by LAB during sessile growth. (A) Bacterial colonies grown on MRSG or MRSS. (B) Analysis of bacterial colonies at cellular level by TEM.

3.2. Analysis of the capability of *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10 to

auto-aggregate adhere and form biofilm. During static growth of MN1 in MRSG, but not in MRSS liquid media, it was observed that the cell biomass accumulated at the bottom of the tube, but for RTF10 this phenomenon was not detected (Fig. 3A). This suggested that only MN1 forms aggregates when grown in MRSG. To test this hypothesis, bacterial cultures were analysed by phase contrast microscopy (Fig. 4). In an attempt to stabilise the potential aggregates, bacteria were grown in Eppendorf tubes and, after sedimentation in the same tubes, concentrated 20 fold by gentle resuspension in PBS. The analysis confirmed that MN1, but not RTF10, forms aggregates upon growth in MRSG (Fig. 4A). Under these conditions, the production of dextran was quantified in the culture supernatants. As expected, production was only detected in cultures of both bacteria grown in MRSS, and after 24 h the concentration of the HoPS reached values of $5.1 \text{ g L}^{-1} \pm 0.1$ for MN1 and $2.9 \text{ g L}^{-1} \pm 0.3$ for RTF10. Thus, the results showed that growth in the presence of sucrose prevented autoaggregation of MN1 cells but had no effect on RTF10.

Figure 3. Detection of growth and biofilm formation of LAB. (A) Photographs of tubes containing cultures grown in MRSG or MRSS media for 24 h at 37 °C. Photographs showing biofilm formation on glass surface (B) or in polystyrene microtiter plates (C). The biofilms were revealed after bacterial growth in MRSG or MRSS 24 h at 37 °C by staining with crystal violet.

In addition, the results suggested that the presence of dextran as well as its *de novo* synthesis could interfere with the interactions between the cells of MN1. Therefore, the addition of sucrose to cell-aggregated MN1 cultures was investigated. To this end, MRSG media was removed from aggregated cultures after sedimentation, substituted by fresh medium (either MRSG or MRSS) and the cultures were further incubated

Figure 4. Detection of cellular aggregation and disaggregation. Bacteria were directly analysed, without fixing, by phase contrast microscopy. (A) *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10 after growth in MRSG or MRSS media for 24. (B) *Lb. sakei* MN1 after growth in MRSG (24 h) and further growth for 3 h in either MRSG or MRSS.

for 3 h prior to analysis by phase contrast microscopy. The results showed that addition of sucrose to an aggregated culture provoked disaggregation, and as expected this process did not take place when glucose was added to the culture (Fig. 4B). In parallel, the dextran production during the 3 h incubation in presence of sucrose was confirmed by analysis of the supernatants by the phenol-sulfuric acid method (around 3 g L⁻¹). Therefore, these results support an anti-adhesive role of dextran on MN1 but not on RTF10.

Thus, the influence of dextran on the potential of the two LAB to form biofilms was evaluated on an abiotic glass surface (Fig. 3B) and polystyrene plates (Fig. 3C). After the assay, the A_{595 nm} in glass tubes were 0.31 ± 0.05 in MRSG and 0.03 ± 0.006 in MRSS for MN1 and 0.01 ± 0.009 in both media for RTF10. The results obtained using polystyrene plates were similar, but values of A_{595 nm} were higher, reaching levels of 2.25 ± 0.20 in MRSG and 0.46 ± 0.01 in MRSS for MN1 and 0.01 ± 0.001 in MRSG and 0.02 ± 0.001 in MRSS for RTF10. Consequently, the results obtained with the two methods showed that MN1 was able to form biofilm in absence and presence of dextran, but the capacity decreased (4- to 10-fold) when the strain was grown in MRSS, the condition for dextran production. The ability of the two LAB to adhere to epithelial intestinal cells was tested *in vitro* performing binding assays of the bacteria to Caco-2 cell lines (Fig. 5). The results revealed that MN1 was able to adhere to the enterocytes with similar levels to that of the probiotic strain *Lb. acidophilus* LA-5 (4.58 ± 0.44 % versus 5.97 ± 0.31 %) in the presence of DMEM medium containing glucose, and the levels of MN1 binding decreased to 0.96 ± 0.28 % when the medium was supplemented with sucrose. By contrast, RTF10 presented low levels of adhesion to Caco-2 cells independently of the absence (0.49%) or presence (0.4%) of sucrose in the assay.

Figure 5. Adhesion of LAB to Caco-2 cells. Binding assays were performed with *Lb. acidophilus* (LA-5), *Lb. sakei* MN1 (MN1) and *Lc. mesenteroides* RTF10 (RTF10) using a ratio bacteria:epithelial cells (10:1) in DMEM (G) or DMEM supplemented with 0.5% sucrose (S) during 1 h. Adhesion levels are expressed as the percentage of cfu. Each assay was conducted in triplicate. Adhesion data were analysed by ANOVA, Tukey's test was employed to determine the statistically significant differences between samples. Differences in adhesion levels of MN1 and RTF10 compared with LA-5 (a, b and d) as well as between MN1G and MN1S (b and c) were significant with a $p < 0.05$.

3.3. Evaluation of *Lb. sakei* MN1 in zebrafish as a potential probiotic in fish. The above results supported probiotic properties for MN1 and influence of dextran on its behaviour. Zebrafish larvae are transparent and fluorescent bacteria can be directly visualized in their gut. Thus, in order to subsequently evaluate MN1 in zebrafish *in vivo* models, the strain was fluorescently labelled with mCherry by transfer of the plasmid pRCR12. As far as we know this is the first time that a *Lb. sakei* strain has been fluorescently labelled. The recombinant strain MN1[pRCR12] showed the same growth

pattern in liquid (Fig. 3SA) and solid (Fig. 3SB) medium as MN1 as well as the same capability for aggregation in MRSG (Fig. 4SA) and disaggregation in MRSS (Fig. 4SB) as its parental strain, when it was analysed by fluorescent microscopy. Furthermore, production of dextran was detected by direct observation upon growth in solid media (Fig. 3SB) and by phenol-sulphuric acid method during growth in liquid media, conditions resembling respectively sessile and planktonic growth. The levels of the HoPS were $4.8 \pm 0.2 \text{ g L}^{-1}$, after growth in MRSS for 24 h. Based on these results we concluded that the presence of the plasmid pRCR12 did not affect the dextran production, nor did HoPS influence on the behaviour of MN1. Following this, the ability of MN1[pRCR12] to colonize the gut of gnotobiotic zebrafish larvae and to compete with the GFP labelled *V. anguillarum* NB10[pOT11] was investigated, taken as an example of fish pathogenic bacteria in this ecological niche. Plate counts of MN1[pRCR12] at 6, 24 and 48 hpi showed a significantly higher level of the LAB in the gut of larvae, when incubation was performed in the presence of glucose instead of sucrose (Fig. 6). A difference in the colony count (between 4- and 10-fold) was observed from the first analysis at 6 hpi and remained after 24 and 48 hpi (Fig. 6). The average colony count per larva decreased with time of treatment in presence of either glucose or sucrose at 6 hpi (40.6 cfu or 8.33 cfu), at 24 hpi (16.33 cfu or 1.07 cfu) and at 48 hpi (5.07 cfu or 0.4 cfu).

Figure 6. Quantification of *Lb. sakei* MN1[pRCR12] prevalence in zebrafish larvae digestive tract.

Colonisation of zebrafish larvae intestines by *Lb. sakei* MN1[pRCR12] in presence of glucose (black bars) or sucrose (white bars) determined by plate count at 6, 24 and 48 h post-infection. Colonisation data were analysed as described in Fig. 5. Differences between the two condition at each time were significant with a $p < 0.05$.

For the competition assays, the fluorescent tag allowed *in vivo* detection of both bacteria in the gut by fluorescence microscopy (Fig. 7A). The results revealed that in the absence of *Lb. sakei* MN1[pRCR12], the cumulative mortality of zebrafish following infection with *V. anguillarum* NB10[pOT11] was 62% (Fig 7B, control). However, the pre-treatment with the LAB decreased this mortality to levels of 27%, when the experiment was performed in the presence of glucose (Fig. 7B, LG-VG). In addition, the replacement of glucose by sucrose during infection with NB10[pOT11] (LG-VS) or during the whole experiment (LS-VS) resulted in a lower decrease in cumulative mortality to values of 37% or 38% (Fig. 7B). These results indicated that MN1 protects zebrafish under conditions of bacterial infection.

Figure 7. Competition between *Lb. sakei* MN1[pRCR12] and *V. anguillarum* NB10[pOT11] in zebrafish larvae digestive tract. (A) Fluorescence microscopy images of 7 dpf infected larvae with *V. anguillarum* NB10[pOT11] and pretreated *Lb. sakei* MN1[pRCR12] in the presence of glucose (LG-VG, left) or sucrose (LS-VS, right). (B) Cumulative mortality (percentage) of larvae: infection with *V. anguillarum* NB10[pOT11] (control); pretreatment with *Lb. sakei* MN1[pRCR12] in the presence of glucose and infection with *V. anguillarum* NB10[pOT11] in the presence of sucrose (LG-VS); pretreatment and infection in the presence of sucrose (LS-VS) or glucose (LG-VG). Mortality data were analysed as described in Fig. 5. Differences were significant with a $p < 0.05$. Arrows indicate locations in which either *Lb. sakei* MN1[pRCR12] (red) or *V. anguillarum* NB10[pOT11] (green) were detected.

4. Discussion

In this work the production of dextran has been investigated in *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10, two LAB isolated from fermented meat (Nácher-Vázquez et al., 2015; Notararigo et al., 2013). TEM analysis of the bacteria detected HoPS attached to the cells upon exposure to sucrose and only during sessile growth in mucous colonies (Fig. 2B) indicating that most of the dextran synthesised by MN1 and RTF10 is in a soluble form. Analysis of their dextran production during planktonic growth in the presence of sucrose showed that the polymers accumulated in the media, reaching at stationary-phase levels of around 2 g L⁻¹ for MN1 and 1 g L⁻¹ for RTF10. In addition, both bacteria had an efficient consumption of sucrose accompanied by production of lactic acid and dextran, with transient accumulation of fructose in the supernatant (Fig. 1). Fructose accumulation has been previously described for dextran producing *Lc. mesenteroides* strains (Dols, Chraïbi, Remaud-Simeon, Lindley & Monsan, 1997; Quirasco, López-Munguía, Remaud-Simeon, Monsan & Farrés, 1999; Santos, Teixeira & Rodrigues, 2000). LAB dextran production is a strain dependent characteristic. Some *Leuconostoc* (Dols, Chraïbi, Remaud-Simeon, Lindley & Monsan, 1997) and *Lactobacillus* (Rühmkorf, Bork, Mischnick, Rübsam, Becker & Vogel, 2013) have a maximal efficiency of EPS production during the exponential-phase of growth like the pattern detected here for MN1 (Fig. 1S), whereas *Lactobacillus reuteri* 121 reaches its maximal production at the end of the exponential-phase and *Lb. reuteri* 180 continues producing EPS during the stationary-phase of growth (van Geel-Schutten, Flesch, ten Brink, Smith & Dijkhuizen, 1998), which is similar to that of RTF10, which maintained the same yield of dextran production during the stationary growth (Fig. 1S).

With regard to the balance between sucrose used for growth and sucrose used for dextran synthesis, MN1 produced 12 mM of the HoPS from 23 mM sucrose present in the medium (Fig 1), which indicated that 52% of the glucose released from the sucrose was used for synthesis of the dextran. The lack of the remaining 11 mM of glucose in the MN1 cytoplasm, could be explained by that, following glycolysis, it would yield 22 mM lactic acid. This level was detected, even before the consumption of the fructose that had accumulated in the supernatant started (7 h) (Fig. 1). In the case of RTF10, the maximum production of dextran was 7 mM (Fig. 1) indicating that 30% of the glucose from the sucrose was present as dextran. This lower efficiency could be the reason why glucose was transiently detected in culture supernatants of this bacterium (Fig. 1). This monosaccharide was presumably transported to the cytoplasm and metabolised, since an increase in lactic acid concentration up to 25 mM was observed but not in dextran (Fig. 1). Thus, this study revealed that production of dextran by both strains never exceeded the synthesis of lactic acid.

In addition, we have constructed the fluorescently labelled *Lb. sakei* MN1[pRCR12], which has the same *in vitro* autoaggregation and abiotic capabilities as the parental MN1 (Figs. 3S and 4S *versus* Figs 3 and 4). We have demonstrated that this bacterium can colonise the digestive tract of gnotobiotic zebrafish larvae (Fig. 6) and compete with the bacterial pathogen NB10[pOT11] (Fig. 7). Colonisation of the gut by the LAB was higher in the presence of glucose than in the presence of sucrose. This correlated with the results obtained *in vitro* which showed an increased capacity of the bacteria, in the absence of dextran synthesis, to adhere to epithelial cell lines (Fig. 5), autoaggregate and form biofilms (Figs. 3S and 4S). Thus, it appears that the presence of dextran decreases the adhesiveness of MN1 while it is not a generalised effect of HoPS and HePS produced by LAB. These bacterial

polymers appear to be involved in biofilm formation, favouring adherence to abiotic and biotic surfaces or causing aggregation of cells (Nwodo, Green & Okoh, 2012). This is the case of the (1-3)- β -D-glucan with branches in position *O*-2, a HoPS produced by LAB isolated from cider, whose presence increases the binding of producing bacteria to Caco-2 cells (Fernández de Palencia et al., 2009; Garai-Ibabe et al., 2010). However, it has also been shown that other HoPS and HePS can have the opposite effect, as it is the case of *Lactobacillus johnsonii* FI9785, a reduced production of its HePS causes an increase in its ability to aggregate, form biofilms and adhere to epithelial cells (Dertli, Mayer & Narbad, 2015). Likewise, in *Lactobacillus rhamnosus* GG, a probiotic bacterium producing a HePS rich in galactose, it has been demonstrated that the presence of the biopolymer on the surface decreases adhesiveness (Lebeer et al., 2009). Probably in these cases, the presence of the EPS hinders access to other surface molecules, such as adhesins, or has an effect on the bacterial surface hydrophobicity affecting the aggregation ability (Del Re, Sgorbati, Miglioli & Palenzona, 2000; Pérez, Minnaard, Disalvo & De Antoni, 1998). In the case of dextrans produced by LAB, the first hypothesis is plausible, because we have previously demonstrated that both RTF10 and MN1 produce (1-6) glucans with approximately 6% substitution (9% for *Lc. mesenteroides* and 3% for *Lb. sakei*), at positions *O*-3, by side chains composed of a single residue of glucose (Notararigo et al., 2013; Nácher-Vázquez et al., 2015), and in this work we have not observed a significant influence of the HoPS in the low levels of the *Lc. mesenteroides* strain adhesiveness. Furthermore, the results obtained for MN1 in the zebrafish larvae gnotobiotic models indicate a protective effect of the LAB on the gut preventing infection with NB10[pOT11]. Although competition for adhesion sites has been widely suggested as a mode of action of probiotics against pathogens, there is currently little evidence in the literature to demonstrate this hypothesis (Kesarcodi-

Watson, Kaspar, Lategan & Gibson, 2008). The protection against NB10[pOT11] detected in this work could be due to a direct effect of MN1 in inhibiting colonisation of *V. anguillarum* by competition, since in the absence of dextran synthesis a marked decrease of mortality (57%) was detected (Fig. 7B). In addition, a clear inhibition of *V. anguillarum* infection (reduction of 40% of larvae mortality) was detected when the experiment was performed in conditions of dextran synthesis (Fig. 7B). This result was unexpected, since a notable decrease of MN1 colonisation was observed when experiments were performed in the presence of sucrose (Fig. 6). Thus, the pretreatment with the LAB could also indirectly reduce the infection by the pathogen, due to a immunomodulation mechanism, in which the dextran could be also involved. This is in agreement with our previous results about the ability of MN1 dextran to inhibit *in vitro* and *in vivo* infection by two types of salmonid viruses presumably connected with its ability to induce *in vivo* production of two interferons (INF-1 and INF- γ), involved in innate and acquired immunity (Nácher-Vázquez et al., 2015). Nevertheless, further experiments should be performed to unravel the mechanism of action of *Lb. sakei* MN1 in the presence and absence of dextran in the gut since it points to a high potential as a probiotic in aquaculture.

5. Conclusions

In conclusion, *Lb. sakei* MN1 successfully colonises and competes with *V. anguillarum*, a fish pathogen, in the zebrafish gut. However this probiotic potential is not linked to the dextran production since it proved to confer *Lb. sakei* MN1 an antiadhesive effect in abiotic and biotic surfaces, allowing bacterial detachment from biofilms. This property of the dextran seems to depend on the ability of the producing bacteria to autoaggregate,

537 since it was not detected for the dextran producing non-aggregative *Lc. mesenteroides*
538 RTF10 strain.

Acknowledgements

This work was supported by the Spanish Ministry of Economics and Competitiveness [grants AGL2012-40084C03-01 and AGL2015-65010-C3-1-R] and by the Agriculture and Fisheries Department of the Basque Government [project VIVAQUA], and II is the recipient of a PhD fellowship from Technological centres foundation-Iñaki Goenaga. We thank Dr Alicia Prieto for help on standardisation and interpretation of the metabolic experiments. We thank Dr Stephen Elson for critical reading of the manuscript. We also thank the Microscopy section of the Central Service for Experimental Research at the University of Valencia (SCSIE) for technical support.

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Figure 1S. Influence of sucrose utilization in yield of dextran and lactic acid production. Bacteria were grown in CDMS medium at 30 °C without shaking. Symbols: ▲, A_{600nm} ; ●, EPS/ A_{600nm} ; ■, lactate/ A_{600nm} .

Figure 2S. Influence of carbon source in growth of *Lb. sakei* MN1 and *Lc. mesenteroides* RTF10. Bacteria were grown at 30 °C without shaking in either CDMG (◇) or CDMS (●) media.

Figure 3S. Growth and dextran production by *Lb. sakei* MN1[pRCR12]. (A) Bacterial cultures after growth in tubes in MRSG or MRSS media at 30 °C without shaking. (B) Bacterial colonies grown on MRSG or MRSS solid media at 30 °C for 24h.

Figure 4S. Cellular aggregation and disaggregation of *Lb. sakei* MN1[pRCR12]. Cells were directly analysed without fixing by optical microscopy. (A) After growth in MRSG or MRSS media for 24 h. (B) After growth in MRSG (24 h) and further growth for 3 h in either MRSG or MRSS media. Overlay of images of the same field obtained by phase contrast and fluorescent microscopy are depicted.