Analysis of technological and probiotic properties of Algerian *L. mesenteroides* strains isolated from dairy and non-dairy products

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Highlights

- Six leuconostoc strains have proteolytic, lipolytic and acidifying activities.
- Five of these strains produce fructose, glucose, lactic acid, dextran and mannitol.
- The CM9 strain has high resistance to acid and stomach-duodenum passage stresses.
- The CM9 strain colonizes the intestinal tract of gnotobiotic zebrafish larvae.

Abstract

Six *Leuconostoc mesenteroides* strains isolated from camel (3) and sheep (1) milk, silage (1) and honey (1) have been investigated. All showed probiotic properties due to their resistance to gastrointestinal tract stresses, antibiogram profiles, hydrophobicity levels and antimicrobial activities against *Staphylococcus aureus* and *Escherichia coli*. Six had proteolytic, lipolytic, acidifying, and coagulation activities and five produced dextran. Metabolic flux analysis during growth in a sucrose-containing medium of two representative dextran-producing strains, and a non-producing strain revealed differences in the levels of the sucrose metabolites: fructose, glucose, lactic acid and mannitol, and a correlation between sucrose consumption and dextran synthesis for the producing strains. CM9 from camel milk showed the highest dextran production and the best pattern of intestinal tract colonization of gnotobiotic zebrafish embryos. These facts together with CM9's technological and probiotic properties indicate that this strain may be useful for the production of functional dairy food.

Keywords

Leuconostoc mesenteroides, technological properties, dextran, metabolic study, probiotic properties, zebrafish.

Abbreviations

CDMS, CDM define medium lacking glucose and supplemented with sucrose; cfu, colony forming unit; CO_2, carbon dioxide; EDTA, ethylenediaminetetraacetic acid; *E. coli*, *Escherichia coli*; EPS, exopolysaccharide; GRAS, Generally Recognized As Safe; KMK, Kempler MacKay agar medium; LAB, lactic acid bacteria; MRSG, Man Rogosa Sharpe broth containing 2% glucose; MRSS, MRS medium containing 2% sucrose instead of glucose; MSE, Mayeux Sandine Elliker agar medium**;** PBS, *phosphate-buffered* saline; *S. aureus*, *Staphylococcus aureus*; PCA, plate count agar medium; TAE, tris acetate buffer; TEM, transmission electron microscopy.

1. Introduction

Lactic acid bacteria (LAB) are a heterogeneous group of strains of different genera including *Leuconsotoc* and belonging to the *Lactobacillales* order, which synthesize lactic acid as the major product of sugar fermentations (Arena, Capozzi, Spano, & Fiocco, 2017). LAB colonize many food matrices such as milk, meat, vegetables and cereals. Moreover, they are members of the digestive tract and vaginal microbiota. This adaptation to various environments is in part due to their metabolism, which results in the production of metabolites which can be exploited in the food and pharmaceutical industries (Mazzoli, Bosco, Mizrahi, Bayer, & Pessione, 2014). Among the LAB, *Leuconostoc* species can produce valuable compounds such as ethanol, antimicrobial agents (Tropcheva, Nikolova, Evstatieva, & Danova, 2014), vitamins (Nuraida, 2015) and mannitol, a polyol which is used in the pharmaceutical industry (Bhatt, Mohan, & Srivastava, 2013) and also included as a low-calorie ingredient in food products (Ghoreishi & Shahrestani, 2009). *Leuconostoc* strains can also produce exopolysaccharides, such as dextran, a homopolysaccharide which has various uses in the food, pharmaceutical, medical or oil drilling industries (Aman, Siddiqui, & Qader, 2012). In the food industry, it is added to bakery products and confectionery to improve softness or moisture retention and to increase viscosity, rheology and texture (Pérez-Ramos et al., 2015). The increased viscosity of EPS-containing foods may increase the time that the ingested fermented milks remain in the gastrointestinal tract and therefore be beneficial for a transient colonization by probiotic bacteria (Hemme, 2012). In addition, we have demonstrated that dextrans synthesized by LAB have beneficial health properties as antivirals and immunomodulators (Nacher-Vazquez et al., 2015; Zarour et al., 2017). Also, *Leuconostoc* produces acids, which preserve several foods by inhibiting the proliferation of undesirable microorganisms (Papagianni, 2012). This acidity also contributes to flavour formation and the coagulation of caseins (the main milk proteins), which modifies the texture of various fermented dairy products (Smid & Kleerebezem, 2014). During the fermentation process, *Leuconostoc* strains contribute in making the final product more edible, safe, and healthy as well as having pleasant texture and aroma. Among the various *Leuconostoc* species, the subspecies *L. mesenteroides* is probably the most used in the dairy field. This is due to its broad utilization as an aroma producer in the dairy industry for different fermented milk. For a long time, *L. mesenteroides subsp. cremoris* strains were the sole starters available on the starter market (Hemme, 2012). *Leuconostoc* species can (i) hydrolyze α-galactosides (Park et al., 2007) and (ii) produce prebiotics oligosaccharides with bifidogenic effects, such as the α-

gluco-oligosaccharides produced by *L. mesenteroides* NRRL-B-18242 (Chung & Day, 2002). In addition, for organoleptic, rheological and functional properties, *L. mesenteroides* specie are involved in various commercial dairy products such as the blue cheeses as well as the fermented milks Dahi, Ymer and Viili, (Yerlikaya, 2014) and non-dairy products like Pulque, a Mexican fermented beverage (Adelfo et al., 2004; Escalante et al., 2016).

This state of the art reveal that new *L. mesenteroides* strains having the aforemention biological properties and able to synthesize products such as dextran able to contribute to their probiotic and technological profile, complemented with tolerance and adaptation to the digestive tract could be good candidates for the development of functional dairy products.

Therefore, in this work we report on six *L. mesenteroides* strains isolated from Algerian milk, silage or honey, which have been evaluated *in vitro* for their probiotic potential and technological properties with a future aim of using them as bioactive starters for the development of Algerian fermented functional dairy products.

2. Materials and methods

2.1. Bacteria and growth conditions. Samples of the following Algerian non-dairy matrices were used for isolation of *Leuconostoc* strains: (i) 6 months fermented silage composed of *Avena sativa* (oat) and *Hordeum vulgare* (barley) and (ii) natural honey collected directly from *Apis mellifera* beehives. Samples were collected from western Algerian (Mascara region) in June 2011 and March 2012, respectively. *Leuconostoc* strains were isolated using agar plates containing the MSE selective medium (Mayeux, Sandine, & Elliker, 1962), supplemented with 10% sucrose and vancomycin (30 µg mL^{-1}) (Mathot, Kihal, Prevost, & Divies, 1994) at 30 °C for 72 h. The cultures were then grown on MRS medium (de Man, Rogosa, & Sharpe, 1960) supplemented with 2% of glucose (MRSG). Three LAB strains were selected on the basis of Gram-staining–and lack of catalase activity (Fortin, Messier, Paré, & Higgins, 2003). In addition, the dextran-producing Algerian isolates *L. mesenteroides* CM9 and CM30 from camel milk collected from south Algerian Sahara (Bechar region) in January 2011 and SM34 from sheep milk of Mascara city in April 2011 (Zarour et al., 2017) were also studied in this work. MRSG was used for routine growth of the LAB, and MRS supplemented with 2.0% sucrose instead of glucose (MRSS) and defined CDM medium (Sánchez et al., 2008) supplemented with 0.8% sucrose (CDMS) were used for analysis of the bacterial metabolic fluxes and to quantify the EPS production. The pathogens used in this study were *Staphylococcus aureus* ATCC 25923 and *Escherichia coli* ATCC 25922. For the

assays, these bacteria were grown in nutrient broth (BD Difco™, USA) at 37 ºC until the phase, or for the times, indicated below in sections 2.4-2.13. Müller-Hinton-agar medium (BD Difco™, USA) was used for the antagonist tests. For long-term bacterial storage at -80 ºC, the adequate medium was supplemented with 20% (v/v) glycerol.

2.2. Phenotypic characterization. The cell morphology of all isolates was characterized by optical and transmission electron microscopies. For the latter, colonies from MRSG-agar plates were treated as previously described (Nácher-Vázquez et al., 2017) and examined using a JEOL 1230 microscope operated at 100 kV at the Electron Microscopy service of the Centro de Investigaciones Biológicas (CIB, Madrid, Spain). Gram-positive, catalase-negative isolates were tested for: (i) the sugar fermentation patterns, using the API 50 CHL system (bioMérieux, France) and subsequent strain identification with the apiweb™ software (BioMérieux, France); (ii) gas production from glucose in Durham tubes containing MRSG (Ortakci, Broadbent, Oberg, & McMahon, 2015); (iii) hydrolysis of arginine on M16BCP medium (Thomas, 1973); (iv) capability to metabolise citrate by growth on KMK-agar medium plates (Kempler & McKay, 1980) and (v) growth in MRSG: (a) at different temperatures (4 °C, 15 °C, 37 °C or 45 ºC); (b) in the presence of NaCl (either at 3% or 6.5%) and (c) at pH values of 4.0 or 6.5.

2.3. Genotypic characterization. Genomic DNA preparations were obtained from LAB cultures as previously described (Nacher-Vazquez et al., 2017b) and the identification of species was achieved by sequencing their 16S rRNA coding genes at Secugen (Madrid, Spain). The PCR reaction specific for detection of *L. mesenteroides* subsp. *mesenteroides* strains was performed using the primers LmmF (5'-CCGTTACCCCTAAATT-3') and LmmR (5'-GACCAAATACAATAGGTTGCG-3') (Sigma-Aldrich) as previously described (Moschetti, Blaiotta, Villani, & Coppola, 2000). The PCR products were analysed in 0.8% agarose gel using Smart Ladder size marker MW-1700-10 (Eurogentec, Osaka, Japón). The amplicons were visualised by staining with GelRed 1X (Biotium Inc., Hayward, CA, USA) and images were captured, digitized and analysed using the Gel Doc 2000 Bio-Rad gel documentation system (West Berkeley, California, USA) and the Quantity One 4.5.2 Bio-Rad software.

2.4. Resistance to gastro-intestinal conditions. Exponential LAB cultures grown in MRSG to an absorbance at 600 nm (A_{600nm}) of 1.0 were sedimented by centrifugation at 12,000 × *g* for 5 min at 5 ºC, washed, resuspended in fresh medium and used as inocula to test at 30 °C, in MRSG medium, survival under gastro-intestinal stresses as described below.

The ability of *L. mesenteroides* strains to resist gastric acidity was determined according to the technique described by Pieniz, Andreazza, Anghinoni, Camargo, & Brandelli (2014) upon growth in medium adjusted to pH 7.0, 3.0 or 2.0. The ability of the strains to resist bile was determined by the method described by Hyronimus et al. (2000) upon growth in medium lacking (control) or supplemented with bile salts at 0.3%, 1% or 2%. Then, the colony forming units (cfu) were determined at the time of inoculation (t_{0h}) and after 3 h incubation (t_{3h}). The results were expressed as percentages of the initial cfu, according to the following Eq. (1). Experiments were carried out in triplicate.

$$\text{Survival rate (\%)} = (\log \text{cfu } (t_{3h}) / \log \text{cfu } (t_{0h})) \times 100 \qquad (1)$$

2.5. **Response to simulated stomach duodenum-passage (SSDP)**. To test the LAB resistance to the stomach-duodenal stimulus containing several barriers, the technique described by Mathara et al. (2008) was used. Briefly, bacterial pre-cultures grown to A_{600nm}=1.0 were diluted ten-fold in Ringer solution (NaCl 9 g, KCl 0.42 g, $CaCl_2$ 0.48 g and $NaHCO_3$ 0.2 g per L) and used to inoculate MRSG medium at pH 3.0 by dilution (1:3). Bacterial cfu were determined at t_{0h} and after 1 h of incubation at 37 °C. Then, 4 mL of reconstituted bile salts in a proportion of 10% followed by 17 mL of a synthetic duodenal secretion ($NaHCO_3$ 6.4 g, KCl 0.239 g, NaCl 1.28 g per L) were added to the cultures. Afterwards, samples were withdrawn after 1 h (t_{1h}) and 3 h (t_{3h}) at 37 ºC and the number of cfu determined as described above. Experiments were carried out in triplicate.

2.6. Bacterial hydrophobicity. This property was analysed according to the method described by Iyer et al. (2010). Briefly, exponential LAB cultures were sedimented as above and, after washing, resuspended in phosphate urea magnesium sulphate buffer (pH 6.5). The initial absorbance at 600 nm ($A_{initial}$) of the cell suspension was adjusted to 1.0. Then, 0.6 mL of xylene was added to 3 mL of the bacterial suspension and the hydrocarbon layers were mixed by incubation at 37 ºC for 10 min and vortex for 2 min. After further incubation for 15 min, the aqueous phase was carefully removed with a Pasteur pipette and the final absorbance (A_{final}) measured. The decrease in the absorbance was taken as a measure of the cell surface hydrophobicity calculated with the Eq. (2).

$$\text{Hydrophobicity (\%)} = (A_{initial} - A_{final} / A_{initial}) \times 100 \qquad (2)$$

2.7. Bacterial sensitivity to antibiotics. This was determined by the disc diffusion method on MRSG-agar plates as recommended in the Performance Standards for Antimicrobial Disk Susceptibility tests (2007). Various antibiotics (Bio-rad, Marnes-la-Coquette, France) were

used (see details in Table 2). Briefly, freshly prepared LAB cultures (A_{600nm}=1.0) were seeded on Müller-Hinton agar plates. Then, discs containing each antimicrobial agent were placed on the surface of the plates. Inhibition zone was assessed after 24 h of incubation at 30 ºC, by measuring the diameter around the discs (mm) (Liasi et al., 2009).

2.8. LAB antagonist activities. The antimicrobial activity of the *L. mesenteroides* strains was evaluated on a solid medium according to the diffusion method (Barefoot & Klaenhammer, 1983). The MRSG pH 6.2 and MRSG pH 7.0 media were consecutively used to discard the acidity effect. Bacterial cultures (A_{600nm}=1.0) were spotted on both types of MRSG-agar media plates. After 24 h of incubation at 30 ºC, the soft Müller-Hinton-agar medium was inoculated with either *S. aureus* ATCC 25923 or *E. coli* ATCC 25922, and poured independently as an overlay on the plates containing the MRSG medium. The plates were then incubated for 24 h to 48 h at 37 ºC. LAB strains with translucent halos around the spots were classified as antimicrobial producing bacteria.

2.9. Acidification activity and coagulation ability. The acidification activity was tested according to Kihal, Prevost, Henni, Benmechernene, & Diviès (2009), in 10% reconstituted skim milk and supplemented with 0.3% of yeast extract. The ability of strains to coagulate milk was revealed by the appearance of a coagulum with the presence of cracks or voids.

2.10. Proteolytic and lipolytic activities. LAB strains were grown in MRSG to A_{600nm}=0.6, prior to testing in solid medium by spotting 10 µL of each bacterial culture. To determine the proteolytic activity, PCA medium (Massa, Caruso, Trovatelli, & Tosques, 1998) agar plates supplemented with 1%, 3% or 5% (v/v) of 10% skim milk were used as previously described (Moslehishad, Mirdamadi, Ehsani, Ezzatpanah, & Moosavi-Movahedi, 2013). After incubation of the inoculated plates at 30 ºC for 48 h, the activities were estimated by measurement of the diameter of the clear zone surrounding the inoculated spots (mm). Lipolytic activity was assessed on MRSG-agar plates buffered to pH 7.0 and supplemented with 1%, 3% or 5% of tween 20 (artificial lipid source) (Samad et al., 1989) or olive oil (natural lipid source). The medium was opacified by addition of 0.5% calcium carbonate. After incubation of the inoculated plates at 30 °C for 48 h, the activity was estimated by measurement of the diameter of the clear zone surrounding the inoculated spots (mm) as previously described (Kalbaza et al., 2018**)**.

2.11. EPS production and quantification. The production of EPS from sucrose by LAB was detected by streaking on MSE-agar medium plates and incubation for 24 h at 30 ºC.

Producing strains were identified by the formation of large, viscous and sticky colonies. The quantification of EPS was carried out in triplicate, from the supernatants of the bacterial cultures grown to A_{600nm}=1.5, on CDM medium supplemented with 2% of sucrose, as sole carbon source, by the phenol-sulfuric method as previously described (Nacher-Vazquez et al., 2015).

2.12. Analysis of metabolic fluxes and dextran synthesis. To determine the metabolic behaviour of CM30, CM9 and CM70 strains in the presence of sucrose as the only carbon source, the bacteria were grown in MRSS, to A_{600nm} of 2. Cells were sedimented by centrifugation (9300 × *g*, 10 min, 4 ºC), resuspended in the same volume of fresh MRSS, diluted 1:100 in CDMS and grown until the beginning of the stationary phase. Batch fermentations of each strain without pH control were performed at 30 ºC in triplicate. The cultures were sampled every hour to monitor growth, by determining A_{600nm} and acidification of the media, by measuring pH. The cultures were finally centrifuged as above, and the supernatants used to assess the amount of EPS by the phenol-sulphuric acid method and the concentration of sucrose, glucose, fructose, mannitol and lactic acid by gas chromatography–mass spectrometry (GC-MS) using *myo*-inositol as internal standard, as previously described (Nácher-Vázquez et al., 2017).

2.13. Colonisation of zebrafish gut by *Leuconostoc* strains. CM30, CM9 and CM70 strains were grown at 30 ºC in MRSG until A_{600nm}=1.0 (5 x 10^8 cfu mL^{-1}). Then, cells were sedimented as above, washed three times with PBS buffer pH 7.0 and resuspended in the sterilized embryo water solution (EW) ($CaCl_2.2H_2O$ 7.35 mg, $MgCl_2.7H_2O$ 3.08 mg, $NaHCO_3$ 1.58 mg and KCl 0.14 mg L^{-1}) to reach 5 x 10^7 cfu mL^{-1} prior to addition to the zebrafish embryos. The zebrafish embryos used in the experiment were provided by the company ZF Biolabs (Madrid, Spain). The embryos obtained by *in vitro* fecundation were washed and treated to produce gnotobiotic zebrafish embryos, modifying the protocol previously described by Oyarbide, Iturria, Rainieri, & Pardo (2015). Embryos were washed five times in EWB (EW solution plus 0.01% (w/v) of methylene blue). Then, embryos were washed ten times in AB solution (EWB containing three antibiotics: kanamycin at 15 μg mL^{-1}, ampicillin at 300 μg mL^{-1} and amphotericin B at 1.25 μg mL^{-1}). After that, the AB solution was removed, and embryos were gently immersed in 0.01% (w/v) polyvinylpyrrolidone (PVP) for exactly 1 min. The PVP was then removed by washing the embryos ten times in EWB followed by ten times in AB solution. Finally, embryos were incubated overnight in AB solution. The following day, the AB solution was removed by washing the embryos ten times

in sterile EWB solution and keeping them in Petri dishes with EW until the experiments were performed. Forty-five gnotobiotic zebrafish embryos of 4 days post-fecundation (dpf) were placed into each Petri dish and 30 mL of the dilution containing the bacterial culture were added. Embryos were incubated at 28 ºC with agitation (25 rpm) . Then, the solution containing the bacteria was removed and the embryos were washed five times with sterile EW and transferred to new sterile dishes. After 6 and 24 h post-infection (hpi), embryos were euthanized with tricaine at 300 mg mL^{-1}, and groups of 5 embryos were placed in 1.5 mL tubes and were washed twice with sterile EW supplemented with 0.1% (v/v) of Tween 20 and washed once with only EW, to remove bacteria loosely attached to the skin (Rendueles et al., 2012). Then, 400 µL of sterile EW were added to embryos for manual homogenisation with a Pellet Pestle. Finally, 200 µL of the homogenate were spotted on each MRSG plate. The initial and final numbers of cfu mL^{-1} were counted after incubation for 24 h at 30 ºC. The colonization rate was determined by expressing the cfu per larva. Two independent experiments were performed and in each one the assays were performed in triplicate.

2.14. Statistical analysis. In the EPS quantification assays as well as probiotic proprieties analyses, the data are expressed as the mean of three independent experiments and the corresponding standard deviation. For the *in vivo* test of LAB in zebrafish embryos two experiments were performed. All the data were subjected to one-way analysis of variance (ANOVA) by using the statistical SAS software. The Tukey's test was employed to determine the significant differences between the variables at $p \leq 0.05$.

3. Results

3.1. Identification of the isolates. Ten Gram positive and catalase negative LAB strains were isolated from four dairy and non-dairy Algerian products (Zarour et al., 2017 and this work). Results of physiological and biochemical identification showed that only three strains from camel milk (CM9, CM30, and CM70), one from sheep milk (SM34), one from silage (E14) and one from honey (M67) were cocci with ovoid shape (CM30 strain in Fig. 1A and 1B) associated in pairs and short chains (Fig. 1A), when observed by optical (Fig. 1A) and electronic (Fig. 1B) microscopies. These six strains were able to produce CO_2 from glucose and unable to hydrolyse arginine, therefore these bacteria should belong to the genus *Leuconostoc.* They were *also able* to grow at 15 °C and 37 °C but not at 4 °C and 45 °C, consequently they are mesophilic bacteria. All strains were resistant to a concentration of 6.5% of NaCl, and to pH 4.0. The formation of blue colonies on KMK-agar medium revealed

that all strains were able to metabolise citrate as a precursor of the aromatic compounds involved in the organoleptic properties of butter and cheese, which is a technological character for the selection of *Leuconostoc* strains (Figure 1S).

Sequencing of 16S rRNA coding genes confirmed the six isolates as *L. mesenteroides* (since the genes showed 99% homology with the *L. mesenteroides* sequences deposited in the GeneBank). The determined sequences have been deposited in the GeneBank with the following accession numbers: KY083048 (CM9), KY082929 (CM30), KY780576 (CM70), KY083047 (SM34), MF977748 (E14) and MF977749 (M67). The genotypic characterization was complemented with a subspecies-specific PCR amplification analysis, and only the genomic DNA of the strains CM30 and E14 served as substrate to generate the expected 0.9-kbp amplicon specific for *L. mesenteroides* subsp. *mesenteroides* strains (results not shown).

In addition, analysis of the sugar fermentation profile of LAB strains with the API 50 CHL biochemical galleries supported that all belonged to the *L. mesenteroides* species. Moreover, since only the CM30 and E14 strains fermented arabinose, this analysis also supported that these bacteria belonged to the subsp. *mesenteroides*.

3.2. Technological properties of the *L. mesenteroides* strains. The acidification activity of the six strains in milk was investigated. The decrease in pH and the evolution of the titratable acidity of the analysed LAB started without a latent phase with two different profiles. Thus, the strains were classified in two groups, the first (GI) included the CM9, CM30, CM70 and SM34 strains isolated from mammalian milk and characterized by rapid and strong acidification, while the second group (GII) included E14 and M67 strains isolated from non-dairy origin, silage and honey, respectively, having a low and slow acidification activity compared to the first group. As an example, the results obtained for one representative of each group are depicted in Figure 2. CM9 strain provoked a rate of pH decrease of -0.12 ± 0.05 pH units h^{-1} (Fig. 2A), whereas M67 cultures displayed a slower rate of -0.07 ± 0.03 pH units h^{-1} (Fig. 2B). Also, the evolution of dornic acidity of these two strains (Figs. 2A and 2B) was inversely proportional to the decrease in pH.

During an 8 h incubation period, the CM9 cultures increased the dornic acidity from 34 ± 1.00 D to 55.33 ± 1.53 D, whereas M67 cultures augmented the acidity from 28 ± 2.00 D to 39.33 ± 0.58 D. Therefore, a difference between the two strains of 31% in total dornic acidity activity was detected. Coagulum formation from milk proteins, after 24 h of incubation, was observed only in GI and not in GII (results not shown).

All strains expressed proteolytic activity, manifested by the appearance of a clear halo around spots (between 12 mm and 21 mm). Only the M67 strain, isolated from honey, exhibited a low activity and generated a halo whose diameter varied between 9 mm and 10 mm. All strains, except CM9, were able to degrade the two lipid sources (natural and artificial) tested (results not shown).

3.3. EPS production and central metabolism. Detection of EPS production by the six strains on MSE-agar medium, showed that only CM9, CM30 and SM34 were able to form viscous and mucous colonies (Fig. 3A). CM70, M67 and E14 were devoid of this phenotype, showing small round and lenticular colonies (Fig. 3B).

The quantification of EPS production by the strains grown on CDMS showed the existence of three categories of producers: (i) high (CM30 and SM34), medium (CM9) and low (E14 and M67) (Fig. 4A). The results revealed EPS production levels of 0.55 ± 0.17 g L^{-1}, 0.63 ± 0.15 g L^{-1}, 2.4 ± 0.13 g L^{-1}, 3.25 ± 0.16 g L^{-1}, 3.52 ± 0.18 g L^{-1} for E14, M67, CM9, SM34 and CM30 strains, respectively (Fig. 4A). The results confirmed the preliminarily classification of CM70 as a non-EPS producer. The culture on CDMS of CM30, one of the high EPS-producers, has the appearance of a dense gel, a property defined as "ropiness" (Fig. 4B).

In a previous work, we identified the EPS produced by CM9 and CM30 strains as α-(1,6) glucans with approximately 9% substitution, at positions *O*-3 (Zarour et al., 2017). These dextrans are synthesised by dextransucrases extracellularly, using sucrose as substrate with a concomitant release of fructose (Leemhuis et al., 2013). Thus, in this work, the EPS-producing CM9 and CM30 strains as well as the non-EPS producer CM70 were subjected to a metabolic study during their planktonic growth in CDMS, containing sucrose as carbon source, determining the concentration of sucrose and its metabolites in the culture supernatants (Fig. 5).

In this medium, the CM9 strain showed the best pattern of growth with a generation time of 91 min and a growth rate of 0.66 ± 0.01 h^{-1}. The CM30 strain had a growth rate of 0.43 $\pm$ 0.02 h^{-1} and a generation time of 140 min. In addition, the CM70 strain presented the longest generation time (205 min). Moreover, the two dextran-producing strains synthesized the polymer, in the exponential phase of growth, in parallel with an increase in A_{600nm} and a decrease of the extracellular pH. The dextran levels in the extracellular medium increased with time and after 24 h of growth reached levels of 2.15 ± 0.15 g L^{-1} and 1.47 ± 0.08 g L^{-1} for CM30 and CM9, respectively.

In addition, after 6 h of incubation, the CM9 strain consumed the sucrose added to the medium (≈ 25 mM), whereas the non-EPS producer CM70 required 17 h before the sucrose was not detected in the supernatant (Fig. 5). Moreover, the three strains accumulated fructose in the culture medium during their consumption of sucrose, and when the extracellular disaccharide reached undetectable levels, the strains began to incorporate the accumulated fructose. Glucose, the primary product of sucrose metabolism, was efficiently metabolized by the three strains without any transient accumulation. At the end of the incubation period, the accumulation of lactic acid in the medium of the EPS-non-producing CM70 cultures (30.53 ± 0.56 mM) was higher than those produced by the two EPS-producing strains (around 19 mM). Finally, mannitol production seemed to be proportional to the consumption of the accumulated fructose. The production rates of CM30, CM9 and CM70 strains at the beginning of the stationary phase were 5.42 ± 0.32 mM, 5.63 ± 21 mM and 2.66 ± 0.17 mM, respectively. Mannitol production was almost stable up to 24 h.

3.3. Probiotic profile of the *L. mesenteroides* strains. The antibiotics recommended by the European Food Safety Authority (EFSA, 2012) were tested to identify bacterial strains with potential acquired resistance to: inhibitors of cell wall synthesis (amoxycillin, clavulanic acid, ampicillin, cefazolin, cefotaxime, cefoxitin, oxacillin, penicillin and vancomycin), inhibitors of nucleic acid synthesis (ofloxacin and pefloxacin) and lately inhibitors of ribosome function (tetracycline) was tested (Table 1). The strains were classified as sensitive (S), intermediate (I) and resistant (R) according to the cut-off values of each antibiotic proposed by the Antibiogram Committee of the French Microbiology Society (Soussy et al., 2000). All strains showed resistance to cefazolin, ofloxacin, oxacillin, pefloxacin and vancomycin as well as sensitivity to cefotaxime, cefoxitin, penicillin and tetracycline. Moreover, only CM70 was resistant to amoxicillin plus clavulanic acid.

Table 1. Antibiotic susceptibility profile of *L. mesenteroides* strains

		Diameter area (mm)											
Antibiotics	**Disc load (µg)**	**CM9**		**CM30**		**CM70**		**SM34**		**E14**		**M67**	
Amoxicillin ± Clavulanic acid	20 µg ± 10 µg	30	S	28	S	18	R	25	S	28	S	31	S
Ampicillin	10 µg	0	R	32	S	27	S	35	S	25	S	22	S
Cefazoline	30 µg	15	R	15	R	13	R	12	R	10	R	14	R
Cefotaxime	30 µg	19	I	23	S	25	S	24	S	27	S	24	S
Cefoxitin	30 µg	23	S	25	S	17	I	22	S	23	S	23	S
Ofloxacin	5 µg	13	R	15	R	14	R	10	R	13	R	09	R
Oxacillin	1 µg	0	R	0	R	0	R	0	R	0	R	0	R
Pefloxacin	5 µg	0	R	0	R	0	R	0	R	0	R	0	R
Penicillin	6 µg/10 IU	31	S	28	S	35	S	32	S	35	S	30	S
Tetracycline	30 µg	29	S	31	S	40	S	25	S	28	S	35	S
Vancomycin	30 µg	0	R	0	R	0	R	0	R	0	R	0	R

(R) resistant, (I) intermediate and (S) sensitive.

In addition, the six strains were able to diminish the growth of *S. aureus* ATCC 25923 and *E. coli* ATCC 25922 indicator strains, having the inhibition halos diameters varying from 17 mm to 22 mm and from 14 mm to 17 mm, respectively. However, the low extracellular pH, produced by acidic metabolites (such as lactate) secreted by LAB, could be responsible for the observed inhibitory effect. To test this hypothesis, the inhibitory activity of LAB against pathogens was evaluated using MRSG-agar buffered at pH 7.0 instead of 6.2, to eliminate the acids effect. The results did not reveal any antagonist activity of the LAB, except for the CM70 strain, which generated halos with smaller diameter, 15 mm and 14 mm against *S. aureus* and *E. coli*, respectively (Table 2S).

The six *Leuconostoc* strains were exposed to conditions resembling the human gastro-intestinal tract. First, the LAB were subjected to the stomach stresses (Fig. 6). Different resistance levels to acidity and bile salts were observed among the various strains. After 3 h exposure to pH 3.0 and 2.0 the survival ranged from 27% to 8% and from 19% to 6% of the initial population. Also, there was observed a resistance to 0.3%, 1.0% or 2.0% bile salts exposure ranging from 29% to 8%, 22% to 5% or 17% to 2%, respectively. In addition, the CM9 strain showed the highest tolerance to acidic and bile salts stresses. As an additional test, the bacterial response to the stomach duodenum-passage was assessed by exposing the bacteria to synthetic duodenal secretions at pH 3.0 for 1 h. Analysis of the bacterial viability after duodenal treatment revealed slight decrease of viability during the incubation time

(Table 1). Moreover, the CM9 strain, as for the other stress treatments, had the highest rate of survival, 33% and 24% after 1 h and 3 h of exposure, respectively.

The hydrophobicity of the cell surface of the bacteria, which is an indicator of their adhesion capability, was tested using xylene. The results presented in Figure 6 showed different levels of hydrophobicity among the six strains, which indicates a poor selectivity of the membrane surfaces for some of them. The highest value (46%) was detected for CM9 and the lowest (13%) for E14.

Moreover, based on their probiotic properties, CM9, CM30 and CM70 were chosen to analyse their ability to colonize the intestine of the gnotobiotic embryos of zebrafish (Fig. 7). The CM9 strain exhibited an average colonization capacity with a value of 16 cfu per larva after 6 h of exposure, compared to the two other strains that showed almost no colonization, with an average of 3 cfu per larva for CM30 and 1 cfu per larva for CM70. After 24 hours, the number of adherent bacteria was reduced to an average value of 2.12 cfu per embryos and 0.33 cfu per larva for CM9 and CM70 strains, respectively, and was undetectable for CM30. Therefore, *in vivo*, the strain CM9 of *L. mesenteroides* showed the best colonization profile of the gut of zebrafish gnotobiotic embryos.

4. Discussion

The form, mode of association, inability to hydrolyse arginine, and heterofermentative metabolism are morphological and biochemical characteristics that distinguish the strains belonging to *Leuconostoc* from other Gram-positive and catalase-negative bacteria (Mozzi, Raya, & Vignolo, 2015), and the six strains studied in this work conform with the definition of this genus. Here, the phenotypic identification of the isolates was conducted according to the results of physiological and biochemical analyses, such as sugar fermentation profiles. The results for the six strains were consistent with the data and identification schemes proposed in several works for *L. mesenteroides* (Björkroth & Holzapfel, 2006; Li et al., 2015 ; Hui & Evranuz, 2016) and sequencing of their 16S rRNA coding gene confirmed this identity. In addition, the use of specific primers for PCR amplification revealed that two out of the six strains were *L. mesenteroides* subsp. *mesenteroides*. These results agree with those of Moschetti et al. (2000) obtained for *Leuconostoc* strains isolated from pizza, field grass, natural whey, mozzarella cheese and sausages.

The most important characteristic for potential starter-fermentation strains is their ability to acidify the fermentation matrix, since the acid production and the accompanying pH decrease

give a specific aroma and extend the lag phase of sensitive organisms including foodborne pathogens (Kostinek et al., 2007). All the strains studied in the current work had a progressive production of lactic acid during a 24 h incubation period, as already reported by Benmechernene et al. (2013) for *L. mesenteroides* species isolated from camel milk. The different acidity profile and coagulation effect observed for the isolates from milks, honey and silage could be due to several factors such as the original habitat, the production and fermentation process and the enzymes involved. It is strongly suggested that dairy leuconostoc is already adapted to this acidic environment, unlike non-dairy leuconostoc. As far as we know, this is the first analysis of acidity carried out with *Leuconostoc* strains isolated from honey. However, several studies report the use of silage as a raw material (Ni, Wang, Cai, & Pang, 2015 ; Wang, Yuan, Dong, Li, & Shao, 2017), which clearly shows the importance of acid production on forage quality.

The proteolytic activity of LAB plays a major role in the growth in milk and contributes significantly to the development of the organoleptic properties of various fermented dairy products (Smid & Kleerebezem, 2014). Our results are consistent with previous works (Idoui and Karam, 2008; Tulini, Hymery, Haertlé, Le Blay, & De Martinis, 2015) indicating that *Leuconostoc* species have low proteolytic activity, which results in limited growth in milk (Alegría, Delgado, Flórez, & Mayo, 2013).

The flavour characteristic of food products may also be due to lipolysis, the incorporation of lipolytic and/or proteolytic enzymes accelerating the formation of aroma (Holland et al., 2005). From the results obtained, it appears that most of the strains have lipolytic activity over lipids from natural and artificial sources. These results are in agreement with those obtained by other authors (Katz, Medina, Gonzalez, & Oliver, 2002), which showed that *L. mesenteroides* species isolated from sheep milk and cheeses could hydrolyse tributyrin.

In order to avoid interference from certain components of complex media, like MRS, in this work, CDM defined medium was used for bacterial growth. We had previously demonstrated that *L. mesenteroides* strains isolated from meat and milk do not produce dextran in the presence of glucose, as the only carbon source (Nácher-Vázquez et al., 2017; Zarour et al., 2017). The production levels obtained under discontinuous growth conditions are consistent with those detected by Notararigo et al., (2013). In addition, this high production of dextran by *L. mesenteroides* strains could be improved by optimizing the culture conditions and it should be noted that, in general, very high values are characteristic of α-glucans.

The analysis of metabolic fluxes performed showed that the CM9 and CM30 strains consumed sucrose and produced dextran with transient accumulation of fructose in the culture

medium as has been described for other *Leuconostoc* and other bacteria producing dextran (Dols, Chraibi, Remaud-Simeon, Lindley, & Monsan, 1997; Santos, Teixeira, & Rodrigues, 2000; Nácher-Vázquez et al., 2017a). However, this is not a general feature for *L. mesenteroides*, since we have not observed this pattern in the RTF10 strain isolated from meat products (Nacher-Vázquez et al., 2017a). The difference between EPS production models is influenced by various factors, including the acidification capacity of the culture medium due to the production of lactic acid by LAB and the optimum pH for the enzymatic activity responsible for the synthesis of the EPS (Rühmkorf et al., 2013). Thus, some strains of *Leuconostoc* (Dols et al., 1997) and *Lactobacillus* (Rühmkorf et al., 2013) have maximum efficacy during the exponential phase of growth. In the case of the CM9 and CM30 strains, the production of EPS seems to be different, as revealed by the calculation of the relative dextran production (the ratio of concentration per biomass estimated from the absorbance values of the culture). CM9 showed a ratio of 0.15 (11 h) and 0.45 (24 h) during the exponential and the stationary phase revealing that the EPS is produced during both phases, whereas the CM30 strain displayed ratios of 0.78 (11 h), 1.01 (13 h) and 1.35 (24 h) correlating with production only during the exponential phases of growth.

CM9, CM30 and the non-EPS-producing CM70 strain were able to consume fructose and produce mannitol, and the beginning of the fructose consumption correlated with the beginning of the production of this polyol, whose levels are low and vary among the three strains. These results are consistent with those detected by several researchers (Patra, Tomar, Rajput, & Singh, 2011; Zahid & Deppenmeier, 2016). Analysis of the pH of the extracellular medium and the production of lactic acid revealed that CM70 had the highest acidifying effect, presumably due to its inability to synthesize dextran, which means that the sucrose would be phosphorylated by the action of a sucrose phosphorylase which would generate glucose-1-phosphate and glucose-6-phosphate, consecutively (Dols, Chraibi, Remaud-Simeon, Lindley, & Monsan, 1997) that would then be converted through glycolysis and by the action of the lactate dehydrogenase into lactic acid. Finally, the efficacy of glucansucrase was estimated for the *in vivo* synthesis of EPS, expressing its concentration in glucose molecules. The results indicated that apparently 50% and 34% of the glucose released by hydrolysis of sucrose had been used for the synthesis of EPS by the CM30 and CM9 strains, respectively.

Being destined for human consumption, the safety of the probiotics is important because their resistance to antibiotics may represent a possible threat. For this reason, in this study,

antibiotics from several groups, with effect on different targets, were tested. Antibiotic resistance in probiotics is not usually a safety issue when mutations or intrinsic mechanisms are responsible for the phenotype. However, the resistance to vancomycin seems to be a common constitutive chromosomal encoded characteristic of *Leuconostoc* strains (Hemme & Foucaud-Scheunemann, 2004), and in fact it was used as a selective agent, for the isolation of the LAB studies in this work. On the contrary, the transmissible resistance to antibiotics, such as the resistance to tetracycline encoded by host range mobilizable plasmids (Teuber, Meile, & Schwarz, 1999) would be a threat, but the studied strains did not bear this resistance.

Moreover, some probiotic strains with intrinsic resistance to antibiotics may be useful for restoring intestinal microbiota after treatment with antibiotics. Among the studied strains, some were resistant to certain antibiotics, and CM70 was the only one resistant to amoxicillin plus clavulanic acid, a combination of a β-lactamase with a β-lactamase inhibitor currently used to combat nosocomial infections.

We also tested the ability of the *Leuconostoc* strains to produce antimicrobial substances evaluating their inhibitory effect against the pathogenic strains *S. aureus* and *E. coli*. As previously commented, this activity is generally due to the acidic environment, competition for substrates or the production of bacteriocidal or bacteriostatic substances (Parente & Ricciardi, 1999). Our results revealed that, apart from CM70, the antagonist action of the studied strains against bacterial pathogens detected in MRS pH 6.2 was abolished by usage of MRS pH 7.0 indicating that the activity was due to the acidic compounds produced. Characterization of a putative production of a bacteriocin by CM70 would require further studies.

It is important to note that the human response to probiotics is difficult to predict only from these tests. Many other factors affect the survival of microorganisms in the upper gastrointestinal tract, such as presence of different enzymes, peristaltic movements, digestion, transit time (from 1 h to 4 h depending on the individual), the diet and other conditions, which may influence the viability of probiotic bacteria (Vizoso Pinto, Franz, Schillinger, & Holzapfel, 2006). However, the *in vitro* and *in vivo* assays performed here provide useful information about the probiotic potential of the studied strains.

The *in vitro* probiotic study showed that strains of *L. mesenteroides* can partially counteract (less than 50%) the stress conditions found in the gastrointestinal tract (acidity, bile stress and stomach-duodenal passage). The decrease in survival rate was presumably due to acid

regulation mechanisms that failed to maintain intracellular pH. This would reduce enzyme activity by damaging certain proteins and DNA (van de Guchte et al., 2002). The number of viable cells fluctuated, but it tended to decrease after 3 h of incubation. Recent studies suggested that some *Leuconostoc* strains are able to grow and survive at these barrier levels (de Paula et al., 2015; Diana, Humberto, & Jorge, 2015; Giles-Gómez et al., 2016).

In addition, the test to evaluate the surface hydrophobicity of bacterial cells with xylene indicated that the CM9 strain is the most adherent (45.6%). Moreover, these strains have a significantly higher adhesion capacity than the probiotic strain *Lb. acidophilus* LA-5, used as a reference in the work of Ng, Koon, Padam, & Chye (2015). According to Giaouris, Chapot-Chartier, & Briandet (2009), *L. lactis* strains with more than 40% affinity for polar solvents are particularly hydrophobic, thus, only the CM9 strain had a hydrophobic surface (Habimana et al., 2007). Recently, we have analysed the ability of the CM30, SM34 and CM9 strains to bind to Caco-2 cells, detecting that, in the presence of glucose, they are able to bind to the enterocytes at similar levels (4.5%-3%) (Zarour et al., 2017). Here, we have used zebrafish embryos to study the capacity of *L. mesenteroides* strains for colonization *in vivo*, since various works support the utility of this model for evaluation of probiotic bacteria, in particular LAB (Nácher-Vázquez et al. 2017a; Wang et al., 2016; Russo et al., 2015). The results revealed that CM9 was the most adhesive among the three strains, as it was detected in a statistical significant higher number in the digestive tract of the embryos 6 h after administration and still detectable together with the other strains at a very low level after 24 h. The lack of permanence of the LAB in the digestive tract is the expected behaviour for probiotic bacteria, which require a daily administration. Since, in addition to these characteristics, *L. mesenteroides* CM9 produces an immunomodulatory dextran with potential anti-inflammatory properties and interesting pseudoplastic rheological properties (Zarour et al., 2017), this strain seems to be a good candidate for its use as a component of functional fermented dairy products.

5. Conclusions

The results of isolation, identification and characterization of strains isolated from dairy and non-dairy habitats permitted the evaluation of dairy *Leuconostoc* for their ability to: (i) produce CO_2, dextran, with potential antiviral and immunomodulatory activity (Nacher-Vazquez et al., 2015; Zarour et al., 2017), the low-calorie compound mannitol used in products for diabetics, as well as lactic acid, which is used mainly for food preservation; (ii)

use citrate, (iii) degrade milk protein and lipid sources and (iv) to resist gastrointestinal stresses. In addition, the probiotic properties of dairy *Leuconostoc*, particularly CM9 isolated from camel milk, suggested that it could be a promising candidate for production of probiotic functional and safe dairy products.

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Legends of figures

Fig. 1. Analysis of bacterial colonies of *L. mesenteroides* CM30 strain by phase contrast optical (A) and transmission electron (B) microscopies

Fig. 2. Acidification kinetics of *L. mesenteroides* CM9 (A) and M67 (B) in skim milk medium supplemented with 0.3% yeast extract. Symbols: ▲, pH and ■, dornic acidity.

Fig. 3. Detection of EPS production by *L. mesenteroides* strains on MSE solid medium. CM9 (A) and CM70 (B) strains.

Fig. 4. Quantification of EPS production by *L. mesenteroides* strains on CDMS liquid medium (A) and ropy appearance of CM30 culture (B). Statistical significances are represented by different letters that mean a $P \leq 0.05$.

Fig. 5. Analysis of central metabolism and EPS production of *L. mesenteroides* strains during planktonic growth. Bacteria were grown in CDMS at 30 ºC. Symbols: ▲, A_{600nm}; ●, Dextran; □, pH; ■, sucrose; ▲, fructose; ♦, glucose; ◊, mannitol and ●, lactic acid.

Fig. 6. Survival of *L. mesenteroides* strains to acidic, biliary salts and gastro-intestinal stresses. Statistical significances are represented by different letters that mean a $p \leq 0.05$.

Fig. 7. Colonization of zebrafish embryos by strains of *L. mesenteroides* in the gastrointestinal tract. (A) Protocol strains prevalence in zebrafish embryos digestive tract. (B) The number of cfu per larva was determined after 6 h (black bars) and 24 h (white bars) post infection. Statistical significances are represented by different letters that mean a $P \leq 0.05$.

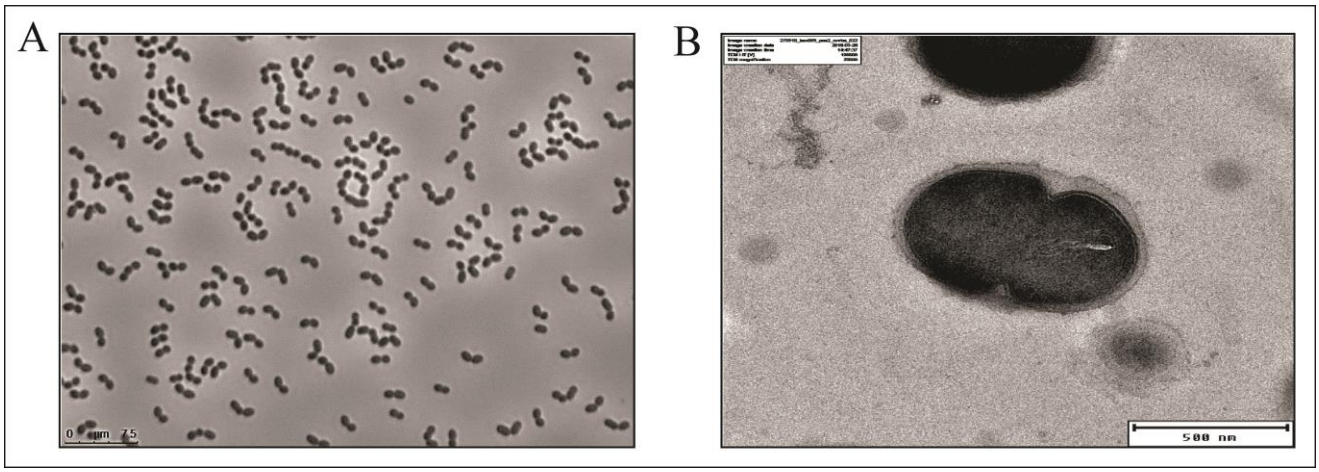

Figure 1

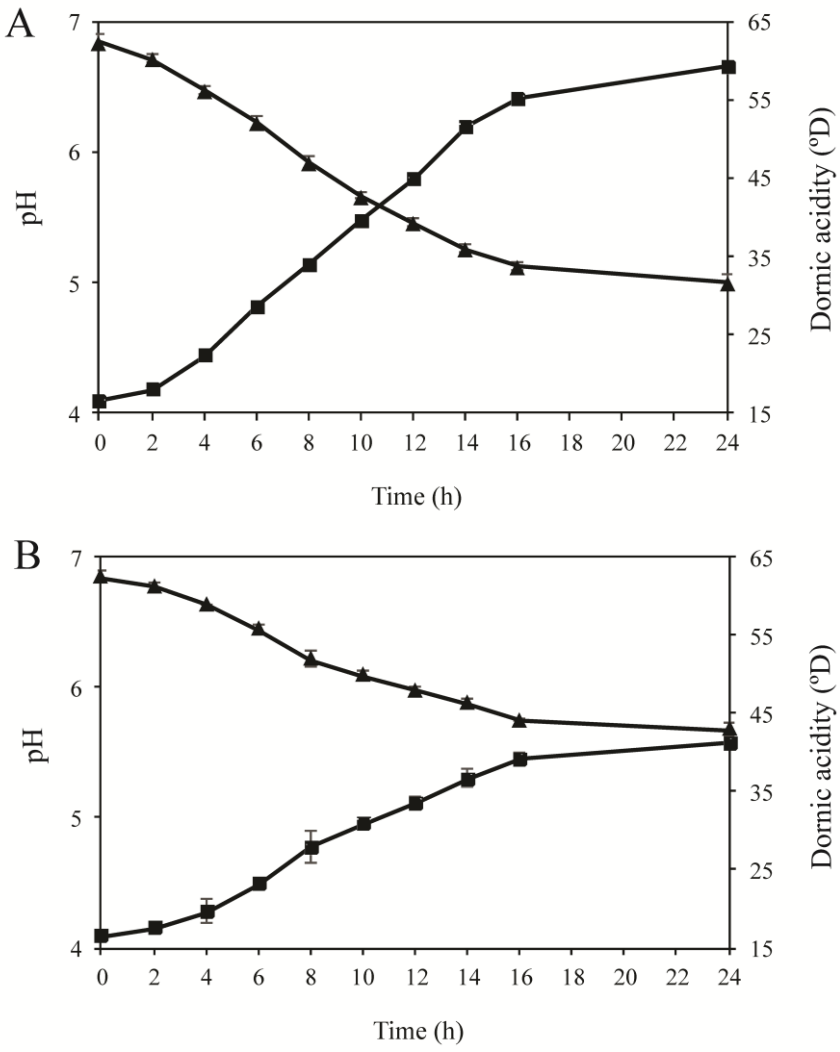

Figure 2

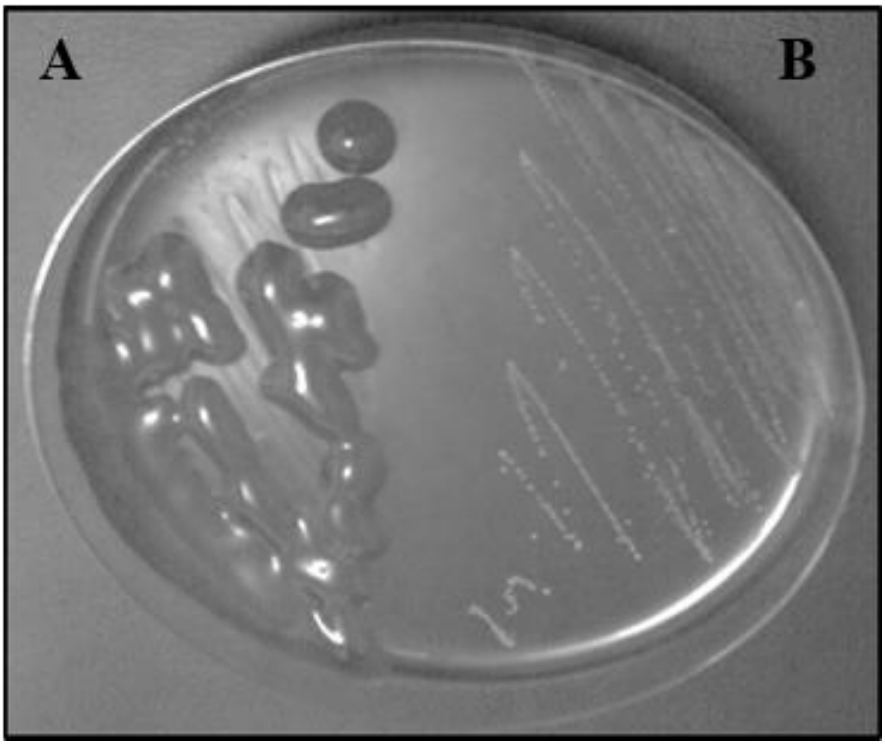

Figure 3

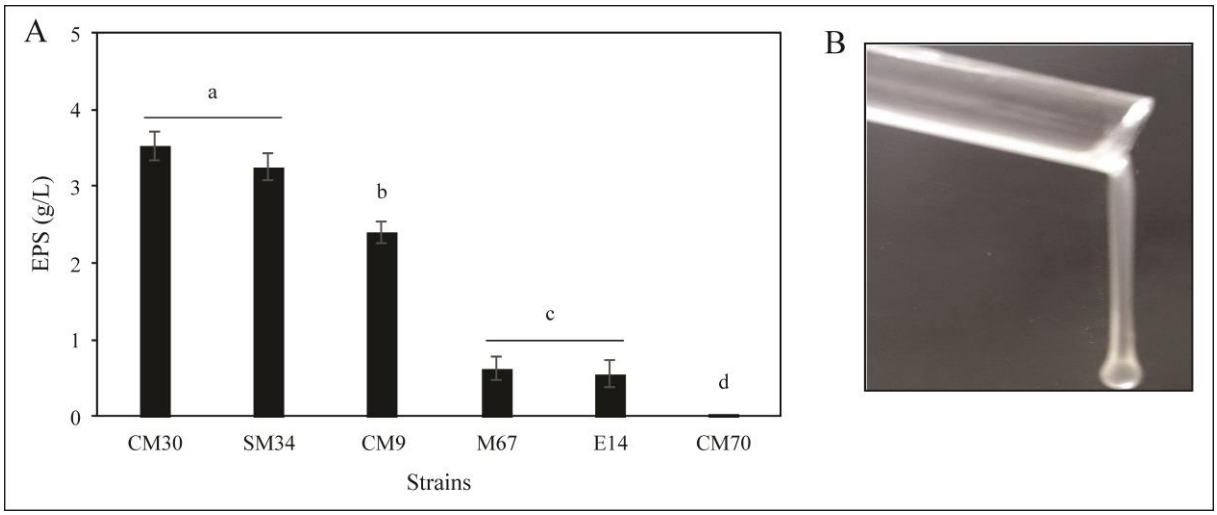

Figure 4

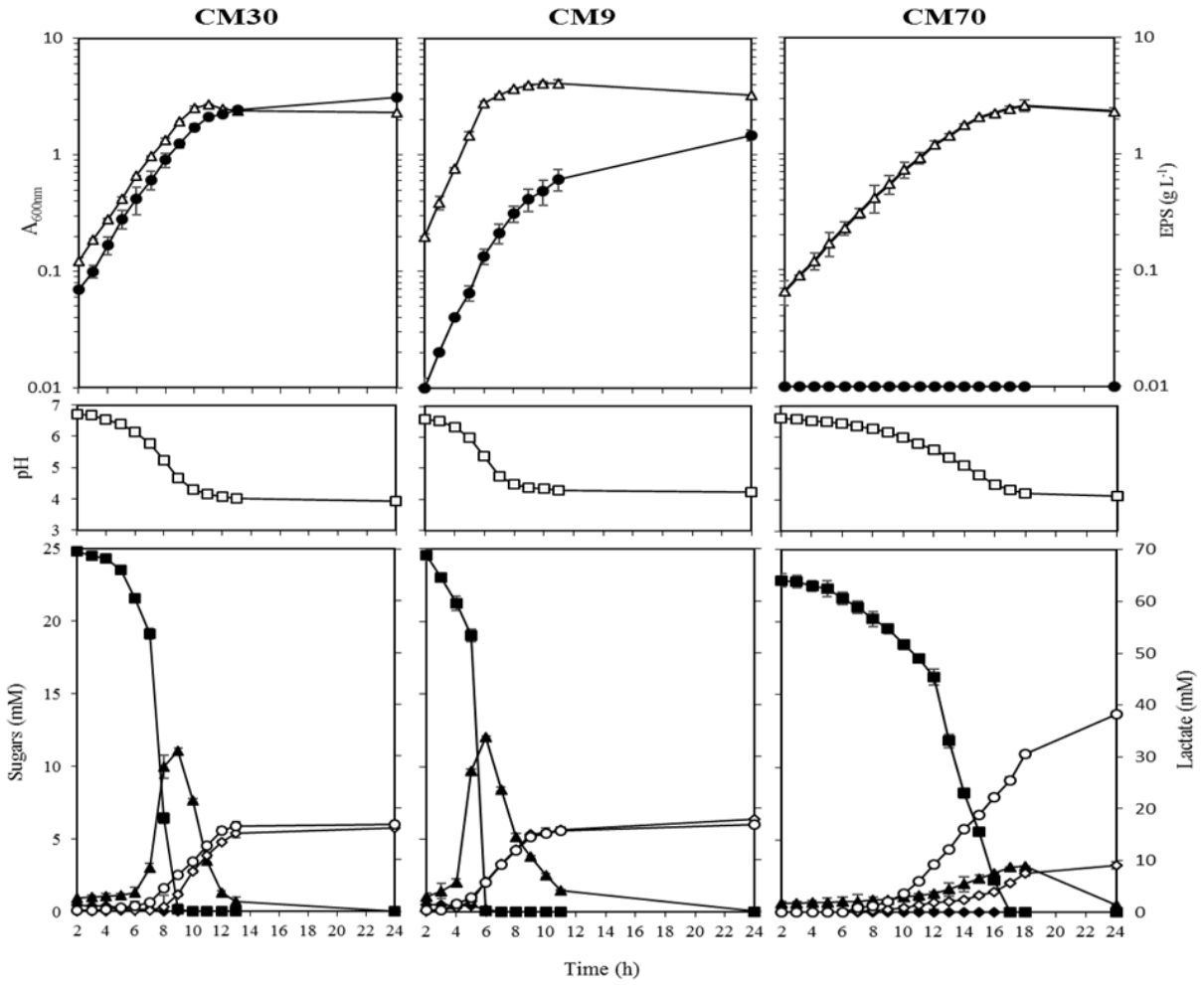

Figure 5

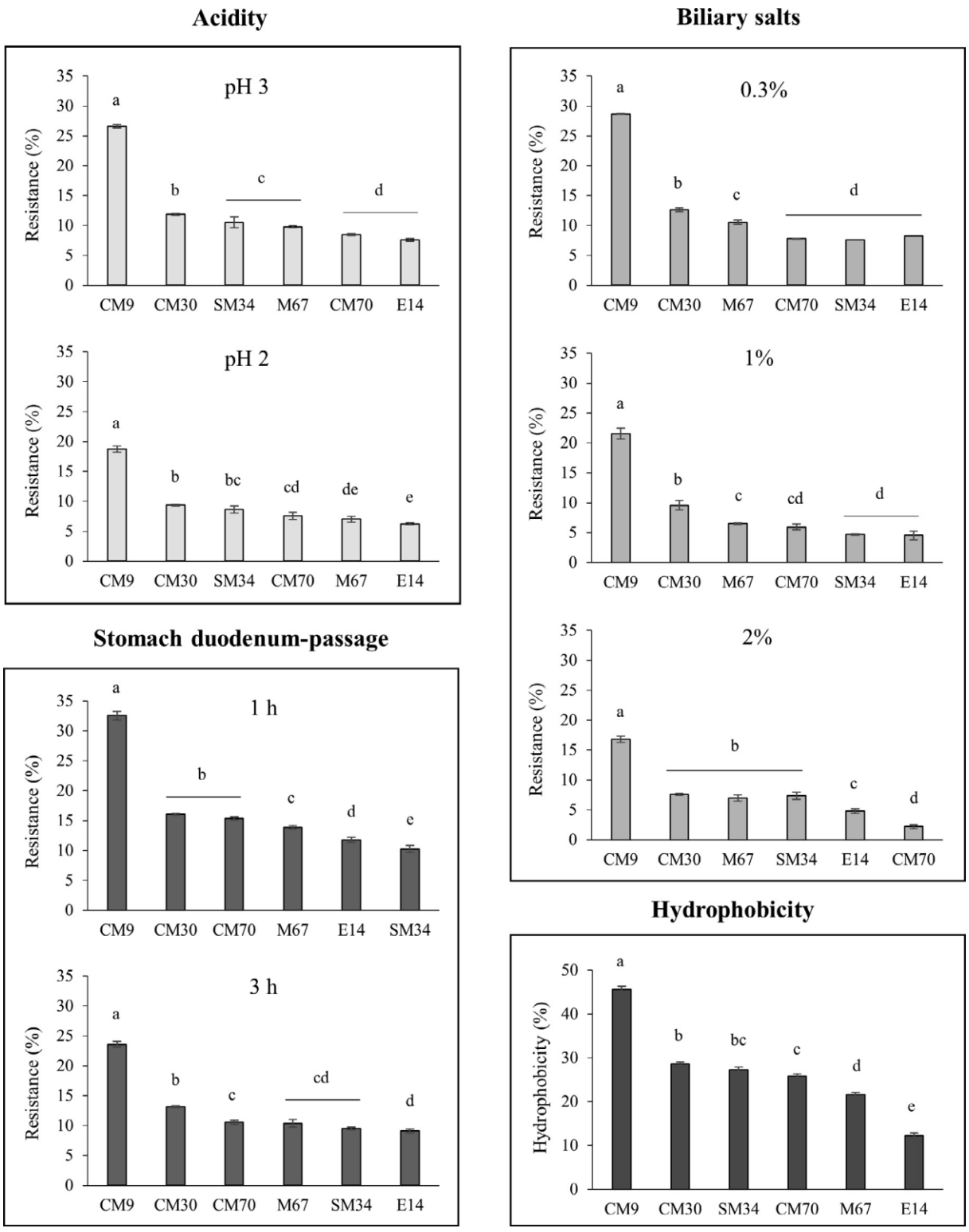
Acidity
pH 3
Resistance (%)
CM9 CM30 SM34 M67 CM70 E14
a b c d
pH 2
Resistance (%)
CM9 CM30 SM34 CM70 M67 E14
a b bc cd de e
Biliary salts
0.3%
Resistance (%)
CM9 CM30 M67 CM70 SM34 E14
a b c d
1%
Resistance (%)
CM9 CM30 M67 CM70 SM34 E14
a b c cd d
2%
Resistance (%)
CM9 CM30 M67 SM34 E14 CM70
a b c d
Stomach duodenum-passage
1 h
Resistance (%)
CM9 CM30 CM70 M67 E14 SM34
a b c d e
3 h
Resistance (%)
CM9 CM30 CM70 M67 SM34 E14
a b c cd d
Hydrophobicity
Hydrophobicity (%)
CM9 CM30 SM34 CM70 M67 E14
a b bc c d e

Figure 6

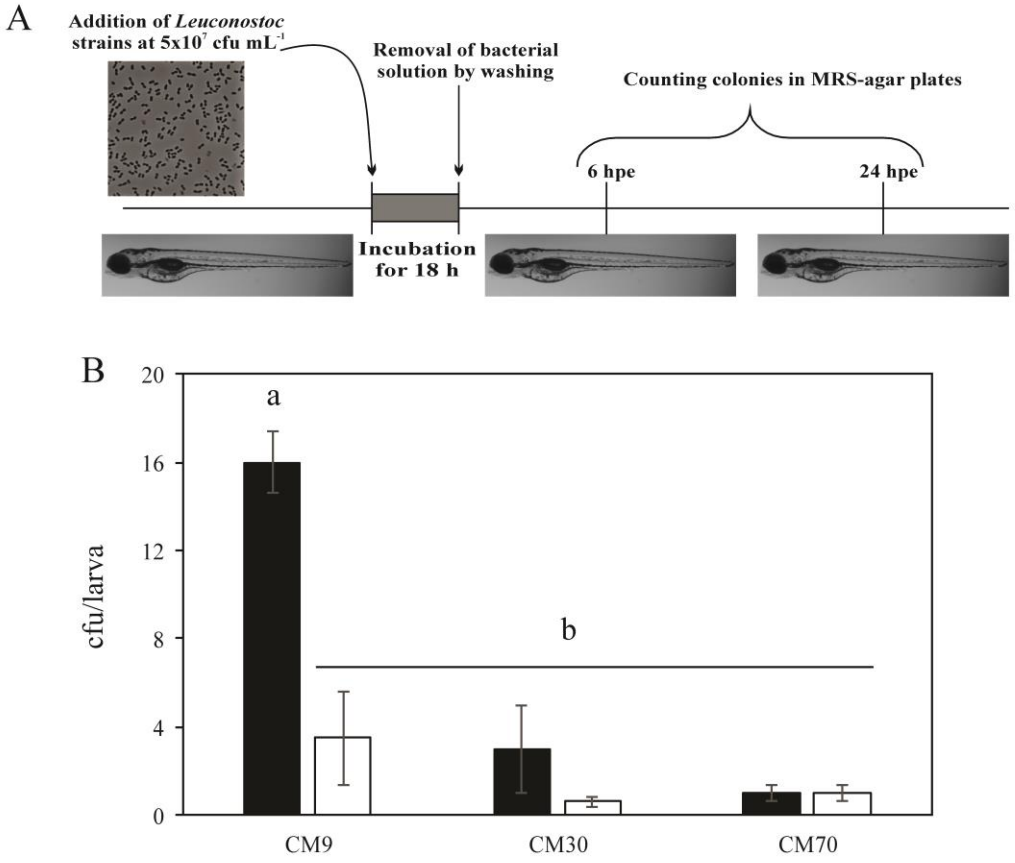
A
Addition of Leuconostoc strains at 5x10^7 cfu mL^-1
Removal of bacterial solution by washing
Counting colonies in MRS-agar plates
6 hpe
24 hpe
Incubation for 18 h
B
cfu/larva
20
16
12
8
4
0
a
b
CM9
CM30
CM70

Figure 7