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Characterization of the heteropolysaccharides produced by *Liquorilactobacillus sicerae* CUPV261 and *Secundilactobacillus collinoides* CUPV237 isolated from cider☆

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ABSTRACT

Some lactic acid bacteria (LAB) strains isolated from alcoholic beverages are able to produce exopolysaccharides (EPS). The present work focuses on the physico-chemical characterization of the heteropolysaccharides (HePS) produced by *Liquorilactobacillus sicerae* CUPV261T (formerly known as *Lactobacillus sicerae*) and *Secundilactobacillus collinoides* CUPV237 (formerly known as *Lactobacillus collinoides*) strains isolated from cider. Genome sequencing and assembly enabled the identification of at least four putative HePS gene clusters in each strain, which correlated with the ability of both strains to secrete EPS. The crude EPS preparation from CUPV261^T contained glucose, galactose and rhamnose, and that of CUPV237 was composed of glucose, galactose and *N*-acetylglucosamine. Both EPS were mixtures of HePS of different composition, with two major soluble components of average molecular weights (M_w) in the range of 10^6 and 10^4 g.mol^{-1}. These HePS were resistant to gastric stress conditions in an *in vitro* model, and they significantly reduced zebrafish larvae mortality in an *in vivo* model of inflammatory bowel disease.

1. Introduction

Some lactic acid bacteria (LAB) produce exopolysaccharides (EPS) that may remain tightly attached to the bacteria, constituting a capsule, or may be released into the environment. EPS are classified as homopolysaccharides (HoPS) or heteropolysaccharides (HePS), depending on whether they are constituted by one or more types of monosaccharides (Werning et al., 2012).

HoPS synthesized by LAB normally contain glucose (glucans) or fructose (fructans), with galactose (polygalactan) being less usual (Torino et al., 2015). The most common HoPS produced by LAB strains isolated from alcoholic beverages is a 2-substituted (1,3)-β-D-glucan (β-D-glucan) (Dueñas-Chasco et al., 1997; Llaubères et al., 1990), synthesized by a single transmembrane glycosyltransferase, the GTF glycosyltransferase, which uses UDP-glucose as donor monosaccharide (Werning et al., 2008; Werning et al., 2006). This polymer is produced by *Pediococcus parvulus* or *Oenococcus oeni* strains in wines (Dols-Lafargue et al., 2008), lactobacilli, *Pediococcus* and *O. oeni* strains in ciders (Dueñas-Chasco et al., 1997; Garai-Ibabe et al., 2010; Ibarburu et al., 2007; Puertas et al., 2018) and lactobacilli in beers (Fraunhofer et al., 2018). The synthesis of α-glucans by *O. oeni* and some other species in wines has also been reported (Dimopoulou and Dols-Lafargue, 2021) and *Liquorilactobacillus mali* (formerly *Lactobacillus mali*) and also *Lentilactobacillus diolivorans* (formerly *Lactobacillus diolivorans*) in ciders (Dueñas-Chasco et al., 1998; Llamas-Arriba et al., 2019b). In addition, some wine *O. oeni* strains produce β-fructans. In the LAB of fermented beverages, α-glucans and β-fructans are mainly synthesized extracellularly from an existing sucrose molecule, which acts as donor of the corresponding monosaccharide by action of a single type of extracellular glycosyl hydrolase (Dimopoulou and Dols-Lafargue, 2021).

HePS from LAB have a great variety of structures. They are composed of a backbone of repeating units with 3–10 monosaccharides,

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derivatives of monosaccharides or substituted monosaccharides (Torino et al., 2015; Zhou et al., 2019). The main components of these polymers are D-glucose, D-galactose and L-rhamnose, and occasionally, they contain amino-sugars (*N*-acetyl-glucosamine, *N*-acetyl-galactosamine), glucuronic acid, glycerol, and also phosphate (Badel et al., 2011). HePS production has been described in lactobacilli, pediococci, streptococci and enterococci strains. In the HePS biosynthetic pathway of lactic acid bacteria (sometimes called the Wzy-dependent pathway), the repeating oligosaccharide unit is synthesized by a consortium of non-processive glycosyltransferases by transfer of monomers to an undecaprenyl phosphate lipid carrier anchored to the membrane (Zeidan et al., 2017). A priming-glycosyltransferase (priming-GTF or p-GTF) catalyzes the first step of the synthesis. Furthermore, a flippase (Wzx) and a polysaccharide polymerase (Wzy) enable translocation and interconnection of the repeating units, respectively. Regulatory enzymes and factors modulate the chain length and the polymer release. The biosynthetic pathways catalyzed by glycosyltransferases lead to lower yields of EPS relative to those catalyzed by glycosyl hydrolases (Dimopoulou and Dols-Lafargue, 2021; Zeidan et al., 2017).

The synthesis of EPS by LAB during the production of fermented foods has shown techno-functional properties (reviewed in (Ryan et al., 2015)). HePS-producing LAB strains have been used in the production of various dairy products, and dextran-producers in cereal-based products because they improve rheology, textural and mouth-feel properties (Torino et al., 2015; Zeidan et al., 2017). In the context of alcoholic beverages, these polymers can contribute to bacterial survival, improve the sensory properties of fermented drinks or, on the contrary, lead to beverage spoilage (Dimopoulou and Dols-Lafargue, 2021; Werning et al., 2006). In addition, *in vitro* experiments performed with human cell lines indicate that some EPS could have *in vivo* immunomodulatory (Zarour et al., 2017), anticancer (Wu et al., 2021), antiviral (Nácher-Vázquez et al., 2015), or prebiotic (Russo et al., 2012) activities. However, non-clinical trials have been performed to prove these beneficial health effects (Angelin and Kavitha, 2020; Oerlemans et al., 2021). EPS from LAB have also promising clinical and pharmaceutical applications such as their use as: (i) intelligent drug delivery systems, (ii) interpenetrating polymer networks and (iii) anticancer drug-targeting (reviewed in (Daba et al., 2021)).

Our group has deeply investigated the genetics, biosynthesis pathway and potential applications of the β-glucan synthesized by LAB isolated from cider, but little is known about the HePS produced by LAB from this beverage. In previous studies, we described EPS production by lactobacilli strains isolated from ropy cider, and the results suggested that most of the strains belonging to *S. collinoides* and *L. sicerae* synthesize HePS (Puertas et al., 2018). This work reports on the physicochemical characterization of the HePS produced by the *S. collinoides* CUPV237 and *L. sicerae* CUPV261 strains, and the identification of potential EPS gene clusters in their genomes. A preliminary characterization of the biotechnological potential of those HePS is also described.

2. Material and methods

2.1. Bacterial strains and culture conditions

The EPS-producing *L. sicerae* CUPV261^T = CECT 8227^T (formerly *Lactobacillus sicerae*) and *S. collinoides* CUPV237 (formerly *Lactobacillus collinoides*) strains were isolated from ropy cider of the Basque Country (Spain) (Puertas et al., 2018; Puertas et al., 2014). The bacteria were routinely cultured at 28 °C in de Man, Rogosa and Sharpe (MRS) medium, pH 5.5 (De Man et al., 1960), under an atmosphere containing 5 % CO_2.

The isogenic, non-ropy strain *S. collinoides* CUPV237NR was generated in this work by mutagenesis with novobiocin (Sigma- Aldrich). The ropy CUPV237 strain was grown in MRS broth with novobiocin (10, 20, 40, 50 μg/mL). After incubation at 28 °C for 48 h, cultures that survived at concentrations above 40 μg/mL were diluted, plated on MRS agar and incubated at 28 °C for 6 days. Individual colonies were scored for ropiness by picking them with an inoculation loop. Non-ropy colonies, which showed a different colony phenotype on MRS agar (Fig. S1), were subcultured in liquid medium several times until we confirmed that the non-ropy character remained stable. Stocks were stored at −80 °C in growth liquid medium containing 20 % (*v*/v) glycerol. For EPS production, a semi-defined medium (SMD; pH 5.5) without peptone, yeast extract and meat extract was used (Dueñas-Chasco et al., 1997) with the aim of avoiding the contamination with polysaccharides present in the MRS medium (Puertas et al., 2018). The SMD contained 20 g/L glucose, 5 g/L Casamino acids, 5 g /L sodium acetate, 6.7 g /L Difco™ Yeast Nitrogen Base, 2 g/L K_2HPO_4, 0.05 g/ L $MnSO_4{\cdot}4H_2O$, 2 g/L diammonium citrate, 1 mL/L Tween 80, and 0.005 g/L adenine, uracil, xanthine and guanine, each.

2.2. Genome sequencing, assembly and identification of EPS gene clusters

The genome sequence of *S. collinoides* CUPV237 was previously described by us and deposited at Genbank under the accession number JYDC00000000 (BioProject reference PRJNA274884; BioSample reference SAMN03333292) (Puertas et al., 2016). *L. sicerae* CUPV261^T was cultured as described above and DNA was extracted following the guidelines of the NucleoSpin tissue DNA extraction kit (Macherey-Nagel, Düren, Germany). After quality and quantity validation by agarose electrophoresis and fluorometry (Qubit, Thermofisher), the DNA sample was sequenced with a 30× coverage at MicrobesNG (Birmingham, UK). Assembly was carried out using SPAdes (Bankevich et al., 2012) and the genome was annotated using both RAST (Aziz et al., 2008; Overbeek et al., 2014) and Prokka (Seemann, 2014). This whole genome shotgun project was deposited at Genbank under the accession number JAHAVQ000000000 (BioProject PRJNA728857; BioSample SAMN19104997).

For the identification of potential EPS gene clusters in the genomes of *L. sicerae* CUPV261^T and *S. collinoides* CUPV237, amino acid sequences of publicly available priming glycosyltransferases or flippases belonging to EPS clusters were used as queries in BLAST analyses against the RAST annotations of both strains. Once identified, adjacent genes were analyzed to determine if the region showed the typical architecture of an EPS gene cluster (Zeidan et al., 2017). The sequences of putative EPS cluster genes and proteins were Blasted in the NCBI database and analyzed in Interpro (Mitchell et al., 2019) to predict their function and determine the conserved domains. The number and coordinates of transmembrane helices (TMH), as well as their orientation in the plasmatic membrane were predicted using TopCons (Tsirigos et al., 2015), TMpred (https://embnet.vital-it.ch/software/TMPRED_form.html) and SACS MEMSAT 2 (Jones et al., 1994). Sequence alignments were performed using ClustalW (Sievers et al., 2011) and TM-Coffee (Notredame et al., 2000). Finally, the presence of probable transcription terminators in mRNAs was detected using Mfold (Zuker, 2003).

2.3. Detection of EPS production at cellular level by transmission electron microscopy

Transmission electron microscopy (TEM) was used to detect EPS location. Samples were processed as described by Gangoiti and colleagues (Gangoiti et al., 2017). Briefly, 10 μL of a 48-h culture in MRS broth were placed on a carbon film copper grid, previously hydrophilized by a glow discharge process. After 1 min, each grid was blotted briefly with filter paper, negatively stained for 30 s with uranyl acetate (2 %) and then blotted quickly and air-dried. Samples were examined at the Microscopy Service of the University of Basque Country (UPV/EHU), using a TECNAI G2 20 TWIN (FEI, OR, USA) microscope operating at an accelerating voltage of 200 kV in a bright-field image mode.

2.4. EPS isolation and quantification

For EPS production, batch fermentations without pH control were carried out in screw-cap flasks fully filled with SMD broth, during 65 h at 28 °C under an atmosphere containing 5 % CO_2. Cells harvested in MRS (pH 5.5) broth at middle exponential phase of growth were washed twice and resuspended in Ringer® solution to prepare the inocula. The flasks were inoculated to give an initial optical density at 600 nm (OD_{600}) of 0.2. After incubation, the cultures were centrifuged at 15,680 ×*g* and 20 °C to remove the cells. The crude EPS from supernatants were isolated as described by Ibarburu and coworkers (Ibarburu et al., 2015). Briefly, EPS were recovered by precipitation with three volumes of cold absolute ethanol, washed three times with 70 % (v/v) ethanol, dialyzed, and lyophilized. The concentration of EPS in the culture supernatant was estimated as neutral carbohydrate content determined by the phenol-sulfuric acid method (DuBois et al., 1956), using glucose as standard.

The EPS used in the *in vivo* zebrafish model were purified as previously described (Llamas-Arriba et al., 2019a). Contaminant DNA, RNA and protein contents were determined in EPS suspensions at 1 mg/mL using specific fluorescent staining kits and the Qubit®2.0 fluorometric detection methods (ThermoFisher Scientific). The crude EPS precipitates contained residual amounts of DNA (0.03 %), RNA (0.07–0.14 %) and protein (6.51–7.38 %) and, after enzymatic elimination of nucleic acids and deproteinization, these values were reduced to below the limit of quantification (<0.01 %, <0.02 % and <1 %, respectively).

2.5. EPS characterization

Weight–average molecular weight (Mw) and polydispersity of EPS were measured by high-performance size exclusion chromatography (HP-SEC) in an Agilent 1100 series device using Chemstation GPC data analysis software (Agilent Technology, Waldbronn, Germany), as in Ibarburu et al. (2015). To characterize the type of EPS isolated from the two strains, neutral sugar composition and linkage types were determined as previously described (Notararigo et al., 2013). Neutral sugars were identified and quantified by gas chromatography, after hydrolysis of polysaccharide samples with 3 M trifluoroacetic acid (TFA) for 90 min and derivatization to alditol acetates. Prior to the methylation analysis, crude EPS were also separated into two fractions using an Amicon® ultrafiltration system (50 kDa cut-off, Merck Millipore, Darmstadt, Germany). Samples were dissolved in NaOH (0.2 M) and centrifuged at 15,680 x*g* for 15 min. The supernatants were filtered following the manufacturer's specifications to obtain two fractions, which were dialyzed using 12–14 kDa cut-off membranes (Medicell International Ltd., London, UK) and lyophilized. To determine linkage types, the polysaccharides were methylated according to Ciucanu and Kerek (Ciucanu and Kerek, 1984), hydrolyzed with TFA (3 M) for 1 h at 120 °C, converted into partially methylated alditol acetates using sodium borodeuteride as the reducing agent and analyzed by gas-chromatography/mass spectrometry. The linkages in the polysaccharides were deduced from the mass spectra and retention time of the peaks, and their relative amount from the area under each peak.

Finally, crude EPS were analyzed by one-dimensional nuclear magnetic resonance (^{1}D, ^{1}H NMR). Samples were weighed (*ca.* 1 mg), dissolved 1:1 (w/v) in 99 % D_2O and their spectra were recorded at 333° K using a Bruker Avance NEO spectrometer (Bruker, Bremen, Germany) operating at 500.13 MHz (^{1}H). Chemical shifts are given in parts per million using the HDO signal (4.4 ppm) as reference.

2.6. EPS digestibility under simulated gastric-stress conditions

An *in vitro* model was used to simulate mouth and stomach environments, following the procedure described by Fernández de Palencia et al. (2009). Briefly, a 0.75 mg/mL solution of each EPS was prepared in a sterile electrolyte (6.2 g/L NaCl, 2.2 g/L KCl, 0.22 g/L $CaCl_2$, 1.2 g/L $NaHCO_3$). An aliquot of 2 mL was withdrawn as a control, exposing the remaining EPS solution to 0.01 % lysozyme and 0.3 % pepsin (Sigma-Aldrich). Gastric-stress (G) conditions were simulated by decreasing the pH first to 4.1. HCl (1 M) was added with that aim, and the solution was incubated at 37 °C for 20 min with shaking (150 rpm). The treatment was then repeated after sequentially decreasing the pH to 3.0 and then to 2.0. Finally, an aliquot of 2 mL was withdrawn as the G sample after incubation at pH 2.0. Control and G samples were centrifuged (15,680 ×*g*) for 10 min, and the EPS present in the supernatants were precipitated with ethanol (1:3, v/v), dialyzed (cut-off 3.5 kDa), lyophilized and analyzed by HP-SEC as described above. The tolerance of EPS to G was evaluated by comparing the fractions with an average molecular weight > 2000 kDa (high) and of approximately 25 kDa (intermediate) in control and in G samples. The results are given as the average recovery of two independent cultures, according to the following expression:

$$Recovery\ (\%) = (peak\ area\ of\ EPS\ fraction\ in\ G\ sample / peak\ area\ of\ EPS\ fraction\ in\ control) \times 100.$$

2.7. Caco-2 cell culture and adhesion assays

Caco-2 human enterocyte cell line samples were obtained from the cell bank at the Margarita Salas Biological Research Centre. Cells were seeded in 96-well culture plates (Falcon Microtest™; Corning, AZ, USA) at a final concentration of 1.25×10^5 cells/mL, growing as monolayers of differentiated and polarized cells, as previously described (Nácher-Vázquez et al., 2017b). Cell concentrations were determined in a counting chamber as previously described (Garai-Ibabe et al., 2010). The bacterial strains used were CUPV261^T, CUPV237 and its isogenic non-ropy strain CUPV237NR, and the probiotic *Lactobacillus acidophilus* LA-5 (Chr. Hansen A/S, Hørsholm, Denmark). For adhesion assays, the strains were first cultured in MRS medium to the middle of the exponential growth phase. Cultures were diluted to a final volume of 1 mL of DMEM (Dulbecco's Modified Eagle Medium; Gibco), supplemented with 0.5 % glucose to give 1.25×10^6 colony-forming units (cfu)/mL, and then were added to Caco-2 cells in a ratio of 10 LAB cells per Caco-2 cell and in a final volume of 0.1 mL per well. Samples were incubated for 1 h at 37 °C in a 5 % CO_2 atmosphere and unattached bacteria were removed by washing twice with 0.2 mL of phosphate buffered saline (PBS) solution at pH 7.2. Caco-2 cells were then detached from the plastic surface by addition of 0.1 mL of 0.05 % trypsin–EDTA per well, and incubation for 5 min at 37 °C. This reaction was stopped by adding 0.1 mL of PBS pH 7.2. The number of LAB cells associated to Caco-2 cells was determined by plating appropriate dilutions onto plates containing MRS medium. All assays were carried out in triplicate, with two biological replicates in each. Data were analyzed as a randomized complete block design, with independent experiments performed on different days as blocks. A two-way analysis of variance without interactions was performed. When differences were significant (p value ≤0.05), mean pairwise comparisons were computed with a Tukey's test. All analyses were done with the R software version 3.6.3 (R Core Team, 2020).

2.8. Protective effect of EPS in a dextran sodium sulfate (DSS)-induced enterocolitis model of zebrafish larvae

Zebrafish embryos were obtained from wild-type adult zebrafish (*Danio rerio*, Hamilton 1822), bred and maintained in the AZTI Zebrafish Facility (REGA number ES489010006105; Derio, Spain) as previously described (Russo et al., 2015) and following standard conditions (Sullivan and Kim, 2008). All experimental procedures were approved by the Regional Animal-Welfare Body. Embryos were cleaned with embryo water (EW: 294 mg/mL $CaCl_2$, 123.3 mg/mL $MgSO_4 \bullet 7H_2O$, 63 mg/mL $NaHCO_3$, and 5.5 mg/mL KCl), and maintained at 27 °C in EW supplemented with 0.01 % (*w/v*) methylene blue. EW medium used in the assays did not contain methylene blue.

Induction of enterocolitis by dextran sodium sulfate (DSS) exposure,

and EPS treatments, were carried out as previously described (Llamas-Arriba et al., 2019a). Pools of 20–30 embryos of 1-day post fertilization (dpf) were distributed in Petri dishes containing EW supplemented with the corresponding EPS concentrations (50, 100 and 150 μg/mL), and incubated at 27 °C. Co-treatment with the EPS and 0.8 % (*w/v*) of DSS (Mw 6500–10,000 Da, Across Organics) extended from 4 dpf to 7 dpf. Treatments were replaced daily, and the dead larvae were counted at 5, 6 and 7 dpf. Plates in which only DSS was added were used as positive controls of mortality. Tests were carried out in triplicate in two independent experiments. The statistical software SPSS-PC 24.0 (SPSS Inc., IBM, NY, USA) was used. Data were subjected to a one-way ANOVA (analysis of variance) followed by a *post hoc* Dunnett's T3 test.

3. Results and discussion

3.1. EPS production by CUPV261^T and CUPV237 strains in semi-defined medium

Fermentation of apple must is a complex microbiological process driven by yeasts, LAB and acetic bacteria (Cousin et al., 2017). Cider produced in the Basque Country is not stabilized and one of the consequences is that LAB are the predominant microbiota at the end of the production process (Puertas et al., 2018). The *L. sicerae* CUPV261^T and *S. collinoides* CUPV237 strains were isolated from spoiled ropy basque cider and selected for the analysis of their EPS as well as the genes potentially controlling the synthesis of those EPS. Both strains showed a characteristic ropy phenotype in MRS broth (Fig. 1A) (Puertas et al., 2018), and TEM analysis of the bacterial cultures revealed the presence of subpopulations of LAB cells with EPS attached to their surface (Fig. 1B). CUPV261^T and CUPV237 yielded, respectively, 57.11 ± 7.45 and 30.26 ± 6.57 mg/L of EPS in the semi-defined medium (n = 2 for each strain), as determined by the phenol-sulfuric method in cultures fermented for 48 h (OD_{600} = 0.893 ± 0.071 and 3.022 ± 0.096, respectively). These levels were similar to those previously reported for other HePS produced by LAB strains isolated from cider, wine and other food matrices (Dimopoulou et al., 2016; Ibarburu et al., 2015; Puertas et al., 2018). The strain CUPV237NR, a non-ropy mutant of CUPV237 obtained by treatment with novobiocin (Section 2.1), produced a considerably lower amount of EPS (8.57 ± 0.03 mg/L). This strain was used for adhesion assays to Caco-2 cells (see Section 3.6).

3.2. Potential EPS gene clusters in the genomes of CUPV261^T and CUPV237

Genomes of strains CUPV261^T and CUPV237 were sequenced, assembled and annotated (see Section 2.2 in Materials and Methods). Assemblies with SPAdes differentiated 419 *contigs* in the case of CUPV261^T and 127 in the case of CUPV237, 156 and 116 after excluding those shorter than 500 nucleotides. These results suggested that, mainly in the case of *L. sicerae*, there is a fraction of the genome (104.41 kb out of 2428.87 kb of the assembly; 4.3 %) that will not be analyzed in this work for the identification of potential EPS clusters.

In a preliminary search for genes potentially involved in the synthesis of EPS, 39 (CUPV237) and 50 (CUPV261^T) clustered open reading frames were identified, respectively, distributed in at least four potential EPS clusters in the genomes of each strain (Fig. 2 and Table S1). Genes coding for proteins predictably involved in regulatory roles, the synthesis of the repeating unit of the EPS, export or polymerization were identified in clusters *CUPV237-1*, *CUPV237-2* and *CUPV237-3*, while cluster *CUPV237-4* may be involved in the formation of dNTP-sugars (Fig. 2A; Table S1; for a reference on the typical structure of HePS clusters of LAB strains, see Zeidan et al., 2017). For brevity of the main text, the nomenclature used to name each EPS-cluster gene, the length of the corresponding proteins, their function, the number of potential TMH-s, their characteristic functional domain, the NCBI entries with the highest conservation (lowest E value) and, finally, the percentage of

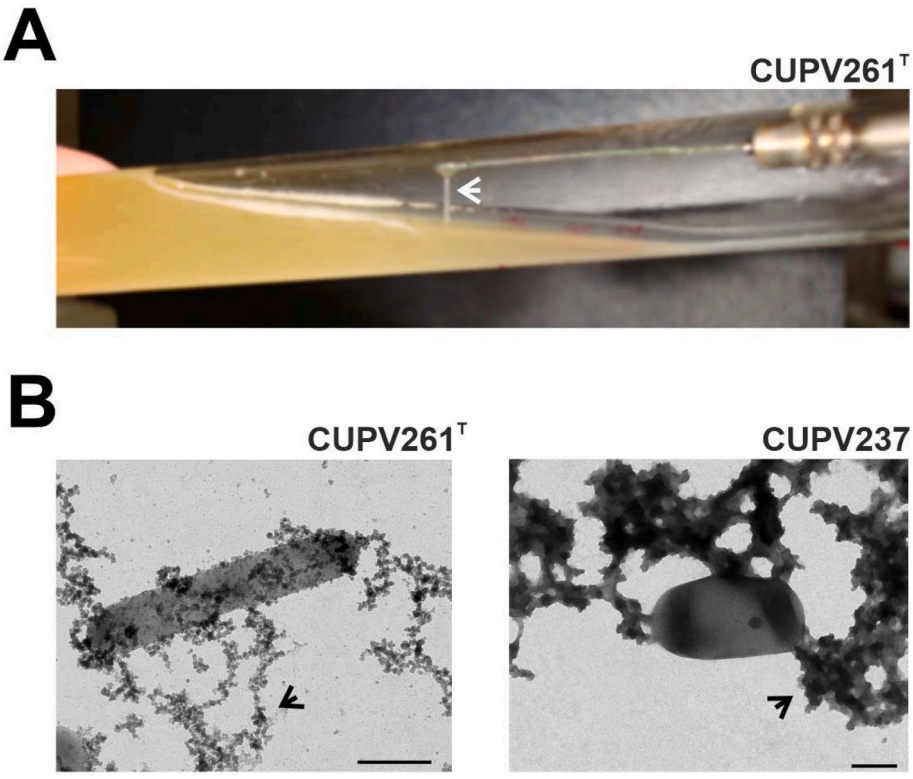

Fig. 1. Visualization of lactic bacteria and their EPS by TEM. A) Example of a LAB culture (CUPV261^T) after 48 h at 28 °C in MRS broth (pH 5.5) under an atmosphere containing 5 % CO_2. The white arrow indicates the ropy phenotype. B) TEM micrographs of *L. sicerae* CUPV261^T (left) and *S. collinoides* CUPV237 (right) in liquid culture. Black arrows indicate EPS produced by each strain. Scale bar = 500 nm.

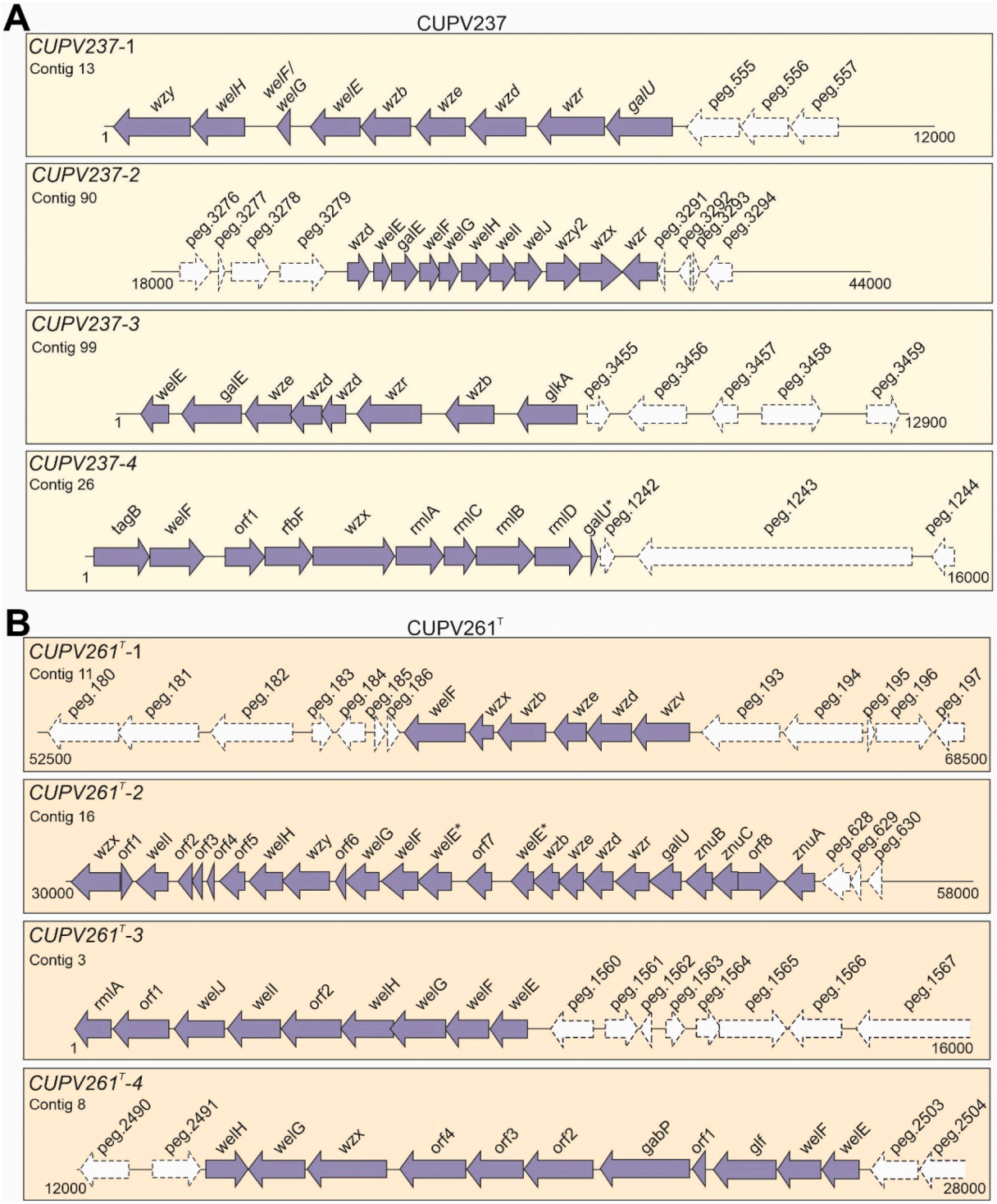

Fig. 2. Potential EPS gene clusters identified in the genomes of strains (A) CUPV237 and (B) CUPV261^T. See also in Table S1 the nomenclature used to name each EPS-cluster gene, the length of the corresponding proteins, their predicted function, the number of potential TMH-s, their characteristic functional domain, the NCBI entries with the highest conservation (lowest E value) and, finally, the percentage of identity with those hits. As a reference of the typical structure of HePS gene clusters of LAB strains, see (Zeidan et al., 2017). In white, adjacent genes predictably not belonging to the EPS gene clusters.

identity with those hits are included in Table S1.

The genome of strain CUPV261^T includes a potential EPS cluster (*CUPV261-2*) with an organization similar to that of cluster *CUPV237-1*, with the difference that additional genes, such as those coding for a putative flippase, an EPS phosphotransferase, an undecaprenyl-phosphate galactose phosphotransferase and a tyrosine protein kinase, would be added to the structure (together with several transposases; Fig. 2B; Table S1). Thus, cluster *CUPV261^T-2* would include genes encoding the same functions as those of cluster *CUPV237-1* and it would provide additional ones characteristic of HePS gene clusters.

Our results agree with those reported for lactobacilli and *O. oeni* strains, in which up to four EPS clusters were reported (Deo et al., 2019; Dimopoulou et al., 2016; Remus et al., 2012; Zivkovic et al., 2015). It is unknown if the potential EPS gene clusters described here are expressed

and functional, or under which culture conditions will play a role, but their presence in the genomes of strains CUPV261^T and CUPV237 correlates with the production by each strain of EPS fractions of different molecular mass (see next section). In addition, it is feasible that the expression of the EPS gene clusters and the activities of their products are coordinated in order to optimize bacterial adaptation to various environmental conditions. This idea is reinforced by the fact that some gene sequences within these clusters are incomplete or have an altered reading frame (asterisks in Table S1), and would, consequently, require equivalent functions from other EPS gene clusters or genomic regions, as previously suggested (Deo et al., 2019). In the future, sequencing of the non-ropy mutant of CUPV237, strain CUPV237NR, may contribute to the determination of which of the potential EPS clusters identified here could be necessary for EPS synthesis. However, the mutation(s) causing the non-ropy phenotype of this strain could be located out of these potential EPS clusters.

3.3. EPS characterization

To determine the molecular weight of the EPS produced by CUPV261^T and CUPV237, crude EPS samples isolated from the bacterial culture supernatants were analyzed by HP-SEC. The analysis revealed the presence of three peaks of different molecular weight in each crude sample (Table 1 and Fig. 3). Previously, we also found three fractions in the EPS recovered from cellular biomass harvested from agar-MRS plates (surface EPS) (Puertas et al., 2018). However, in the case of EPS from the supernatants (Table 1), the fraction with the highest Mw was larger than 2000 kg/mol (out of calibration). The intermediate and the low Mw-polymer exhibited similar average molecular weight compared to the surface EPS, around 10^4 g/mol and 10^3 g/mol, respectively, with the second fraction being the most abundant (b in Fig. 3). The simultaneous production of EPS with different molecular weights and composition is a common feature of LAB strains isolated from cider and other fermented foods (Garai-Ibabe et al., 2010; Ibarburu et al., 2015; Mozzi et al., 2009), as occurs with the strains characterized in this work. Furthermore, it has been described that the quantity of each EPS fraction is altered depending on the ratio of carbon and nitrogen sources in the culture medium (De Vuyst and Degeest, 1999), the main carbohydrate available (Ibarburu et al., 2015), or the temperature (Bengoa et al., 2018).

The ^{1}H NMR spectra of the crude EPS of both CUPV261^T and CUPV237 strains were different, with several signals in the anomeric region (between 4.5 and 5.6 ppm) which give an idea of the complexity of the samples, more evident in CUPV261^T, and support the presence of both α- and β-anomers (Fig. 4). Moreover, a signal around 2 ppm, characteristic of acetyl groups, was also detected in both samples, which reinforces the possibility that they contain different proportions of *N*-acetylated amino sugars.

HePS synthesis by LAB strains isolated from cider and wine has been reported. We previously found that *O. oeni* and *P. ethanolidurans* strains produce mostly a 2-substituted (1,3)-β-D-glucan together with a small quantity of HePS. *O. oeni* I4 synthesized two HePS, constituted by galactose and glucose, and one of them also contained rhamnose (Ibarburu et al., 2007). The EPS from *P. ethanolidurans* CUPV141 was composed of glucose, galactose, glucosamine, and glycerol-3-phosphate (Llamas-Arriba et al., 2018). In addition, two *Paucilactobacillus suebicus* strains synthesized a mixture of complex HePS, one of them acidic, containing glucose, galactose, and phosphate, and other composed of glucose and *N*-acetylglucosamine (Ibarburu et al., 2015).

For a more detailed characterization of the crude EPS produced by strains CUPV261^T and CUPV237, their monosaccharide composition was determined after acid hydrolysis, derivatization to alditol acetates and gas chromatography analysis. The chromatograms revealed the presence of glucose, galactose, rhamnose and small amounts of glucosamine in the case of *L. sicerae* CUPV261^T, and glucose, galactose and glucosamine in the case of *S. collinoides* CUPV237. The crude samples were also subjected to methylation analysis to determine the type and percentages of the *O*-glycosidic linkages (Tables 2 and 3). The results confirmed the complexity of the samples, with 13 different residues identified in CUPV261^T and 11 in CUPV237. This is in good agreement with the HP-SEC data, which indicated the presence of two major soluble components in the range of 10^6 and 10^4 Da in both crude EPS (Table 1 and Fig. 3). Thus, taking advantage of the wide Mw difference between these two main components, we tried to separate them by centrifugation in tubes with a 50 kDa-cutoff Amicon® membrane. To induce solubilization, the crude EPS samples of both strains were first treated with a 0.2 M NaOH solution. The CUPV237 crude EPS was completely dissolved, while 41 % of the CUPV261^T sample remained insoluble. Filtration of the soluble material allowed recovering the >50 kDa (retentate) and < 50 kDa (filtrate) fractions, which were subjected to methylation analysis together with the insoluble material isolated from CUPV261^T, (Tables 2 and 3).

The linkage types deduced for the three fractions from *L. sicerae* CUPV261^T are gathered in Table 2. The insoluble material was mainly composed of galactopyranose and glucopyranose (around 2:1 proportion), without any representation of the rhamnose and galactofuranose units detected in the crude EPS sample. The galactose residues were mostly 1,3-linked (34 %), and the detection of similar amounts of 3,6-substituted and terminal Gal*p* units (15 % and 12 %, respectively) indicated a branched structure. Glucose is found as 1,3- and 1,4-linked residues, and it remains to be determined whether glucose and galactose are part of one or several insoluble polymers. On the other hand, the two soluble fractions represented 14 % (>50 kDa) and 44 % (<50 kDa) of the crude EPS and were very similar to each other, although clearly different from the insoluble material. They were both rich in galactofuranose 1,3- (~19 %) and 1,6-linked (6.8–10.3 %), galactopyranose 1,3-linked, and had 1,2- and 1,3-linked rhamnose, with the last residue amounting to 18 % in the <50 kDa fraction. The identification of 2,3-substituted galactose residues (16.6–20.4 %) points to a branched structure and is compatible with 1,3-linked galactose located in the main backbone and branched at positions *O*-2. However, with the available data it was impossible to ascertain if these galactose branching points corresponded to pyranose or furanose rings, since both give the same product (1,2,3-*O*-triacetyl-4,5,6-*O*-methyl galactose) after chemical derivatization in methylation analysis. Low quantities of 1,4-linked glucosamine and galactosamine were also detected.

Table 1
Molecular weight (Mw) and polydispersity index (PDI) values of each EPS fraction in crude EPS and EPS samples autoclaved at 120 °C (20 min).

Strain	Crude EPS				Crude EPS after the autoclaving process		
	Fraction	Mw (g/mol)	PDI	Ratio of fractions a:b:c	Mw (g/mol)	PDI	Ratio of fractions a:b:c
CUPV261^T	a	$>2 \times 10^6$	1.57	3.8:7.4:1.0	$>2 \times 10^6$	1.45	3.6:7.2:1.0
	b	2.23×10^4	1.18		1.97×10^4	1.18	
	c	4.84×10^3	1.08		5.06×10^3	1.04	
CUPV237	a	$>2 \times 10^6$	1.74	5.5:8.0:1.0	$>2 \times 10^6$	1.60	5.2:6.6:1.0
	b	3.12×10^4	1.29		2.57×10^4	1.17	
	c	5.61×10^3	1.03		5.69×10^3	1.03	

Mw/Mn is a measure of polydispersity index (PDI) where Mw and Mn are the weight average and number average molecular weight, respectively.

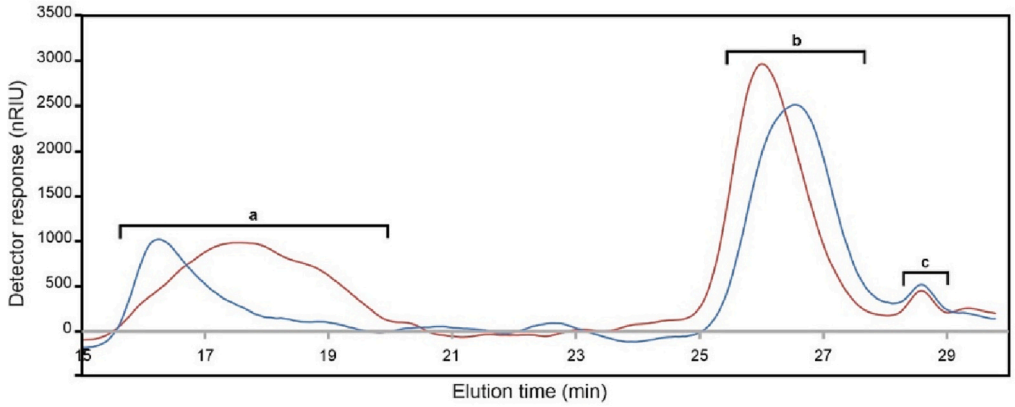

Fig. 3. High-performance size exclusion chromatograms of the crude EPS isolated from the supernatant produced by *L. sicerae* CUPV261^T (blue line) and *S. collinoides* CUPV237 (red line). The letters indicate each of the three fractions detected in both EPS. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

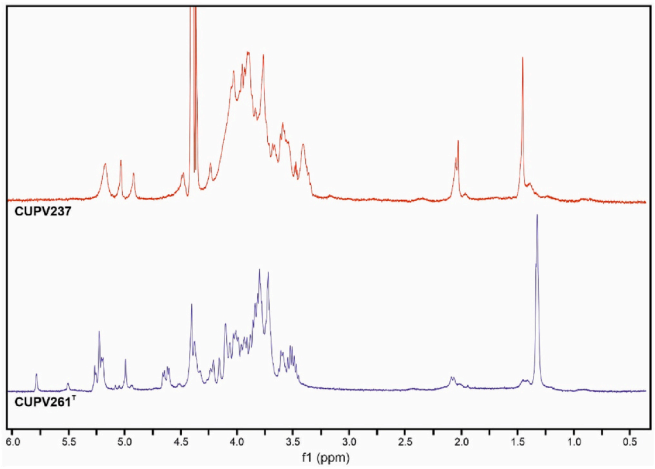

Fig. 4. ^{1}H NMR (500 MHz) spectra of crude EPS. The blue line corresponds to *L. sicerae* CUPV261^T and the red line to *S. collinoides* CUPV237. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

In *S. collinoides* CUPV237 samples (Table 3) the fractions represented 58.5 % (>50 kDa) and 41.5 % (<50 kDa) of the crude EPS, and their composition was different. The higher Mw material contained only 1,4-linked glucose and 1,4 (or 1,5)-linked galactose units, both partly branched at positions *O*-6, as could be deduced from the presence of 4,6-linked hexoses and terminal glucopyranose and galactofuranose residues. Nevertheless, the proportion of branching points detected in this sample was much higher than that of terminal residues. This could be due to: (i) an incomplete methylation of the sample (even after several methylation cycles), (ii) destruction of labile terminal units during hydrolysis, or (iii) the presence of non-carbohydrate substituents not detected with the methodologies used. Finally, the <50 kDa fraction includes the same types of components as the >50 kDa fraction but in lower proportions, which may be caused by incomplete separation of low Mw polymers from the very polydisperse high Mw material (see Fig. 3). On the other hand, terminal glucopyranose residues were not quantitatively correlated with a proportional amount of branching points. A significant proportion of them were 4,6-linked glucosamine units, marking a clear difference from the other fraction, that lacked amino sugars.

As deduced from these results, *L. sicerae* CUPV261^T and *S. collinoides* CUPV237 produce, respectively, at least three and two different EPS under the assayed conditions.

Table 2
Linkage types of the monosaccharides of the EPS fractions of strain CUPV261^T, deduced from methylation analysis, and their relative proportion. Rha: rhamnose; Glc: glucose; Gal: galactose; $GlcNH_2$: glucosamine; $GalNH_2$: galactosamine; *p*: pyranose; *f*: furanose.

Rt[a] (min)	Linkage type	Fragments (*m/z*)	Relative proportion in EPS fraction (%)			
			Crude	Insoluble	> 50 kDa	< 50 kDa
5.64	→2)-Rha*p*-(1→	89, 115, 131, 190	10.2	0.6	10.5	9.2
5.91	→3)-Rha*p*-(1→	89, 101, 118, 131, 202, 234	8.1	0.0	7.1	18.4
6.38	Glc*p*-(1→	87, 88, 102, 118, 129, 161, 205	11.7	3.7	5.2	4.3
6.53	Gal*f*-(1→	89, 102, 118, 162, 205, 278	0.0	0.0	2.4	4.0
6.78	Gal*p*-(1→	87, 88, 102, 118, 129, 161, 205	7.3	12.4	1.2	0.7
7.21	→2,3)-Rha*p*-(1→	89, 131, 202, 262	2.0	0.0	1.3	0.0
8.19	→3)-Gal*f*-(1→	118, 306	16.8	0.0	19.8	19.4
8.28	→3)-Glc*p*-(1→	101, 118, 129, 161, 234	2.9	19.7	2.1	1.4
8.55	→4)-Glc*p*-(1→	87, 102, 113, 118, 129, 131, 162, 173, 233	0.0	13.2	0.0	1.7
8.75	→3)-Gal*p*-(1→	101, 118, 129, 161, 234	21.8	34.8	15.6	9.1
9.56	→6)-Gal*f*-(1→	88, 101, 102, 117, 118, 127, 159, 233	3.5	tr.[b]	6.8	10.3
10.46	→2,3)-Gal*p*/*f*-(1→	101, 129, 161, 202, 262	10.3	tr.[b]	16.6	20.4
12.28	→3,6)-Gal*p*-(1→	118, 129, 189, 174, 234	4.4	15.1	0.9	0.0
16.43	→4)-Glc*p*NH_2-(1→	117, 159, 233	0.7	0.0	1.0	1.0
17.27	→4)-Gal*p*NH_2-(1→	117, 159, 233	tr.[b]	0.0	1.0	0.8

[a] Retention time.
[b] <0.2 %.

Table 3
Linkage types of the monosaccharides of the EPS fractions of strain CUPV237, deduced from methylation analysis, and their relative proportion. Glc: glucose; Gal: galactose; Man: mannose; Hex: hexose; $GlcNH_2$: glucosamine; *p*: pyranose; *f*: furanose.

Rt[a] (min)	Linkage type	Fragments (m/z)	Relative proportion in EPS fraction (%)		
			Crude	>50 kDa	<50 kDa
6.38	Glc*p*-(1→	87, 88, 102, 118, 129, 161, 205	8.2	9.0	51.1
6.78	Gal*f*-(1→	89, 102, 118, 162, 205, 278	0.0	1.2	0.0
8.16	→2)-Man*p*-(1→	87, 88, 101, 129, 130, 161, 190	2.4	0.0	1.8
8.36	→4 or 5)-Gal-(1→	87, 102, 113, 118, 129, 131, 162, 173, 233	40.3	17.9	5.5
8.53	→4)-Glc*p*-(1→	87, 102, 113, 118, 129, 131, 162, 173, 233	9.1	25.8	8.0
8.96	→6)-Glc*p*-(1→	87, 88, 102, 118, 129, 162, 189	tr.[b]	0.0	2.1
10.11	→3,4)-Glc*p*-(1→	118, 129, 305	1.55	2.5	0.0
11.32	→4,6)-Hex*p*-(1→	118, 201, 261	8.0	22.4	6.9
11.53	→4,6)-Hex*p*-(1→	118, 201, 261	28.2	22.1	6.2
13.92	Glc*p*NH_2-(1→	117, 129, 145, 159, 161, 203, 205	tr.[b]	0.0	2.6
16.43	→4)-Glc*p*NH_2-(1→	117, 159, 233	tr.[b]	0.0	2.4
18.20	→4,6)-Glc*p*NH_2-(1→	117, 159, 189, 233	1.0	0.0	11.0

[a] Retention time.
[b] <0.2 %.

3.4. EPS digestibility under simulated gastric-stress conditions

Previously, other authors have shown that EPS from LAB exhibited potential prebiotic properties such as high resistance to gastric and intestinal digestion and enhancement of beneficial gut bacteria (Hongpattarakere et al., 2012; Salazar et al., 2016; Tang et al., 2020). The tolerance of EPS from the CUPV237 and CUPV261^T strains to digestive tract stress was investigated in an *in vitro* gastric-stress model by comparing the high and intermediate fractions in the control and G samples (see Materials and Methods, Section 2.7). As shown in Table S2, recoveries after G treatment were total (≈100 %) in the case of the high molecular weight (>2000 kDa) fractions. However, a decrease in the recovery values of the intermediate molecular weight fractions (~25 kDa; see Table 2) was observed, being 81.03 ± 4.18 % in the case of CUPV261^T and 91.86 ± 1.10 % in the case of CUPV237. In addition, the comparison of the polydispersity index values showed that only the higher Mw fraction from CUPV237 was affected. This could indicate an increase in the dispersion of the molecular weight distributions (Sheu, 2001) due to partial polymer degradation by the low pH conditions. According to these assays, the EPS from both LAB withstood simulated gastric stress conditions and therefore would presumably reach the small and large intestine and could be utilized by the gut microbiota.

3.5. Caco-2 cell-culture and adhesion assays

The capacity of *S. collinoides* CUPV237, its isogenic non-ropy strain CUPV237NR, and *L. sicerae* CUPV261^T to interact with human epithelial cells was assessed using the enterocyte-like Caco-2 cell line (Fig. 5). The three strains showed lower capacity to bind the enterocytes than the probiotic *L. acidophilus* LA-5 strain (used as control), which showed a 6.0 % ± 0.4 (n = 3) of adherence. *L. sicerae* CUPV261^T showed the highest adhesion capability among the three strains assayed (2.3 % ± 0.3; n = 3; $p < 0.05$ compared to *L. acidophilus* LA-5). This value is in accordance with the adhesion values detected for other cider isolates (Llamas-Arriba et al., 2019b; Llamas-Arriba et al., 2018). In the case of the CUPV237 strain, it appears that the presence of HePS drastically inhibits adhesion to enterocytes. Adherence values for this strain (not depicted in Fig. 5) were <0.001 % for all measurements, compared to significantly higher values for strain CUPV237NR (1.1 % ± 0.5; n = 3; $p < 0.05$).

The decrease in their adhesion capacity in correlation with EPS production is a feature that has also been reported for other species and types of EPS (Llamas-Arriba et al., 2019b; Nácher-Vázquez et al., 2017a; Zarour et al., 2017). On the contrary, the production of β-glucans or HePS has also been described as useful to interact with eukaryotic cells (Garai-Ibabe et al., 2010; Živković et al., 2016). Some authors have tried to elucidate the mechanism by which these cells were able to adhere to the enterocytes, and a protein-mediated adherence has been discussed (Coconnier et al., 1992). Others have demonstrated that this adherence is mediated by surface-associated lipoteichoic acids (Granato et al.,

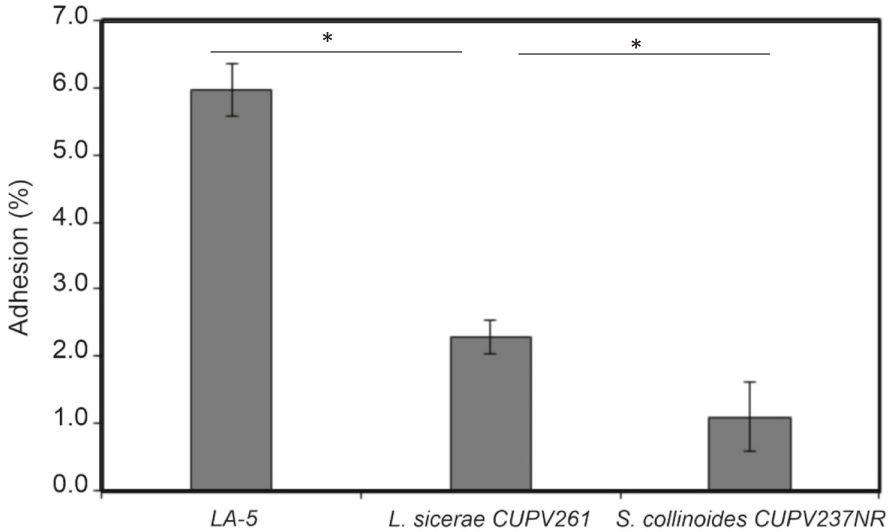

Fig. 5. Adhesion of LAB strains to Caco-2 cells. Values are expressed as the percentage of the cfu added in the assay. *p* values were below 0.05 (*) in the comparisons between strains LA-5 and CUPV261^T, or CUPV237 and its isogenic, non-ropy strain CUPV237NR. Strain CUPV237 showed adherence values below 0.001 % in all measurements and, therefore, it is not included in the graph. All assays were carried out in triplicate, with two biological replicates in each.

1999) or by factors promoting mucus adhesion (Miyoshi et al., 2006).

3.6. Protective effect of the HePS in an in vivo zebrafish DSS-enterocolitis model

The zebrafish (*Danio rerio*) model was used to evaluate the anti-inflammatory effect of the purified HePS preparations isolated from the two LAB strains, CUPV261^T and CUPV237. Pérez-Ramos and colleagues (Pérez-Ramos et al., 2018) revealed that, when it was evaluated in an induced inflammation model of *Tg(mpx:GFP)* zebrafish, the β-glucan produced by *Pediococcus parvulus* 2.6 (with a molecular weight higher than 10^3 kDa) exhibited an anti-inflammatory effect (Notararigo et al., 2014). However, a limited response to the EPS (45.065 kDa) of *Lactobacillus* HNUB20 strain was found in a study using a healthy zebrafish model (Ma et al., 2020). Here, we used an *in vivo* enterocolitis model of zebrafish induced by the chemical agent dextran sodium sulfate. Three different HePS concentrations (50, 100 and 150 μg/mL) were tested to assess any dose-dependent effect of the polymers. Similar to the HePS from two *Bifidobacterium* strains (Llamas-Arriba et al., 2019a), both HePS analyzed in this work reduced the negative effects caused by DSS. A mortality rate higher than 65 % (68.0 ± 3.7 %; tests carried out in triplicate in two independent experiments) was observed after 7 days of exposure to 0.8 % DSS (Fig. 6). At this time point, the highest reduction in larval mortality was achieved at an EPS concentration of 100 μg/mL. The HePS of strain CUPV261^T reduced larval mortality to 30.51 ± 4.5 % ($p < 0.05$), while treatment with the HePS of strain CUPV237 exerted a slightly milder effect on the reduction of mortality (34.31 ± 5.12 %; $p < 0.05$) (Fig. 6). The mechanism by which these polymers reduce zebrafish mortality is still unknown. It has been described that the anti-inflammatory activity of the EPS of specific *Bifidobacterium* strains could be mediated by the induction of Treg cells in mesenteric lymphoid nodes (Hidalgo-Cantabrana et al., 2016) but further immunological assays should be performed to confirm this hypothesis. Overall, the results suggest that the EPS of the strains analyzed in this work could have a protective effect in this zebrafish DSS-enterocolitis model.

4. Conclusions

L. sicerae CUPV261^T and *S. collinoides* CUPV237 strains produce a mixture of HePS of different molecular weights, composition, structures and thermal stability. The diversity of these polymers correlates with the presence in their genomes of multiple potential EPS gene clusters. CUPV261^T showed adherence capacity to Caco-2 enterocyte-like cells but not at the level of reference probiotic strains. CUPV237 showed the lowest adherence capacity. The EPS from both strains resisted simulated gastric stress conditions, which suggests that they would reach the small and large intestines. Furthermore, purified HePS preparations of both strains significantly reduced zebrafish mortality caused by DSS.

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CRediT authorship contribution statement

Conceptualization, P.L., A.P. and M.T.D. Methodology, P.L., A.P., M. A.P, I.B. and M.T.D. Investigation, A.I.P, M.G.L-A., and O.E. Original draft preparation, A.I.P., M.G.L-A., O.E. and M.T.D. All authors reviewed and edited the manuscript. Funding acquisition, P.L., A.P., O.E. and M.T. D. All authors have read and agreed to the published version of the manuscript.

Declaration of competing interest

The authors declare no conflict of interest.

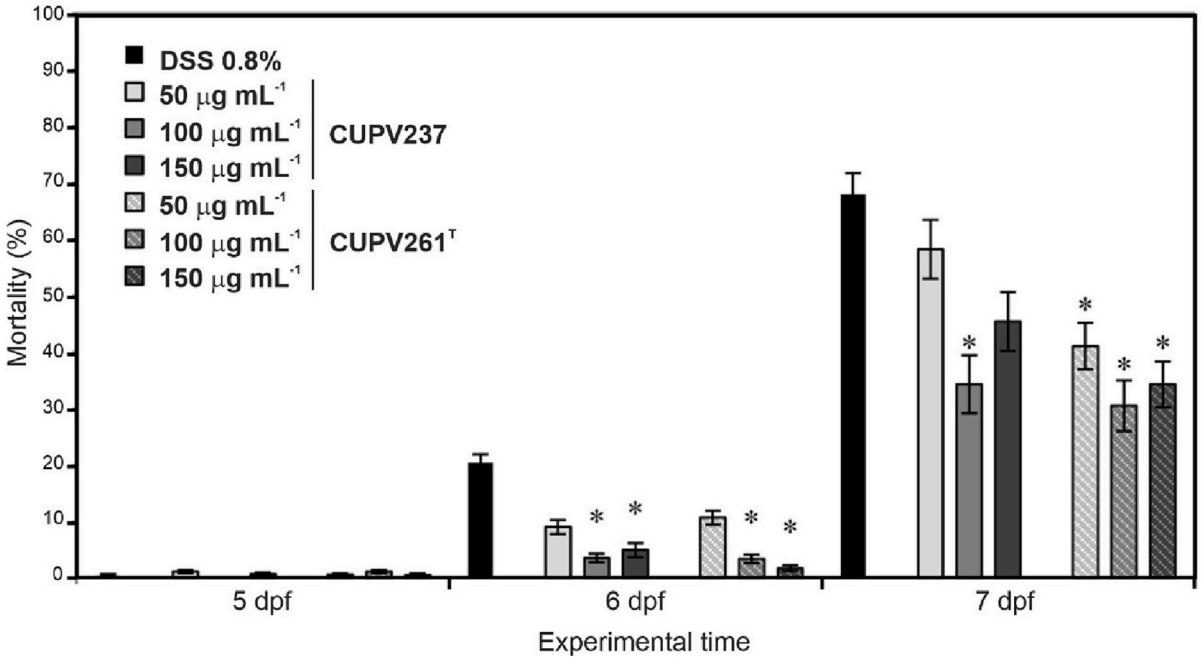

Fig. 6. Protective effect of HePS in an *in vivo* DSS-induced enterocolitis zebrafish model. Analysis of the mortality of zebrafish larvae treated with DSS (0.8 %) and DSS with variable quantities (50, 100 and 150 μg/mL) of the HePS produced by strains CUPV237 and CUPV261^T. The results are expressed as mean ± SD. Tests were carried out in triplicate in two independent experiments. Data were subjected to a one-way ANOVA followed by a *post hoc* Dunnett's T3 test with significances of $p < 0,05$ (*).

Data availability

No data was used for the research described in the article.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ijfoodmicro.2023.110199.

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