Disclosing diversity of exopolysaccharide-producing lactobacilli from Spanish natural ciders

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ABSTRACT: Lactic acid bacteria (LAB) are naturally occurring bacterial populations in traditional cider from the Basque Country (Spain), which is spontaneously fermented without starters. Some LAB are able to produce exopolysaccharides (EPS) thus causing viscosity, a cider spoilage called “ropiness” or “oiliness”. A total of 41 isolates recovered between 1992 and 2009 from ropy ciders of different cider factories were included in the present study. Identification by 16S rRNA gene sequencing, biochemical traits (API), RAPD-PCR using four universal primers (M13, M13V, P1 and P2) and 16S-23S ISRs amplification profiles revealed that the spoiler species were *L. collinoides*, *L. diolivorans*, *L. sicerae* and *L. suebicus*. EPS production in *L. collinoides* and *L. sicerae* strains approached by PCR amplification of *eps* genes encoding for homopolysaccharide and heteropolysaccharide revealed that these strains synthesize heteropolysaccharides and in addition, *L. collinoides* CUPV231 synthesizes a 2-branched (1,3)- β -D-glucan. The molecular weight of their EPS determined by HP-SEC revealed the presence of three polymers with average molecular weights (M_w) of around 10^6 g/mol, 10^4 g/mol and 10^3 g/mol. The middle-weight fraction was the most abundant except in *L. sicerae* CUPV261 which mainly produced the high M_w polymer.

KEYWORDS: *cider*, *Lactobacillus*, *exopolysaccharides (EPS)*, *ropy isolates*

1. Introduction

Natural cider from the Basque Country (Spain) is usually produced in small cider factories by using traditional techniques, and it is spontaneously fermented by indigenous LAB present in the musts (Dueñas et al., 1994, Del Campo et al. 2008, Sánchez et al., 2010). Microbiological stabilization is not performed after malolactic fermentation (MLF) or before bottling and as result, LAB becomes predominant in the cider microbiota. Certain LAB isolated from cider and wine are able to produce exopolysaccharides (EPS), and their release to the culture medium strongly modifies the beverage rheological characteristics that might render products unpleasant to the palate. This alteration, called “ropiness” or “oiliness”, has also been described in red and white wines from the Bordeaux region in France. It was reported that *Pediococcus parvulus*, *Oenococcus oeni*, *Lactobacillus suebicus*, and *Lactobacillus diolivorans* strains isolated from ropy cider and wine (Dueñas-Chasco et al., 1997, Ibarburu et al., 2007, Garai-Ibabe, 2010a, Dols-Lafargue et al., 2008) synthesize a characteristic homopolysaccharide (HoPS) 2-substituted- β -D-glucan. This β -glucan accumulates around the cells and forms a protective barrier, which confers to the ropy *P. parvulus* strains resistance to wine stress conditions (low pH, high ethanol or SO₂ concentrations) (Dols-Lafargue et al., 2008) and lysozyme treatment (Coulon et al., 2012). The β -glucan also increases the adhesion of the β -glucan producing strains to abiotic and biotic surfaces (Dols-Lafargue et al., 2008, Fernández de Palencia et al., 2009, Garai-Ibabe et al., 2010b) and could provide a selective advantage for adhesion to fruit surfaces and beverage making equipment. LAB cider isolates can also synthesize other types of EPS [heteropolysaccharides (HePS) or other HoPS such as dextrans, which are α -glucans] (Dueñas et al., 1998; Ibarburu et al., 2015). Despite of spoilage, LAB EPS can also contribute to sensory characteristics such as mouth-feel and foaming of cider, in addition to the polysaccharides from raw material and yeasts (Mangas et al., 1999).

The aim of this work was to disclose the EPS-producing *Lactobacillus* isolated from cider which, despite causing spoilage in this beverage, might contribute to enhance organoleptic characteristics of cider or be useful for other food applications. On one hand, identification and

genotypic differentiation was approached to trace the occurrence of spoiler strains. On the other hand, characterization of EPS produced by newly detected naturally occurring LAB species in ropy cider was carried out aiming at deeply analyzing the structural polysaccharides and genes associated with EPS synthesis.

2. Material and methods

2.1. Bacterial strains and culture conditions

The bacterial strains used in this work included 41 isolates from Spanish natural ciders and they belong to the genus *Lactobacillus*. Some of them were previously characterized: *L. diolivorans* G-77 (CUPV 218) (Dueñas et al., 1998); *L. suebicus* (CUPV 221, CUPV225, and CUPV226) (Garai-Ibabe, 2010a; Notararigo et al., 2013; Ibarburu et al., 2015; and *L. sicerae* (CUPV 261^T and CUPV 262) (Puertas et al., 2014). In addition, 12 reference strains representing 11 species (apart from *L. sicerae*) were also included for comparative purposes. Table 1 reports the species names and their sources. The isolates were recovered between 1992 and 2009 from ropy ciders of different factories that showed occurrence of spoilage. *Lactobacilli* included in this study were selected on the basis of the ropy phenotype exhibited by using the method described in Puertas et al. (2014); their colonies formed long filaments when touched with the loop, and also exhibited a ropy appearance in MRS broth. Strains were routinely cultured in de Man, Rogosa and Sharpe broth (MRS, De Man, Rogosa, & Sharpe, 1960) at pH 5.5 for 48 h at 28 °C in an atmosphere containing 5% CO₂, and stored in growth liquid medium containing 20% (v/v) glycerol at −80 °C.

For EPS isolation and quantification, strains were grown in a semi-defined-medium (SMD) at an initial pH of 5.5, according to Ibarburu et al. (2015). Batch fermentations without pH control were carried out in fully filled 250 mL screw-cap flasks for 65 h at 28 °C in an atmosphere containing 5% CO₂. Growth was monitored spectrophotometrically at 600 nm

(OD600). Analysis of the fermentation products was carried out by HPLC as described by Ibarburu et al. (2015).

2.2. Identification and genotypic characterization

LAB strains were characterized by biochemical tests and PCR-based techniques. Carbohydrate utilization was determined with the API 50CH system (API bio-Mérieux, Marcy l'Etoile, France). API galleries were incubated for up to 7 days at 28 °C. Gas production from glucose and gluconate was determined in a modified MRS broth without citrate or meat extract and provided with 2% (w/v) of the carbohydrate and Durham tubes (Mañes-Lázaro et al., 2008).

Identification of isolates was approached by 16S rRNA gene partial sequencing as previously described in Erhman et al. (2003). Genomic DNA was extracted and purified with the DNeasy® Blood & Tissue kit (Qiagen, Hilden, Germany) supplementing the enzymatic lysis buffer with lysozyme (30 mg/mL) and mutanolysin 2 U/μL (Sigma-Aldrich). The PCR products were purified using the QIAquick PCR purification kit (Qiagen, Hilden, Germany), according to the manufacturer's instructions. Sequences were obtained at the Sequencing and Genotyping Service (SGIker) of the University of the Basque Country (UPV/EHU) and they were analyzed using the EzTaxon-e server (<http://eztaxon-e.ezbiocloud.net/>; Kim et al., 2012).

Genomic diversity of the strains was analyzed by the randomly amplified polymorphic DNA (RAPD) method using four universal primers: M13 (5'-GAA ACA GCT ATG ACC ATG-3') (Pinto et al., 2005), P1 (5'-ACG CGC CCT-3') and P2 (5'-ATG TAA CGCC-3') (Simpson et al., 2002), and M13V (5'-GTT TTC CCA GTC ACG AC-3') (Ehrman et al., 2003). In addition, amplification of 16S-23S ISRs with primers LA2-16S1F (5'-CACCCAAAGTCGGTTCGG-3') and LA2-ITSMR (5'-GTTCTCGGCTTAATTACTG-3') was performed (Suzuki et al., 2004) to differentiate between *L. collinoides* and *L. paracollinoides*. PCR amplifications were conducted in duplicate with a final volume of 50 μL. Amplification products (20 μL) were electrophoresed on 1.9% (w/v) agarose gels at 80 V for 90 min. Gels were stained with ethidium bromide and

photographed under U.V. light. Gel images were recorded using a Gel-Doc system (Bio-Rad) and stored as TIFF files.

The banding patterns of each strain obtained after the five amplifications were analysed with BioNumerics 2.5 software (Applied Maths) using the Pearson coefficient and the unweighted pair group method with arithmetic averages (UPGMA). Clustering of multiple data sets, combining banding patterns (RAPD M13, P1, P2, M13V and 16S-23S ISR) and API results on carbohydrate fermentations, was calculated taking averaged similarity values from the individual experiments with the aid of the “Composite data set” tool in BioNumerics.

2.3. PCR detection of the *eps* genes

Amplification reactions of the *eps* genes were performed using different primers targeting HoPS (*gtf*, *deg*, *lev*) and HePS (*epsD/E*, *epsA*, *epsB*, *epsEFG*, *epsF*) coding genes as previously described (Ibarburu et al., 2015). The PCR conditions were carried out according to the literature. The amplified products were purified by using the QIAquick Gel extraction kit (Qiagen, Hilden, Germany) in accordance with the manufacturer's recommendations. Both DNA strands of amplicons were sequenced and the predicted amino acid sequences were searched against the National Center of Biotechnology Information (NCBI) database by means of the BLASTP program available at the NCBI (<http://www.ncbi.nlm.nih.gov/>). Amino acid alignments were done using the ClustalW program (<http://www.ebi.ac.uk/Tools/msa/clustalw2/>).

2.4. Exopolysaccharide isolation and quantification

For quantification of EPS released to the culture media, *Lactobacillus* strains were grown in the SMD medium under the conditions described above. Bacterial cells were removed by centrifugation (12,000 x g, 20 °C, 30 min), and the EPS present in the supernatant was precipitated and recovered as previously described in Ibarburu et al. (2015). The concentration of

EPS was estimated as neutral carbohydrate content determined by the phenol-sulphuric method (Dubois et al., 1956), using glucose as standard. All determinations were made in triplicate. In addition, to obtain the EPS attached to the bacterial surface, strains were grown on agar-MRS for 7 days in an atmosphere containing 5% CO₂. The crude-EPS was collected from the biomass and purified as previously described by López (2012). The EPSs were frozen at -80 °C and lyophilized.

2.5. EPS molecular weight

The weight-average molecular weight (M_w) of the crude-EPS was determined by high-performance size exclusion chromatography (HP-SEC) on an Agilent 1100 system (Hewlett-Packard, Germany) equipped with a PL-aquagel-OH guard column (8 µm, Agilent Technologies) and PL-aquagel-OH 40, 50, and 60 (Agilent Technologies) columns in series as described in Ibarburu et al. (2015).

3. Results and discussion

3.1. Identification and characterization of EPS-producing *Lactobacillus* strains

The ropy isolates included in this study are part of the CUPV collection, and were recovered from Spanish natural cider producers that suffered cider spoilage episodes (Table 1). Spoiled beverages from 2009 were additionally tested for the presence 2-substituted-(1,3)-β-D-glucan producing bacteria in cider (Ibarburu et al., 2010). Four ropy ciders gave a negative result, which indicated no β-glucan production, while 14 isolates showed ropy phenotype and are included in the present study.

The 41 cider strains, 6 previously identified and 35 new isolates, were obligate heterofermentative, producing CO₂, DL-lactate, acetate and ethanol from glucose. They were preliminarily identified by 16S rRNA coding gene sequencing (500 pb), and the isolates showed sequence similarity above 98% with *L. diolivorans* (17) and *L. collinoides*/*L. paracollinoides* (19). Since many LAB species have high 16S rRNA gene sequence similarity, i.e. *L.*

collinoides/*L. paracollinoides*, their proper identification required additional tests. Therefore, 16S-23S ISRs amplification profiles and RAPD-PCR using four universal primers (M13, M13V, P1 and P2) were further approached to aid identification as well as to analyze the intraspecific genetic diversity of *Lactobacillus* isolates. The clustering analysis including reference strains and cider isolates was calculated individually for each RAPD and ISR-PCR profile and also by combining electrophoretic profiles and API results. The highest intraspecies differentiation was obtained with the combined RAPD, ISR-PCR and API profiles. Figure 1 shows the dendrogram derived from UPGMA clustering including ropy cider isolates and reference strains. At 50% similarity four clusters were formed grouping cider isolates. Cluster I includes 17 isolates and the type strain of *L. diolivorans* LMG 19667^T; they were confirmed as members of the species by 99% 16S rRNA gene sequence similarity. Cluster II includes 2 isolates of *L. sicerae*, a new species described in another study (Puertas et al., 2014). Cluster III groups 19 isolates together with the type strain of the species *L. collinoides* CECT 922^T. This result corroborates their ascription to this species in addition to the ISR-PCR profiles, which allowed differentiation from *L. paracollinoides*. Cluster IV includes 3 isolates, the HePS-producing strains CUPV225 and CUPV226 (Ibarburu et al., 2015) and the O2-substituted (1,3)- β -D-glucan producing strain CUPV 221 (Garai-Ibabe et al. 2010b) previously identified as *L. suebicus*, and the reference strain *L. suebicus* CECT 5917^T. The RAPD analysis assessed the correct identification of isolates, which revealed that spoiler species were *L. diolivorans* in 1992, *L. collinoides* in 2000, these two species and *L. suebicus* in 2003, and *L. collinoides* and *L. sicerae* in 2009. The isolates analyzed were recovered from spoiled cider under demand of cider factories and only factory F was sampled in 2003 and 2009, the latter rendering the new species *L. sicerae* isolates. Therefore, there is no possibility to trace back the LAB species occurrence in each factory (Figure S1). Nevertheless, *L. collinoides* and *L. diolivorans* were the main isolates recovered in three and two years, respectively, and they are the species most frequently associated with alterations and organoleptic defects in cider. Both species were related with bitterness production (via glycerol dehydratase pathway), which is characterized for an unpleasant bitter taste that is associated with the presence of acrolein combined to polyphenols (Garai-Ibabe et al., 2008). *L. collinoides*

species was also implicated in phenolic off-flavour defects of ciders (Bouron et al., 2012) that are due to volatile phenols (4-ethylcatechol, 4-ethylphenol and 4-ethylguaiacol). In addition, *L. collinoides* strains isolated from Spanish and French ciders showed the ability to produce biogenic amines (Garai-Ibabe et al. 2007; Coton et al. 2010). Intraspecific diversity analyzed by RAPD revealed differences between *L. suebicus* isolates, recovered from different cider factories, and separated *L. collinoides* isolates in three groups that clustered apart from the type strain; one included 2 isolates from 2000 and one from 2003, another 11 from 2009, and a third one, grouped 4 from 2000 and one from 2009.

3.2. PCR screening for HoPS and HePS genes

The presence of EPS coding genes in the ropy *L. collinoides* (12) and *L. sicerae* (2) isolates recovered in 2009 was assessed by PCR. No dextran or levan producers could be detected using the primers targeting glucansucrase (*deg*) or levansucrase (*lev*) genes. PCR was also used to detect the presence of the *gtf* gene encoding the GTF glycosyltransferase, which catalyzes the synthesis of the 2-branched (1,3)- β -D-glucan (Werning et al., 2006). The expected amplicon was only detected in *L. collinoides* CUP V231, recovered in 2000. This strain agglutinated in the presence of anti-serotype 37 antibodies targeting the β -glucan (Garai-Ibabe et al., 2010; Werning et al., 2014), suggesting that it was a 2-substituted-(1,3)- β -D-glucan producer. NMR and methylation analyses confirmed the β -glucan production (data not shown) along with other polysaccharides.

In addition, the presence of genes implicated in an essential step of bacterial HePS biosynthesis was sought using several sets of *eps* primers (*epsD/E*, *epsA*, *epsB*, *epsEFG*, *epsF*) previously described (Ibarburu et al. 2015). DNA from *L. collinoides* (12) and *L. sicerae* (2) isolates recovered in 2009 yielded only a 189-bp PCR product with the *epsD/E* primers, which amplifies the genes encoding the priming-glycosyltransferase (p-GTF) (Provencher et al., 2003). This enzyme catalyzes the transfer of the first phospho-sugar residue from an activated nucleotide sugar to the undecaprenyl phosphate-lipid carrier on the cytoplasmic face of the membrane in the

first step of repeating unit synthesis of HePS (Lebeer et al., 2009). The predicted amino acid sequences of PCR products from *L. sicerae* strains showed identities above 77% (see Table S1 in the supplemental material) with respect to the partial sequence of EpsE protein of the HePS producer *Lactobacillus helveticus* CRL1176 (accession no. ABB59702.1, Mozzi et al., 2006). Nevertheless, *L. collinoides* strains showed identities ranging from 66% to 83% with EpsE protein of the HePS producer *L. fermentum* TDS030603 (accession no. BAI67356.1, Dan et al., 2009).

Similar to that described in HePS-producers belonging to the genera *Lactobacillus* and *Bifidobacterium* (Provencher, et al. 2003; Ruas-Madiedo, 2007), the predicted amino acid sequences of the PCR products showed parts of two conserved blocks (Figure 2), which are localized at the C-terminus of the p-GTF. Block B is involved in the interaction with the lipid carrier (Wang et al., 1996), and all sequences of block C of the HePS-producers isolated from cider contain a glutamate residue, which has been proposed as one of the catalytic residues in the p-GT of *Lactococcus lactis* (Krannenburg et al., 1999).

Accordingly, all these data suggested that *L. collinoides* and *L. sicerae* strains recovered in 2009 synthesize HePS. In addition, *L. collinoides* CUPV 231 recovered in 2000 synthesizes the 2-branched (1,3)- β -D-glucan.

3.3. EPS production and molecular weight determinations.

In previous studies, we described the EPS production by *L. diolivorans* G-77 (CUPV 218) and *L. suebicus* strains isolated from cider. *L. diolivorans* G-77 and *L. suebicus* CUPV221 synthesize the characteristic O-2-substituted (1,3)- β -D-glucan. Besides β -glucan, *L. diolivorans* G-77 synthesizes a (1,6)(1,2)- α -D-glucan (Dueñas et al. 1998). More recently, the HePS produced by two *L. suebicus* strains (CUPV 225 and CUPV226) were characterized (Notararigo et al., 2013; Ibarburu et al., 2015).

In the present study, we screened the EPS production and molecular weight of EPS produced by the ropy *L. collinoides* (12) and *L. sicerae* (2) isolates recovered in 2009. The EPS-producing *L. collinoides* and *L. sicerae* strains showed the characteristic ropy phenotype in MRS broth. However, some polysaccharide components of this complex medium, such as glucomannans in yeast extract, interfere with the EPS isolation procedure. Indeed, the analysis by HP-SEC of an extract from fresh MRS broth obtained by ethanol precipitation showed a very broad peak with a M_w of $1.29 \cdot 10^4$ g/mol and a polydispersity index of 4.89 (M_w/M_n , where M_w is the weight-average molar mass and M_n is the number-average molar mass). Due to this interference, the EPS producing ability of the *L. collinoides* (12) and *L. sicerae* (2) strains was tested in a SMD semi-defined-medium (Table 2) previously used to quantify EPS production (Ibarburu et al., 2015). Three strains (*L. sicerae* CUPV 261, CUPV 262 and *L. collinoides* CUPV 2371) were able to grow (Table 2) and to increase the viscosity of the medium as it was observed when touched with the loop. These strains yielded between 68.7 and 38.0 mg/L of EPS determined by the phenol-sulphuric method. The other strains exhibited a cell deposit that formed a long strip upon agitation. Therefore, due to the interference of the components of the MRS medium in the EPS-extraction, and the poor growth obtained in the semi-defined medium for the majority of the *L. collinoides* strains (Table 2), the EPSs were collected from cellular biomass harvested from agar-MRS plates (surface EPS).

Analysis of the surface EPS samples by HP-SEC revealed the presence of three peaks of different molecular weights (Table 2). The molecular weight distribution of the crude EPS was a high M_w -EPS with average M_w of around 10^6 g/mol, a second polymer with an intermediate M_w (around 10^4 g/mol), and a low M_w -polymer of around 10^3 g/mol. In most of EPS the second fraction was the most abundant, although *L. sicerae* CUPV261 produced mainly the high M_w polymer.

The presence of several polymers in EPS has also been reported in other *Lactobacilli* (Baruah et al., 2016; Ibarburu et al. 2015). It is also the case of the cider isolates *L. diolivorans* G77 and *O. oeni* I4 which, besides β -glucan, synthesize a (1,6) (1,2)- α -D-glucan (Dueñas et al.

1998), and two HePS (Ibarburu et al., 2007), respectively. In wine, HePS and β -glucan production has been also described for *O. oeni* strains as well as the HoPS synthesis from sucrose (dextrans or levans, which are β -fructans) (Dimopoulou, et al., 2014; 2016). These authors showed the protective effect of either cell-linked HePS or dextrans during freeze drying of the malolactic *O. oeni* strains, and suggested that these EPS may help them to better survive during production and conservation.

4. Conclusions

In the present study, a deeper analysis on ropy cider *Lactobacillus* revealed diversity in species and EPS type of naturally occurring populations associated with cider spoilage in the Basque Country (Spain). EPS production was screened in *L. collinoides* and *L. sicerae* strains and molecular weight of their surface polysaccharides were determined. The results of PCR screening to detect *eps* genes suggest that these strains synthesize HePS. This study contributes to improve our current knowledge about the EPS-producing *Lactobacillus* involved in the cider-making process and opens a new research line for biological control during cider manufacture. Further studies are in progress on the structural analysis of novel HePS synthesized by selected *L. collinoides* and *L. sicerae* strains and genes associated with HePS synthesis.

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Table 1. Strains used in the study.

Species	Source	Origin	Cider producer	Year of isolation
<i>Lactobacillus brevis</i>				
CECT 4121 ^T	CECT	Human faeces		
<i>L. collinoides</i>				
CECT 922 ^T	CECT	Fermenting apple juice		
CUPV238, CUPV239, CUPV2311, CUPV2312, CUPV2371	This study	Ropy cider	D	2009
CUPV236	CUPV	Cider	K	2003
CUPV231, CUPV232, CUPV234, CUPV235, CUPV2119, CUPV2328	CUPV	Spoiled cider	G	2000
CUPV2313, CUPV2315	This study	Ropy cider	H	2009
CUPV2317, CUPV2320, CUPV2322, CUPV2323, CUPV2324	This study	Ropy cider	I	2009
<i>L. diolivorans</i>				
LMG 19667 ^T	LMG	Maize silage		
CUPV213	CUPV	Ropy cider	A	1992
CUPV211, CUPV219, CUPV2112	CUPV	Cider	B	1992
CUPV212, CUPV214, CUPV215, CUPV216, CUPV217, CUPV 218=G77, CUPV2110, CUPV 2111, CUPV2113	CUPV	Ropy cider	C	1992
CUPV2117, CUPV2118	CUPV	Spoiled cider	E	2003
CUPV2114, CUPV2115	CUPV	Cider	K	2003
<i>L. hilgardii</i>				
CECT 4786 ^T	CECT	Wine		
<i>L. mali</i>				
CECT 4149	CECT	Wine must		
<i>L. paracollinoides</i>				
CECT 5926,	CECT	Beer		
DSM 15502 ^T	DSMZ	Brewery environment		
<i>L. satsumensis</i>				
CECT 7371 ^T	CECT	Grape must		
<i>L. sicerae</i>				

CECT 8227 ^T =CUPV261, CUPV262	CUPV	Ropy cider	F	2009
<i>L. suebicus</i>				
CECT 5917 ^T	CECT	Apple mash		
CUPV225	CUPV	Ropy cider	E	2003
CUPV221	CUPV	Ropy cider	F	2003
CUPV226	CUPV	Ropy cider	J	2003
<i>L. uvarum</i>				
CECT 7335 ^T	CECT	Grape must		
<i>L. vini</i>				
CECT 5924	CECT	Fermenting grape must		

CUPV, Colección de la Universidad del País Vasco (Spain); CECT, Colección Española de Cultivo Tipo (Spanish Type Culture Collection, University of Valencia, Burjassot Spain); DSMZ, Leibniz Institute DSMZ-German Collection of Microorganisms and Cell Cultures (Braunschweig, Germany); LMG, BCCM/LMG Belgian Coordinated Collection of Microorganism (Brussels, Belgium).

Table 2. Growth characteristics and EPS production of *L. collinoides* and *L. sicerae* strains grown in SMD medium, and the molecular weight of their EPS fractions isolated from the cell biomass harvested from the surface of MRS-agar plates.

Strain	OD ₆₀₀	EPS (mg L ⁻¹)	pH	Mw (g/mol)			Ratio of fractions
				High	Medium	Low	
				(x10 ⁶)	(x 10 ⁴)	(x10 ³)	
<i>L. sicerae</i>							
CUPV261 ^T	1.36±0.06	64.11±4,66	4.48	2.26	1.69	4.49	4.9: 3.0: 2.0
CUPV262	1.72±0.06	68.69±3,65	4.21	-	2.77	4.10	0.0: 5.7: 4.3
<i>L. collinoides</i>							
CUPV2371	1.13±0.07	37.98±2.75	4.88	1.07	1.95	4.35	1.6: 6.5: 1.8
CUPV238	0.37±0.01	7.91±3.97	5.44	1.22	1.93	4.85	3.6: 6.6: 1.6
CUPV239	0.33±0.01	7.14±1.95	5.52	2.08	2.10	4.78	5.0: 4.6: 0.9
CUPV2311	0.28±0.01	8.28±3.83	5.66	1.76	2.07	4.21	5.0: 4.0: 1.0
CUPV2312	0.51±0.01	7.91±2.28	5.59	2.07	2.05	5.14	3.6: 4.8: 1.6
CUPV2313	0.26±0.07	11.65±3.57	5.55	1.41	2.26	5.04	2.5: 6.5: 9.4
CUPV2314	0.45±0.03	10.84±3.08	5.60	1.58	2.01	4.75	2.0: 6.9 : 1.0
CUPV2317	0.76±0.01	10.77±2.00	5.54	1.65	2.02	4.87	2.5: 6.3: 1.2
CUPV2320	0.45±0.01	10.91±3.12	5.39	1.29	2.05	4.97	4.0: 5.0: 1.0
CUPV2322	0.44±0.05	9.36±5.44	5.43	1.21	2.02	4.76	3.2: 5.8: 1.1

CUPV2323	0.49±0.05	8.72±2.28	5.56	1.36	2.02	4.82	3.8: 5.2: 1.0
CUPV2324	0.32±0.05	8.62±2.25	5.51	1.89	2.00	4.48	2.7: 6.5: 0.8

Values are given as mean values $\pm$ standard deviations; Mw, weight-average molecular weight

Figure 1. Dendrogram derived from UPGMA cluster analysis based on the combined similarity matrix obtained from 16S-23S ISRs amplification profiles and RAPD-PCR using four universal primers (M13, M13V, P1 and P2) and API results corresponding to EPS-producing *Lactobacillus* isolates and reference strains. Strips show the combined representative profile for each cluster or strain after conversion, normalization, and subtraction of the background. The scale measures the percentage of similarity. Clusters are numbered.

Figure 2. Alignment of deduced amino acid sequences of PCR products obtained with PG primers. The letter X indicates an undetermined amino acid. Dark shading indicates identical amino acids, and light shading indicates conserved amino acids (only two different amino acids between strains); black arrow indicates a putative catalytic residue; dark and light line indicate two of the blocks in which the p-GT is divided (Kranemmburg et al., 1999).

Figure S1: Dendrogram derived from UPGMA cluster analysis based on the combined similarity matrix obtained from 16S-23S ISRs amplification profiles and RAPD-PCR using four universal primers (M13, M13V, P1 and P2) and API results corresponding to EPS-producing *Lactobacillus* isolates and reference strains. Strips not shown. The scale measures the percentage of similarity. The dendrogram also includes the isolation year and the cider factory where they were isolated.

Table S1: Results of BLASTP comparison of predicted amino acid sequences of PCR products obtained with PG primers (Provencher et al 2003).

Isolate	Nucleotide size (pb)	Protein	Organism	C %	Identity (%)	e-value	Conserved domain / Superfamily	e-value
CUPV261 [†]	197	EpsE	<i>L. helveticus</i> CRL1176	94	81	2e ⁻²⁹	Bac_trans/TIGR03022	1,04e ⁻¹⁹
CUPV262	161	EpsE	<i>L. helveticus</i> CRL1176	88	77	1e ⁻²⁰	Bac_trans/TIGR03022	5,2e ⁻¹¹
CUPV2371	146	EpsF	<i>L. fermentum</i> TDS030603	96	66	2e ⁻⁹	Bac_trans/TIGR03022	8e ⁻⁹⁰
CUPV238	142	EpsF	<i>L. fermentum</i> TDS030603	99	68	6e ⁻¹⁷	Bac_trans/TIGR03022	6e ⁻¹⁷
CUPV2311	142	EpsF	<i>L. fermentum</i> TDS030603	97	70	3e ⁻²⁵	Bac_trans/TIGR03022	4,22e ⁻⁹
CUPV2312	145	EpsF	<i>L. fermentum</i> TDS030603	99	67	5e ⁻¹⁷	Bac_trans/TIGR03022	3,77e ⁻⁸
CUPV2317	144	EpsF	<i>L. fermentum</i> TDS030603	89	72	3 ⁻¹⁶	Bac_trans/TIGR03022	5e ⁻⁸
CUPV2320	139	EpsF	<i>L. fermentum</i> TDS030603	99	70	9e ⁻¹⁷	Bac_trans/TIGR03022	1,03 ⁻⁸
CUPV2322	96	EpsF	<i>L. fermentum</i> TDS030603	90	83	2e ⁻⁷	Bac_trans/TIGR03022	3,22e ⁻³
CUPV2323	143	EpsF	<i>L. fermentum</i> TDS030603	94	71	1e ⁻¹⁶	Bac_trans/TIGR03022	4,22e ⁻⁷
CUPV2324	138	EpsF	<i>L. fermentum</i> TDS030603	100	67	6e ⁻¹⁶	Bac_trans/TIGR03022	6,61e ⁻⁸
CUPV 2313	120	EpsF	<i>L. fermentum</i> TDS030603	100	75	2e ⁻¹⁵	Bac_trans/TIGR03022	4,52e ⁻⁷
CUPV 2315	144	EpsF	<i>L. fermentum</i> TDS030603	78	71	2e ⁻¹⁶	Bac_trans/TIGR03022	6,85e ⁻⁸
CUPV 231	121	EpsF	<i>L. fermentum</i> TDS030603	94	81	2e ⁻²⁹	Bac_trans/TIGR03022	1,04e ⁻¹⁹

The amino acid sequences were searched in the National Center of Biotechnology Information (NCBI) data base by means of the BLASTP algorithm and analysed for the presence of conserved domains (CD) using the conserved domain database (CDD) available at the NCBI (<http://www.ncbi.nlm.nih.gov/>). C.%, represents the percentage of coverage of the sequence which corresponds to the reference protein.