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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 “oilness”. 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 heteropolysacccharides 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 Mw polymer.

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 (Del Campo et al., 2008; Dueñas, Irastorza, Fernández, Bilbao, & Huerta, 1994; 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 “oilness”, 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 (Dols-Lafargue et al., 2008; Dueñas-Chasco et al., 1997; Garai-Ibabe et al., 2010a; Ibarburu et al., 2007) 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 SO_2 concentrations) (Dols-Lafargue et al., 2008) and lysozyme treatment (Coulon, Houlès, Dimopoulou, Maupeu, & Dols-Lafargue, 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 mouthfeel and foaming of cider, in addition to the polysaccharides from raw material and yeasts (Mangas, Moreno, Rodríguez, Picinelli, & Suárez, 1999).

The aim of this work was to disclose the EPS-producing *Lactobacillus*

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Table 1
Strains used in the study.

Species	Source	Origin	Cider producer	Year of isolation
Lactobacillus brevis				
CECT 4121^T	CECT	Human faeces		
L. collinoides				
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
L. diolivorans				
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
L. hilgardii				
CECT 4786^T	CECT	Wine		
L. mali				
CECT 4149	CECT	Wine must		
L. paracollinoides				
CECT 5926, DSM 15502^T	CECT DSMZ	Beer Brewery environment		
L. satsumensis				
CECT 7371^T	CECT	Grape must		
L. sicerae				
CECT 8227^T = CUPV261^T, CUPV262	CUPV	Ropy cider	F	2009
L. suebicus				
CECT 5917^T	CECT	Apple mash		
CUPV225	CUPV	Ropy cider	E	2003
CUPV221	CUPV	Ropy cider	F	2003
CUPV226	CUPV	Ropy cider	J	2003
L. uvarum				
CECT 7335^T	CECT	Grape must		
L. vini				
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).

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 et al., 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% CO_2, 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% CO_2. 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., 2009).

Identification of isolates was approached by 16S rRNA gene partial sequencing as previously described in Ehrmann, Muüller, and Vogel (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, Chenoll, & Aznar, 2005), P1 (5′-ACG CGC CCT-3′) and P2 (5′-ATG TAA CGCC-3′) (Simpson, Stanton, Fitzgerald, & Ross, 2002), and M13V (5′-GTT TTC CCA GTC ACG AC-3′) (Ehrmann et al., 2003). In addition, amplification of 16S-23S ISRs with primers LA2-16S1F (5′-CACCCAAAGTCGGTT CGG-3′) and LA2-ITSMR (5′-GTTCTCGGCTTAATTACTG-3′) was performed (Suzuki, Koyanagi, & Yamashita, 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