Section: Food Microbiology and Safety

Short version of title

EPS PRODUCTION BY WINE *L. MESENTEROIDES*

Evaluation of Exopolysaccharide Production by *Leuconostoc mesenteroides* Strains Isolated from Wine

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ABSTRACT: Exopolysaccharide (EPS)-producing lactic acid bacteria are responsible for the alteration of wine and other fermented beverages. The potential to produce EPS was investigated for *Leuconostoc mesenteroides* strains isolated from Spanish grape must and wine. Most strains were able to produce EPS from sucrose containing media. Based on their EPS-producing phenotype and on their EPS monosaccharide composition, the *L. mesenteroides* strains analyzed could be arranged in two groups. One group comprises mucoid strains producing a glucan polymer, and a second group includes strains producing a fructan polymer. The presence of a glucosyltransferase encoding gene in the glucan producing *L. mesenteroides* strains was assayed by PCR. Two primer sets, PF1-PF8 and GTFF-GTFR, were used to amplify internal fragment of known glucosyltransferase genes. None of the glucan-producing strains gave a positive amplicon by the primer sets used. Therefore, new tools need to be developed to broaden the range of potentially spoiling agents detected by PCR in fermented beverages.

Keywords: lactic acid bacteria; exopolysaccharide; glucan; fructan; PCR detection method.

Introduction

During vinification, lactic acid bacteria (LAB) perform malolactic fermentation; they transform malic acid into lactic acid with a decrease of wine acidity and taste improvement. Nevertheless, they are also regularly responsible for the spoilage of wine and other fermented beverages. Exopolysaccharide (EPS)-producer LAB are responsible for an alteration known as “ropiness” or “oilness”, characterized by a viscous, thick texture and oily feel, which although not appreciably altering the taste, renders the products unpleasant to the palate. This alteration was first described by Pasteur, and has been described in wine, ciders, beers, and other fermented beverages (Lonvaud-Funel and Joyeux 1988). Ropy wines are sometimes encountered either during vinification or after bottling. Such wines cannot be sold due to their abnormal viscosity and this presents a serious problem to wine producers and merchants, resulting in considerable economical loss.

It has been described that some *Pediococcus* and *Lactobacillus* strains isolated from spoiled wine and cider produce an identical exopolysaccharide synthesized by the same glucosyltransferase. This EPS is a D-glucan consisting of a trisaccharide repeating unit with a (1→3) linked backbone in which every other residue is substituted at O-2 by one single unit of D-glucose (Llaubères and others 1990; Dueñas-Chasco and others 1997, 1998). In *P. parvulus* 2.6 (formerly named *P. damnosus* 2.6), *P. damnosus* IOEB8801, and *Lactobacillus diolivorans* G77, and *Oenococcus oeni* strains isolated from cider and wine, EPS-production seems to be linked to the presence of a glucosyltransferase gene (Walling and others 2001; Werning and others 2007). However, EPS-producing *Lactobacillus collinoides* IOEB0203 and *Lactobacillus hilgardii* IOEB0204 strains do not

contain any sequence similar to that encoding for the glucosyltransferase responsible for the EPS production in the above mentioned strains (Walling and others 2005).

As early as 1954 it was reported that the formation of high molecular weight dextran by *Leuconostoc mesenteroides* may occur in ciders and in fruit wines particularly when sucrose has been added, being the active enzyme a dextransucrase (Stacey 1954). Dextran production is a frequent characteristic in *L. mesenteroides* strains. Dextrans from *L. mesenteroides* are usually D-glucans with backbone structures in which (1→6) linkages predominate (Monsan and others 2001). However, in 1967 was described a *L. mesenteroides* strain unusual in that it formed a fructan from sucrose (Lewis 1967).

The aim of this study was to know the ability to EPS-production by wine *L. mesenteroides* strains.

Materials and Methods

Strains and growth conditions

Sixteen strains belonging to the *L. mesenteroides* species were isolated from grape must and wine samples from three different Spanish regions at the Instituto de Fermentaciones Industriales CSIC (Moreno-Arribas and others 2003). By sequencing their 16S rDNA, they were classified as *L. mesenteroides* subsp. *cremoris*.

L. mesenteroides strains were grown microaerobically at 30 ºC in a semi-defined medium (SDM) since complex media contain complex nutrients like beef extract, peptone, and yeast extract which interfere with EPS quantification (Kimmel and Roberts 1998). SDM medium contains dextrose, Tween 80, ammonium citrate, sodium acetate, magnesium sulphate, manganese sulphate, potassium sulphate, yeast nitrogen base, and bacto-casitone. EPS production was assayed in SDM containing 100 g/l of sucrose instead of the 20 g/L

glucose present in the original SDM medium. The pH was adjusted to 5.7 and the medium was autoclaved. In the preparation of SDM, the sugar source was sterilized by filtration. The medium was solidified with 20 g/L agar when appropriate.

Screening for EPS production

The determination of EPS producing phenotypes was carried out as described by Vescovo and others (1989). All wine *L. mesenteroides* strains were grown on sucrose-containing SDM plates for 3 days at 30 ºC under aerobic conditions. Plates were scored for mucoid or ropy phenotype. Ropy phenotype was considered when a string of 5 mm or more was detected in a wire-inoculating loop touched colony.

For EPS synthesis and purification, sucrose-containing SDM (200 mL) was inoculated with strains pregrown in SDM medium. After 3 days of incubation, cultures were centrifuged, and two volumes of cold (4 ºC) ethanol were added to one volume of culture supernatants, and the mixtures were stored overnight at 4 ºC. After precipitation with two volumes of cold ethanol and centrifugation, pellets were resuspended in distilled water. In order to eliminate monosaccharide residues, EPS was membrane dialysed (cut off 3500 Da) overnight at 4 ºC against distilled water, freeze dried, and stored.

Total amount of EPS was determined, by means of the total carbohydrate content of the precipitates, by the phenol-sulphuric acid method using sucrose as standard (Dubois and others 1956). Briefly, 200 µL EPS sample aliquots were prepared, and 200 µL of 5% phenol aqueous solution and 1 mL of sulphuric acid 95% (v/v) were added. After vigorously mixing, samples were incubated in a boiling bath for 30 min, and absorbance at 490 nm was measured. The concentration of EPS was determined in triplicate.

Determination of EPS size and purity by size exclusion chromatography

Dry EPS was dissolved in 0.3 M NaOH and centrifuged to eliminate insoluble material. The supernatant was loaded into a column (60 cm x 2.6 cm) of Sepharose CL-6B equilibrated with 0.3 M NaOH, which was also used as eluent (0.3 mL/min). Fractions were collected, and monitored for carbohydrate content by the phenol-sulphuric acid method (Dubois and others 1956). A calibration curve was obtained by using standards (Dextran Blue, T70, T10, and vitamin B_{12}). From this curve, the apparent molecular size (M_r) of the EPS was estimated.

Determination of monosaccharide composition

Samples were hydrolyzed with trifluoroacetic acid (TFA) for 1 h at 120 ºC at two different concentrations: 0.15 M to release fructofuranose residues avoiding their degradation, and 3 M to release any other sugars. The reducing sugars obtained after hydrolysis were measured by the dinitrosalicilic (DNS) method using glucose as standard (Bernfeld 1955). The glucose obtained from the EPS hydrolysis with 3M TFA was enzymatically determined by using the Glucose Monoliquid kit (Biotecnica, Spain) following the manufacturer instructions. The minimum glucose detected by this enzymatic method is 2mg/dL.

The monosaccharides obtained from hydrolysis were converted into their corresponding alditol acetates (Laine and others 1972) and then identified and quantified by gas-liquid chromatography (GLC) in an Autosystem (Perkin-Elmer) using an SP-2380 fused silica column (30 m x 0.25 mm I.D. x 0.2 µm film thickness) with a temperature program (210 ºC to 240 ºC, initial time 3 min, ramp rate 15 ºC/min, final time 7 min) and a flame ionization detector.

134

135 **PCR amplification of glucosyltranferase gene**

136 *L. mesenteroides* DNA was isolated from overnight cultures using a protocol
137 previously described (Vaquero and others 2004). For the detection of the gene responsible
138 for the glucan synthesis we used two primers sets previously described, and based on
139 glucosyltransferase genes found in LAB isolated from ropy wines and ciders. Primer set
140 PF1 (5´-GATTGTAATAAAATAAAAAGACCC) and PF8 (5´-
141 CATATGATAACACGCAGGGC) amplifies a 981-bp DNA fragment (Walling and others
142 2005); and, primer set GTFF (5´- CGGTAATGAAGCGTTTCCTG) and GTFR (5´-
143 TCTAGATTAATCATTCCAATCAACTG) it is predicted to give an amplicon of 417 bp in
144 some glucan-producing wine lactic acid bacteria (Werning and others 2006). PCR reactions
145 were performed in 0.2 mL microcentrifuge tubes in a total volume of 25 µL containing 1µL
146 of template DNA (aprox. 10 ng) and using conditions previously described (Walling and
147 others 2005; Werning and others 2006). DNA fragments were resolved on 1.2% agarose
148 gels.

149

150 **Results and Discussion**

151

152 **Screening for EPS production by wine *L. mesenteroides* strains**

153 Screening for EPS synthesis by LAB is usually carried out on agar plates, where the
154 strains could show different EPS-producing phenotypes. It has been described that some
155 LAB can express both ropy and mucoid phenotypes depending on culture conditions (Ruas-
156 Madiedo and de los Reyes-Gavilán 2005). Since sucrose has been described to be an
157 excellent substrate for abundant EPS synthesis (van Geel-Schutten and others 1998), we

158 grew the cultures on agar plates containing high concentrations of this sugar. A high
159 proportion of the wine isolates of *L. mesenteroides* showed a mucoid phenotype (Table 1),
160 although their appearance differed among the strains analyzed. Four strains (RM45, RM47,
161 RM48, and RM49) presented a more apparent mucoid phenotype than the other isolates
162 analyzed. None of the isolates investigated presented a ropy phenotype.

163 In order to test EPS production by the *L. mesenteroides* strains, we grew them in
164 liquid sucrose-containing SDM medium. After 3 days of incubation, the EPS was
165 precipitated, dialysed, and freeze-dried. Table 1 shows that EPS was produced in relatively
166 large amounts in all *L. mesenteroides* strains analyzed, with exception of RM57 strain that
167 did not produce EPS. The EPS production reached more than 0.5 g/L, being the highly
168 mucoid strains the major EPS-producers (Table 1).

169 Representative strains showing the high-mucoid (RM48) and less-mucoid
170 phenotypes (RM54) were selected for further analyses. The EPSs from these strains were
171 analyzed by gel filtration chromatography and the eluate was monitorized for
172 carbohydrates, confirming that the material analyzed contained, in each case, a single
173 polysaccharide with an apparent M_r higher than 1000 kDa (data not shown).

174

175 **Chemical analysis of the EPS**

176 *L. mesenteroides* strains mainly produce a glucan-type EPS (Stacey 1954; Beech
177 and Carr 1977). However, some strains could form a fructan from sucrose (Lewis 1967).
178 Monosaccharides with furanose ring structures, as fructose, are very acid-labile and are
179 removed by mild acid hydrolysis to avoid their degradation (Pazur, 1986; Peng and Tian,
180 2001). Then, to determine their composition, the EPSs were subjected to two different
181 hydrolytic conditions. 0.15 M TFA was used to release the fructofuranose residues, but

these soft conditions do not hydrolyze the more resistant linkages of pyranoses (Politi and others, 2006; Domenech and others 1999). 3 M TFA was necessary to release non-furanosidic components, but leaves to destruction of fructose and other furanoses (Politi and others, 2006; Domenech and others 1999; Prieto and others 2007).

From the results displayed on Table 1 it can be deduced that the *L. mesenteroides* strains here investigated could be divided into two groups. One group comprises strains RM45, RM47, RM48, RM49, and RM70, showing a more apparent mucoid phenotype, and EPSs with low fructose content (less than 11%) and rich in glucose. The second group comprises strains RM43, RM44, RM50, RM51, RM52, RM53, RM54, RM55, RM60, and RM61, which did not show a very obvious mucoid phenotype, and produced fructose-rich EPSs.

GLC analysis of the EPS produced in sucrose-containing SDM medium by representative strains of each *L. mesenteroides* group (RM54 and RM48) confirmed the previous results. Hydrolysis of the EPS from strain RM54 with 3 M TFA gave only 3% of glucose due to destruction of the fructan, while hydrolysis with 0.15 M TFA gave similar amounts of mannose (30%) and glucose (36%) arising from fructose after borohydride reduction, as expected for a polymer composed of fructose (Politi and others 2006). Despite the gentle hydrolytic conditions (0.15M TFA), some destruction of the fructan can take place, being responsible for the low recovery of monosaccharides. On the other hand, hydrolysis with 3 M TFA of the EPS from strain RM48 released 80% of glucose, as expected for a glucan, while only small amounts of mannitol and glucitol were detected after mild hydrolysis with 0.15M TFA, confirming that it contains only a small amount of fructose.

The experimental approaches used in this study corroborate that *L. mesenteroides* can produce two types of EPS: a glucan, correlated with high mucoid strains, and a fructan correlated with less mucoid strains. These results are in contrast with previous reports describing EPS production by *L. mesenteroides* strains. It is generally assumed that *L. mesenteroides* are mainly dextran producing bacteria (Monsan and others 2001), being scarcely reported the production of fructans by strains from this species (Lewis 1967). However, in this study, among the 16 strains analyzed only five were glucan-producing. Since the *L. mesenteroides* strains analyzed previously were not isolated from a wine-related source, it could be suggested that substrate composition will direct the prevalence of some specific EPS-producer strains.

Presence of a glucosyltransferase gene in the glucan-producing *L. mesenteroides* strains

Werning and others (2006) demonstrated the absence of the glycosyltranferase gene in two dextran-producing *L. mesenteroides* strains from culture collection. By using GTFF and GTFR oligonucleotides, these strains gave a negative PCR response. In order to investigate the presence of known glycosyltransferase genes in the glucan-producing strains analyzed in this study, PCR experiments were performed. Two primer sets, PF1-PF8 and GTFF-GTFR, were used to amplify internal fragment of know glucosyltransferase genes. These primers set have been successfully used to detect glucan producing LAB strains (Walling and others 2005; Werning and others 2006).

None of the strains assayed were able to produce a positive amplicon by the primer sets used (data not shown). This result is in agreement with those previously reported in two glucan-producing *L. mesenteroides* strains (Werning and others 2006).

EPS from LAB could be synthesized either extracellularly from exogenous substrates or intracellularly from sugar nucleotide precursors (Monsan and others 2001), however, the currently available PCR methods only detect the presence of intracellular membrane-bound glycosyltransferases. This could be the reason why two different ropy *Lactobacillus* species isolated from ropy beverages, were not amplified using primers PF1 and PF8 targeting the glycosyltransferase gene (Walling and others 2005). Both the glucan-producing *Lactobacillus* species reported by Walling and others (2005) and the wine *L. mesenteroides* strains analyzed in this study could produce EPS by action of an extracellular glucansucrase not detected by the currently available PCR methods. A similar situation has been described on three of the ten EPS-producing strains isolated from dairy and cereal products whose corresponding genes could not be detected (Van der Meulen and others 2007).

In spite of the facts that the *L. mesenteroides* strains analyzed in this study were not isolated from ropy wines, and that in the conditions assayed they did not show a ropy phenotype, they could constitute a problem during winemaking since they are potentially-EPS producers. In alcoholic beverages a clear product is required, and when a high molecular weight EPS is formed it gives an appreciable haze (Lonvaud-Funel and Joyeux, 1988). In addition, in ciders, *Leuconostoc* strains have been reported as the cause of ropiness (Beech and Carr 1977). It is already assumed that EPS synthesis is a direct response to some environmental conditions. In EPS-producing LAB strains, factors such as nitrogen and glucose and fructose concentration, temperature, pH, etc. seem to influence EPS production (Dueñas and others 2003; Wailling and others 2005). To date, the factors that favour or inhibit the production of EPS by these wine *L. mesenteroides* strains are not understood enough to predict the appearance of haze or ropiness in wines.

253

254 **Conclusions**

255 In this work we found that most of the *L. mesenteroides* strains isolated from grape
256 juice or wine are EPS-producers. These strains could be grouped based on the composition
257 of the EPS produced. Glucan-producing *L. mesenteroides* strains could not be detected by
258 the currently available PCR detection methods targeting glycosyltransferase genes.
259 Therefore, new tools need to be developed to broaden the range of potentially spoiling
260 agents detected by PCR in fermented beverages.

261

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(Table 1. Montersino, Prieto, Muñoz, & de las Rivas)

Table 1. Characteristics of the of the EPS produced by the wine *L. mesenteroides* strains analyzed in this study

				EPS composition [b]	
	Phenotype			0.15M	3M
Strain	Ropy	Mucoid[a]	EPS[c]	Fru (%)[d]	Glc (%)[e]
RM43	-	+	609	100	0
RM44	-	+	617	100	0
RM45	-	+++	811	5	65
RM47	-	+++	816	10	92
RM48	-	+++	823	6	71
RM49	-	+++	840	6	72
RM50	-	+	591	100	0
RM51	-	+	720	100	0
RM52	-	++	873	100	0
RM53	-	+	692	100	0
RM54	-	+	812	100	0
RM55	-	+	676	100	0
RM57	-	-	n.d.[f]	n.d.	n.d.
RM60	-	+	767	100	0
RM61	-	++	825	100	0
RM70	-	+++	904	11	74

[a]Glistering colonies showing a diameter of 3 mm (+), 3-4 mm (++), and >4 mm (+++).
[b]Determined by trifluoroacetic acid hydrolysis at two different concentrations.
[c]Determined by the phenol/sulphuric acid method, in mg/L
[d]Determined by the dinitrosalicilic (DNS) method.
[e]Determined by an enzymatic method.
[f]n.d., not detected.