Heterofermentative Lactobacilli
Causing Ropiness in Basque Country Ciders

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ABSTRACT

Ropy strains of heterofermentative Lactobacillus spp. were isolated from bottled spoiled ciders produced in the Basque Country. The isolates were able to produce slime experimentally on glucose-containing medium and apple must. The production of ropy slime occurred during exponential growth and the increase in viscosity was correlated with production of polysaccharide. They were characterized with regards to growth at different pH, sulfuring (SO2), ethanol and temperatures and showed similar behavior. All ropy strains grew in the presence of high ethanol concentrations (15%) and at low temperatures (10 and 15°C). In addition, these were tolerant to 10, 25 and 50 mg/l of total SO2 (pH 3.8), but with longer lag times as the concentration of SO2 increased. However, the ropy lactobacilli studied were affected by the pH of the medium and in general pH below 3.7 limited their growth seriously.

Key Words: Lactobacilli, ropiness, cider

Ropiness is a microbiological disorder common to ciders, beers and wines. The cider becomes heavy, it ropes when poured and flows without any noise. This defect is caused by certain strains of lactic acid bacteria (LAB), but is not necessarily caused by the same organism in all the beverages. This alteration is characterized by the production of a polysaccharide substance, which thickens the consistency of the cider. Nevertheless, the LAB not only are responsible for this alteration but they also carry out the malolactic fermentation and contribute to the organoleptic properties of alcoholic beverages.

Ropy Pediococcus damnosus isolated from Bordeaux spoiled wines increased the viscosity of the beverage, due to the production of an exocellular polysaccharide, a D-glucan. Further investigations showed that plasmid deoxyribonucleic acid (DNA) of a ropy strain contained the plasmid encoding for polysaccharide synthesis. In ciders, strains of lactic acid bacteria belonging to the genus Leuconostoc, Lactobacillus and Pediococcus have been reported as the cause of ropiness.

The residual sugars together with low amounts of tannins, alcohol and acidity favor the development of the ropy lactic flora. Although the prevention of the growth of lactic acid bacteria is difficult, different techniques such as low temperature, low pH, sulfiting and microbiological stabilization before bottling are used.

Ciders from the Basque Country are not subject to any treatment before bottling and the apparition of this alteration is very frequent, causing great economic loss. This study was designed with the aim of preventing this disorder and determining the active ropy strains and their role in the development of ropiness, as well the factors that control their growth such as pH, temperature and the concentrations of sulfur dioxide and ethanol.

MATERIALS AND METHODS

Microbial count and isolation of LAB strains causing ropiness

Seventeen samples of bottled spoiled ciders collected from Basque Country cider producers were examined. Lactic acid bacteria were counted by spreading on modified Carr agar medium containing (g/L) in distilled water: yeast extract, 4; Casaminoacids, 5; DL malic acid, 5; glucose, 20; KH2PO4, 0.5; KCl, 0.425; CaCl2 2 H2O, 0.125; Mg SO4, 0.125; MnSO4, 0.0025; pH adjusted to 4.8 with 10 N sodium hydroxide (NaOH). Fifty mg/L of pimaricin was added to eliminate yeast and molds. Plates were incubated at 25°C under anaerobic conditions (GasPak™ bioMerieux, France) for 8 to 10 days. After incubation, the roping ability of the colonies was estimated visually; they were touched with a loop and ropy colonies were isolated. They were picked and streaked for isolation on the same medium.

Acetic acid bacteria and yeast were counted by spreading on apple-juice agar supplemented with 1% yeast extract, and 1% (vol/vol) of 0.5% pimaricin solution to inhibit the growth of yeast and molds, and 1% (vol/vol) of penicillin (250,000 IU stock solution) were added, to prevent the growth of lactic acid bacteria.

Analytical methods in spoiled bottled ciders

The following parameters were measured in accordance with the O.I.V. methodology: pH, volatile and total acidity, density at 20°C, ethanol, reducing sugars and total phenols. Kinematic viscosity of spoiled ciders was determined by using an Ubbelohde Viscometer at 25°C.

Identification of LAB strains causing ropiness

Fifteen ropy strains were isolated from spoiled bottled ciders. General morphological, physiological and biochemical characteristics were determined according to Bergy's Manual of Systematic Bacteriology. The homo- or heterofermentative characteristic was tested by the methods of Salih and Pilone. The production of ammonia from arginine was examined in heterofermentation-arginine broth and in de Man Rogosa...
Sharpe (MRS) medium (pH 4.8) with and without the addition of 3 g/L L-arginine hydrochloric acid (HCl) (8). After incubation for 8 days, the presence of ammonia was detected with Nessler’s reagent (20). The configuration of lactic acid produced from glucose was determined enzymatically using the method specified by Salih (22). Dextran formation from sucrose was examined on MRS agar at pH 4.8 using sucrose (5%) instead of glucose. The ability to utilize L-malate was tested by growing the isolates in MRS broth (pH 4.8) containing 0.4% L-malate during 14 days and the residual L-malic acid was analyzed enzymatically.

Carbohydrate fermentation tests were performed by the tube method and by the Analytab Products 50 CHL system (5041 API system, France). Using the test tube system 20 carbohydrates were tested: D-arabinose, L-arabinose, ribose, D-xyllose, galactose, D-glucose, D-fructose, D-mannose, mannitol, sorbitol, esculin, salicin, cellobiose, maltose, lactose, sucrose, trehalose, melezitose, D-raffinose and gluconate. Cultures were inoculated in tubes containing MRS broth without meat extract and glucose (pH 6.0) with 0.04% (wt/vol) chlorophenol red as an indicator. Filter-sterilized solutions of the test carbohydrates were added to a final concentration of 1% (23). Hydrolysis of esculin was examined using the medium specified by Carr (5). They were incubated at 28°C for 21 days. The API 50 CHL identification system was used according to the manufacturer’s instructions to determine the acid production from carbohydrates.

Characterization of strains

Growth at different pH, temperatures, ethanol and SO₂ concentrations was evaluated by inoculating into duplicate test tubes containing MRS broth with the following modifications: a) media adjusted to pH 3, 3.3, 3.5, 3.7, 4.0 or 4.8, the pH value was adjusted before sterilization and confirmed afterwards; b) media incubated at 4, 10, 15 or 28°C (pH 4.8); c) media incubated with 0, 4, 7, 10 and 15% (vol/vol) ethanol (pH 4.8). Growth at different concentrations of total SO₂ was determined on 10 ml MRS (pH 3.8) in screw-capped glass tubes, which was adjusted to contain 0, 15, 25, 50, 75 and 100 mg/L total SO₂ by addition of filter-sterilized potassium metabisulfite solutions just prior to introducing the cells and the concentration of total SO₂ was determined using the aeration-oxidation method (19). All media were incubated for a period of 14 days at 28°C except for the test of SO₂ influence, where cell growth was observed during 80 days. The growth was followed by determining the density of the cultures spectrophotometrically at 600 nm and compared to control samples.

Production of ropiness

The ability of the strains isolated to produce ropiness was tested in modified Carr broth (pH 4.8) and apple juice supplemented with 1% yeast extract. One ropy strain (No. 5) was used to demonstrate the kinetics of growth, polysaccharide production and increase in viscosity in Carr liquid medium containing glucose (2%). Flasks containing 200 ml of medium were inoculated with a culture of ropy strain grown for 48 h in the same Carr broth medium. The inoculum was standardized to an optical density of 0.1 at 600 nm. Incubation was at 25°C for 13 days. After removal of the cells by centrifugation at 10,000 × g (15 min, 4°C), the polysaccharides were harvested from the supernatant solution by ethanol precipitation (18). Polysaccharide concentration was analyzed by the phenol-sulfuric acid method (10) with glucose as standard. Glucose and L-malic acid were determined by enzymatic methods (Boehringer Mannheim, Spain). Culture viscosity was measured using a Cannon-Fenske glass capillary viscometer at 25°C after removal of the cells.

RESULTS

Physicochemical and microbiological parameters in ropy ciders

The main population was constituted by lactic acid flora which reached levels of 10⁶ to 10⁷ colony forming units (CFU/ml) in all samples. However, the population of yeasts were present in very low levels, around 10⁴ CFU/ml, and in most of the sampled ciders, acetic acid bacteria were not found, except for one sample in which 10⁵ CFU/ml were detected. Table 1 shows the average data of the different physicochemical parameters studied. As can be seen, ciders were bottled with a small amount of residual sugars about 1.5 g/L, which could be utilized as nutrient by the predominant lactic population. Ciders have undergone malolactic fermentation as can be deduced from the lack of L-malonic acid. The pH and titratable acidity presented normal values. However, the acetic acid levels were very high, which could be due to the lack of microbial control (sulphiting, filtration) during cider making and bottling.

Bacterial strains causing ropiness

Fifteen ropy strains were isolated in solid medium from bottled spoiled ciders. Colonies were scored for ropiness by picking with an inoculation loop. Ropy colonies (ropy+) could be easily distinguished from non-ropy colonies (ropy-) by the occurrence of long ropy filaments and the slime-producing trait was also confirmed, when these isolates were cultivated in liquid medium (Carr, MRS and apple must with 1% yeast extract). Some changes in the physical characteristics, such as an increase in viscosity, consistency and long ropy strands that fell from a pipette tip were observed (7). The ropy character remained persistent after several transfers under laboratory conditions. This differs from other researchers’ observations (5,11).

Growth kinetics of a ropy strain in liquid medium, together with the production of polysaccharide, and the viscosity increase on glucose-containing medium were investigated (Fig. 1). One non-ropy reference strain belonging to the Spanish Type Culture Collection Lactobacillus brevis (CECT 216) was used as the control strain. Growth experiments have shown that the increase in viscosity and the production of polysaccharide by ropy strain occurred.

### TABLE I. Chemical analysis of spoiled ciders.

<table>
<thead>
<tr>
<th>Analytical parameter</th>
<th>Mean</th>
<th>Standard deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total sugar (g/L)</td>
<td>1.54</td>
<td>0.84</td>
</tr>
<tr>
<td>Ethanol (% vol/vol)</td>
<td>6.6</td>
<td>0.32</td>
</tr>
<tr>
<td>Phenolic compounds (g/L)</td>
<td>1.26</td>
<td>0.40</td>
</tr>
<tr>
<td>Total acidity (g acetic acid/L)</td>
<td>5.84</td>
<td>0.75</td>
</tr>
<tr>
<td>Volatile acidity (g acetic acid/L)</td>
<td>3.37</td>
<td>0.49</td>
</tr>
<tr>
<td>Density at 20°C</td>
<td>999.5</td>
<td>0.93</td>
</tr>
<tr>
<td>Viscosity (cst)*</td>
<td>1.1630</td>
<td>0.0183</td>
</tr>
<tr>
<td>L-malic acid (g/L)</td>
<td>(traces)</td>
<td>3.82</td>
</tr>
</tbody>
</table>

* cst: centistokes

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During exponential growth. According to our results, there was a correlation between the increase of polysaccharide production and the increase in viscosity. Although the control strain provoked a slight increase in medium viscosity, this could not be compared to the great increase of the viscosity that the ropy bacteria produce. On the other hand, as Fig. 1 shows, glucose was exhausted from the medium during active growth and the initial L-malate was consumed partially during the 13 days of observation.

**Identification of ropy strains**

The strains were gram-positive, catalase-negative, nonsporing rods. They produced gas from glucose and dextran from sucrose, although production of ammonia from arginine was not detected. They grew at 15°C but not at 45°C. They formed the D(-) (between 0.64 and 1.72 g/L) and L(+) (between 0.96 and 1.84 g/L) isomers of lactic acid and utilized malic acid (between 70 and 100%) in the presence of glucose.

Using the test tube system to evaluate fermentation responses for different carbohydrates, all strains utilized L-arabinose, ribose, D-xyllose, galactose, D-glucose, D-fructose, maltose and gluconate. None of the strains produced acid from D-arabinose, mannitol, sorbitol, salicine, trehalose or melezitose. Most of these strains utilized sucrose (12 strains), esculin (12 strains), D-raffinose (13 strains) and a few strains utilized D-mannose (1 strain), cellobirose (1 strain), lactose (5 strains exhibited a weak response). When the API 50 CHL system was used, all strains were found to ferment L-arabinose, ribose, D-xyllose, D-glucose, D-fructose, maltose, melibiose and gluconate. Most strains utilized β-methyl-xiloside (13 strains), galactose (11 strains), D-raffinose (10 strains) and D-arabitol (12 strains). A few strains utilized D-mannose (2 strains), a-methyl-D-glucoside (1 strain), lactose (2 strains) and 5-ketogluconate (5 strains). The strains failed to ferment other substrates in the API system. When comparing both methods similar results were observed, except for the sucrose and esculin. In the API test, sucrose was utilized only by three strains and esculin by none, while in the tube system they were used by 12 and 13 strains, respectively.

In terms of carbohydrate fermentation, these lactic rods resemble quite closely the species called *Lactobacillus collinoides* and *L. brevis* (23). As noted by Kandler and Weiss (23), *L. brevis* is often difficult to distinguish clearly from *L. collinoides* by simple physiological tests, especially carbohydrate fermentation reactions. In consequence, in order to carry out a complete identification of these strains it would be necessary to perform other identification methods, such as DNA/DNA homology or electrophoretic mobility of lactic acid dehydrogenases. The results for the production of ammonia from L-arginine were surprising because these heterofermentative lactobacilli gave a negative result in both media used. This test is usually positive for heterolactic LAB (23), but there are reports in the literature that some heterofermentative lactobacilli from cider (*L. collinoides*) cannot hydrolyze arginine (6). On the other hand, these negative results could be explained by poor test sensitivity for the production of ammonia from L-arginine metabolism using Nessler's reagent (27).

**DISCUSSION**

Various physico-chemical factors are known to affect the growth of the LAB. All of these factors are at the tolerance limit of the bacteria and their negative effects can be additive.

Ten strains of ropy lactobacilli were tested for their abilities to grow at different pH values, temperature and SO₂ and ethanol concentrations. Among chemical factors, ethanol is generally regarded as the principal inhibitor of bacterial growth and generally ethanol concentrations above 13% limit bacterial growth severely (13). All ropy strains grew well in the presence of ethanol even with a concentration of 15% and small differences were noted in the growth of all the strains tested at the different ethanol concentrations studied (Fig. 2). However, the growth of non-ropy reference strain 216 CECT was moderate at 4 and 7% no growth was detected at higher concentrations. Therefore, the ethanol concentrations found in Basque Country ciders can not act as inhibitors of these strains (Table 1). Our results are in agreement with Vaughn (25) who noted that the heterofermentative spoilage lactobacilli exhibited the greatest resistance to ethanol compared to other wine spoilage bacteria. This ethanol resistance has been also reported in other ropy strains of *P. damnosus* (16).

It is widely recognized that LAB are mesophilic organisms with a temperature optimum in the neighborhood of 25°C (2,13). But the growth of all ropy strains used in this study is not limited by low temperatures and, as can be observed in Fig. 2, they grew well at 10 and 15°C. Further laboratory testing confirmed that only temperatures above 42°C seriously limited their growth. On the other hand, at 28°C the development was also strong, so we can assert that these bacteria tolerate a wide range of temperatures. In addition, we have also observed that the loss of viscosity in the culture medium is faster at high-incubation temperatures than at temperatures of 10 to 15°C. In consequence, the low storage temperatures of the Basque Country cider producers will not prevent the development of this alteration.
The pH is another factor affecting LAB growth, this factor being one of the most important that controlled the development. The optimum pH for growth is between 4.3 and 4.8 (2) that is, higher than the normal pH of cider. A pH of 3 or lower practically prevents all growth; its increase always favors it (13). All ropy strains showed similar behavior at the different pHs tested. They exhibited
good development at pH 4, but the best growth was obtained at pH 4.8 (Fig. 2). However, pHs below 3.7 affected growth seriously and lower pH, around 3 to 3.3 completely inhibited their development. Hence, our results are in agreement with data from several authors who have indicated that the growth of lactobacilli strains does not occur in wines of pH below 3.5. Values of pH usually found in ciders vary from 3.8 to 4 and so the growth of these strains will not be affected by this factor.

Sulfur dioxide is widely used in wine and cidemaking as an antioxidant, as well as to control the growth of wild yeasts and bacteria (1,27). Sulfur dioxide can also be bactericidal depending greatly on the pH, concentration and the organism (3,9). In our study, the tolerances of the strains to SO2 were examined at 0, 15, 25, 50, 75 and 100 mg/L at pH 3.8. As showed in Fig. 3, large differences between ropy strains with regard to SO2 tolerance were not observed. All strains were inhibited by SO2, as evidenced by bacterial growth delays that increased with the SO2 concentration. In the presence of 10 mg/L total initial SO2, the growth started with a delay of between 5 and 9 days when compared to the corresponding control. Logarithmic growth was achieved by all strains within 12 to 15 and 23 to 32 days after inoculation at 25 and 50 mg/L total SO2, respectively. We also observed a slight decrease in growth achieved at 25 and 50 mg/L total SO2. At higher concentrations (75 and 100 mg/L total SO2), all strains were unable to grow after 80 days.

CONCLUSION

Ropy strains of heterolactic Lactobacillus spp. were isolated from bottled spoiled ciders produced in the Basque Country. They produced a polysaccharide that increased the viscosity of liquid medium. The bacteria tested grew in the presence of 10, 25 and 50 mg/L of total SO2 (pH 3.8) under the conditions examined. All ropy strains tolerated high ethanol concentrations and grew well at low temperatures (10 and 15°C). In consequence, as the ciders from the Basque Country, which are not microbiologically stabilized, have low ethanol concentrations (about 6%) and are stored at room temperature, they can undergo significant spoilage. However, the ropy lactobacilli studied were affected by the factor pH, and in general a pH below 3.7 limited their growth seriously. Therefore, this disorder could be prevented by adequate sulphiting, increasing the acidity of low-acid juices and ciders by either blending or fruit selection, and sterile filtration or pasteurization.

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