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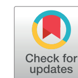
# *Leuconostoc mesenteroides* in the brewing process: A controversial role



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ABSTRACT

The aim of this study was to characterize 29 *Leuconostoc mesenteroides* isolates obtained from a brewing process in order to determine their potential contributions to this process and the product. In order to achieve this aim different enzymatic activities, the exopolysaccharide (EPS) production, the antagonistic activity, the beer spoilage ability and the production of lactic acid and diacetyl in beer, were assessed. *Ln. mesenteroides* isolates showed weak acidifying activity, and only 28% of them produced decreases in pH higher than 0.5 units after a 24 h period of incubation. The isolates were also scarcely proteolytic and did not produce EPS. Results from enzymatic activity assay showed that some of the isolates produced β-galactosidase and α- and β-glucosidases while none produced β-glucuronidase.

All the isolates inhibited the growth of both *Escherichia coli* and *Staphylococcus aureus*, although none of them inhibited *Pediococcus acidilactici*. When *Lactobacillus brevis* was used as an indicator, 44.8% of the isolates showed antagonistic activity. The beer-spoilage ability assay showed that only 5 of the 29 isolates were able to grow in beer but none of them harboured *horA* or *horC* genes. However, the growth of these isolates in beer did not produce an excess of D-lactic acid or diacetyl.

These findings show that the presence of any of the characterized isolates during the brewing process or in the bottled beer would not have a negative impact. On the contrary, the findings reveal interesting and potentially beneficial effects.

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## 1. Introduction

Beer has been recognized for centuries as a safe beverage. However, the presence of microorganisms, both bacteria and yeasts, different from those used to carry out the fermentation, has been displayed at various stages of the production process. Among the bacteria, both Gram (+) and Gram (−) are present, with lactic acid bacteria (LAB) being isolated at almost every stage of the malting and brewing process (Bokulich & Bamforth, 2013). The role of LAB in this process is controversial and they are recognized as the most hazardous bacteria in breweries, responsible for approximately 70% of the microbial beer-spoilage incidents. During growth, they produce an excess of turbidity and acidity as well as off-odours, due to the production of diacetyl and hydrogen sulfide (Fernandez & Simpson, 1995; Hartnett, Vaughan, & van Sinderen, 2002; Sakamoto & Konings, 2003), which negatively affect the quality of beer, producing important economic losses for the brewing industry (Back, 1994; March, Manclús, Abad, Navarro, & Montoya, 2005; Sakamoto & Konings, 2003; Vaughan, Eijsink, O'Sullivan, O'Hanlon, van Sinderen, 2001).

However, some authors affirm that it is not categorically possible to define LAB as having a negative impact as they may perform some necessary and beneficial functions (Bokulich & Bamforth, 2013; Haikara, Uljas, & Suurnäkki, 1993). Through their ability to produce antimicrobial substances, these bacteria may prevent the growth of harmful organisms improving safety of beer (Boivin & Malanda, 1997; Haikara & Laitila, 1995). Positive effects of LAB in food, including some beverages, are well known (Okada, 2002), but these bacteria have rarely been explored in the brewing process.

LAB in beer belong mainly to the *Lactobacillus* and *Pediococcus* genera (Bokulich & Bamforth, 2013; March et al., 2005; Sakamoto & Konings, 2003), but the presence of isolates belonging to *Lactococcus* and *Leuconostoc*, albeit in lower frequency, has also been shown (Garofalo et al., 2015; Justé et al., 2014; Suzuki, Asano, Iijima, & Kitamoto, 2008).

*Leuconostoc* is a genus whose presence in some fermented foods contributes to the organoleptic properties since it produces

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acetaldehyde, diacetyl and acetoin from lactate and citrate (McSweeney & Sousa, 2000). In addition, it possesses other capabilities, which enable it to produce dextrans from sucrose (Vedamuthu, 1994) and different enzymes, such as proteinases, lipases and aminopeptidases (Macedo & Malcata, 1997). In the brewing process, *Leuconostoc* species have been described to be present in both malt (Kaur, 2009; Vaughan et al., 2001) and during the malting process (Bokulich & Mills, 2012; Justé et al., 2014) yielding an improvement in malt quality, which may shorten lautering and wort filtration time (Haikara et al., 1993). On the other hand, Giles-Gómez et al. (2016), in a study carried out on a Mexican alcoholic beverage different from beer, reported that the presence of *Leuconostoc* species can be beneficial due to their probiotic properties. However, Suzuki et al. (2008) reported their potential spoilage ability, as ropy slime producers; though it should be noted that the frequency of spoilage incidents by isolates of *Leuconostoc* in beer is lower than 1%.

A recent study by Poveda, Ruiz, Seseña, and Palop (2017) showed the presence of *Leuconostoc mesenteroides* strains in samples taken at different stages during a craft brewing process and the objective of this study was to assess these strains in order to understand their potential positive and negative impact on the process or on the product.

## 2. Material and methods

### 2.1. Bacterial isolates and growth conditions

Twenty-nine *Leuconostoc* (*Ln.*) *mesenteroides* isolates were assessed. They had been previously isolated from samples taken at different stages during a craft brewing process (Poveda et al., 2017) and were maintained frozen (−80 °C) in MRS (de Man, Rogosa, & Sharpe, 1960) broth (Scharlab, Barcelona, Spain) containing 20% (v/v) glycerol. Isolates were revitalized by cultivation in the same broth and incubated at 30 °C for 48 h in aerobic conditions.

### 2.2. Characterization of isolates

#### 2.2.1. Enzymatic activities

The acidifying and proteolytic activities were tested as described by Nieto-Arribas, Seseña, Poveda, Palop, and Cabezas (2010). For the acidifying activity, pH was measured using a Crison pH-meter (Crison, Barcelona, Spain) after 4 h and 24 h of incubation at 30 °C, and the values were expressed as ΔpH. For the proteolytic activity, the o-phthaldehyde (oPA) method as described in Church, Swaisgood, Porter, and Catignani (1983) was used and the results were expressed as mM glycine/L.

For the autolytic activity, cells from exponentially growing cultures (Optical Density (OD) at 650 nm = 0.7–0.8) in MRS broth were harvested by centrifugation at 10,000×g for 10 min at 4 °C and the pellet washed and resuspended in 20 mM sodium phosphate buffer (pH 6.8). The cell suspension was incubated at 30 °C and the lysis was monitored after 30 min, 4 h and 24 h of incubation by recording the decrease in $OD_{650}$ using a Beckman DU-530 spectrophotometer. The percentage of lysis was determined as 100 - (A1/A2 x 100), where A1 and A2 were the lowest and the highest value of the $OD_{650}$ measured during incubation, respectively.

For all tests, three cultures of each isolate were analysed and the measurements were performed in duplicate.

The presence of another 19 enzymatic activities was assessed using the API-ZYM galleries (BioMérieux, Marcy l'Etoile, France) in accordance with the manufacturer's instructions. After incubation, ZYM A and ZYM B reagents were added and a positive reaction was recorded when there was a change of colour. The intensity of the colour was rated as light (+), medium (++) or dark (+++).

#### 2.2.2. Exopolysaccharides (EPS) production

Exopolysaccharide (EPS) production was tested as described by Ribeiro et al. (2013) using both, MRS agar medium containing 2% (w/v) of one of the following carbon sources: sucrose, glucose, lactose or fructose (all purchased from Oxoid) and Mayeux agar (BIOKAR-Diagnostics, Solabia Group, Francia).

Five microlitres of the revitalized isolate in MRS broth were inoculated in each of the plates as a drop and plates were incubated at 30 °C for 48 h, both in aerobic and anaerobic conditions (GenBox/GenBag BioMérieux). Mucoidy of colonies was determined by visual appearance and isolates producing slimy colonies were regarded as positive (Smitinont et al., 1999).

#### 2.2.3. Antagonistic activity against potential spoilage bacteria

The spot test, as set forth in Garcia et al. (2016) with slight modifications, was used. Five microliters of an overnight culture in MRS broth of the analysed isolate were spotted on the surface of MRS agar plates and incubated at 30 °C for 24 h in aerobic conditions.

Simultaneously, cultures of the bacteria used as indicators in an appropriate media were prepared and incubated in optimal conditions until reaching absorbance values at 600 nm (BECKMAN DU 530 spectrophotometer) between 0.8 and 1 (approximately 8 log CFU/mL). The strains used as indicators and the conditions used for their growth were as follow: *Lactobacillus* (*Lb.*) *brevis* CECT 4121$^T$ and *Pediococcus* (*P.*) *acidilactici* CECT 5765$^T$, both cultivated in MRS broth at 30 °C, *Escherichia* (*E.*) *coli* CECT 45 and *Staphylococcus* (*St.*) *aureus* CECT 86$^T$, both cultivated in Tryptone Soy Broth (TSB) at 37 °C. All were purchased from the Spanish Type Culture Collection (CECT).

An adequate volume of the indicator bacteria culture was mixed with 7 mL of soft (0.7% w/v agar) MRS or Trypticase Soy Agar (Pronadisa), depending on the species, to reach a final viable count of $10^6$ CFU/mL and the mixture was poured over the spot-inoculated MRS agar plates. The plates were again incubated in optimal conditions for each indicator, and the antagonistic activity was recorded as the diameter (mm) of growth inhibition zones around each spot. As reported by Rouse, Sun, Vaughan, and Sinderen (2007) five possible results were recorded: (−) no inhibition halo was observed; (+) diameter of halo $\leq$ 1 cm; (++) diameter of halo between 1.1 and 1.9 cm; (+++) diameter of the inhibition halo between 2 and 2.9 cm and (++++) diameter of inhibition halo $\geq$ 3 cm.

#### 2.2.4. Beer spoilage ability

The beer spoilage potential was tested as described by Suzuki (2002). Degassed Ale beer (pH 4.4; 4.8% (v/v) alcohol content; 25 IBU) was adjusted to pH 4.2 with 1 M NaOH and sterilized by filtration (0.45 μm pore size filters; Sartorius Stedim Biotech, Goettingen, Germany). Fifteen millilitres of the beer were dispensed in sterile polypropylene tubes and inoculated with a culture of the isolate analysed to reach approximately $10^5$ cells/mL. The inoculated beers were incubated anaerobically at 30 °C for up to 60 days. Non-inoculated beer was used as a control.

A sample of beer was taken weekly and serially diluted in sterile saline solution, in order to be plated in duplicate on MRS agar. Plates were incubated aerobically at 30 °C for 48 h and the counts expressed as colony forming units (CFU) per mL of beer.

In order to confirm that colonies grown on plates corresponded to the inoculated isolates, 10% of the total number of colonies from countable MRS agar plates were picked at random and together with a colony of the inoculated isolates grown on the same medium typed by RAPD-PCR (Randomly Amplified Polymorphic DNA

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