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Occurrence of biogenic amine-forming lactic acid bacteria during a craft brewing process

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ABSTRACT

The aims of this study were to assess the lactic acid bacteria population present during a craft brewing process and to determine their potential to produce biogenic amines (BA). Samples at different stages were taken and the isolates grown on MRS agar plates were genotyped by randomly amplified polymorphic DNA-PCR and identified by MALDI-TOF as belonging to *Leuconostoc mesenteroides* (80%), *Lactobacillus brevis* (15%) and *Enterococcus faecium* (5%).

To assay the aminobiogenic capacity of the strains, both a qualitative assay, using a synthetic medium, and a quantitative assay, using wort and beer, were carried out and contents of different BA and free amino acids (FAA) were measured by RP-HPLC. Presence in strains of *tdc*, *hdc*, *odc* and *ldc* decarboxylase genes was assayed by multiplex PCR.

Tyramine was the only BA produced by all the strains in the qualitative assay while the 90% did it significantly in wort and beer. Concentrations of the remaining BA analyzed did not change significantly. In contrast, all the strains harboured the *tdc*, *hdc*, *odc* decarboxylases genes. Total BA content in the beers ranged between 30.3 and 48.3 mg/L. None of the strains produced any significant change in total FAA content in wort or beer.

1. Introduction

Craft beers are beverages elaborated using traditional methods in small, independent breweries which interpret historic styles with unique twists and also develop new styles that have no precedent. Craft beer is generally made with traditional ingredients like malted barley though interesting and sometimes non-traditional ingredients are often added for distinctiveness. Fermentation process can also vary between breweries but in essential it comprises the steps shown in Fig. 1 (malting of barley, maceration, boiling of the wort obtained in the previous step, cooling and addition of hop and yeasts fermentation). Currently in Europe, and specifically in Spain, the production of craft beer is experiencing a strong growth and in the last decade several hundred microbreweries have emerged.

For centuries, beer has been considered a safe beverage, since it constitutes an unfavourable medium for the growth of most microorganisms due to its low pH, low oxygen and nutrient contents, high CO_2 content and the presence of ethanol and antibacterial hop compounds. In spite of that, some undesirable microorganisms still manage to grow, either during brewing or in beer, causing an increasing of turbidity and unpleasant sensory changes in the beer (Li, Wang, & Liu, 2017). Among beer spoilage bacteria, both Gram-positive and Gram-negative bacteria are found, but hop-resistant lactic acid bacteria (LAB) of *Lactobacillus* and *Pediococcus* genera, are reported as the most frequent and harmful beer spoilage microorganisms (Bokulich & Bamforth, 2013). However, the presence of other LAB of the genera *Leuconostoc*, *Lactococcus* and *Weissella* has been described, with *Leuconostoc* being an abundant genera both in malt (Kaur, 2009, p. 230) and during malting (Bokulich & Mills, 2012; Justé et al., 2014).

One matter of concern related with the presence of LAB in beer is the production of biogenic amines (BA), low molecular weight compounds formed by decarboxylation of amino acids with the participation of substrate specific enzymes. It is well-known that the ability of LAB to decarboxylate amino acids is a strain-

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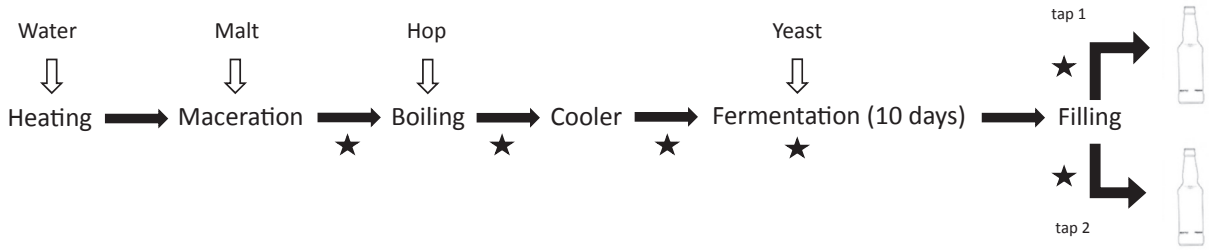

Fig. 1. Craft brewing process. Stars in the figure indicate where samples were taken.

dependent property, and that factors such as the environmental conditions can influence in this reaction (Ladero et al., 2012). BA are compounds that can cause health problems (Ladero, Calles-Enríquez, Fernández, & Álvarez, 2010) even if ingested in relatively low amounts, and their presence has been reported in many fermented foods including meat, cheese, wine, cider and beer (Guo, Yang, Peng, & Han, 2015; Jairath, Singh, Dabur, Rani, & Chaudhari, 2015; Ladero et al., 2011; Pérez-Martín, Seseña, Izquierdo, & Palop, 2014; Poveda, Molina, & Gómez-Alonso, 2016; Prádenas, Galarce-Bustos, Henríquez-Aedo, Mundaca-Uribe, & Aranda, 2016). At this respect, special attention should be paid to alcoholic beverages since the activity of monoamine oxidase, the enzyme responsible of detoxification of BA in the organism, can be suppressed by ethanol (Ten Brink, Damink, Joosten, & Huis in 't Veld, 1990). Tyramine is, together with histamine, one of the most toxic and commonly found BA in foods, causing different toxicological effects such as headaches, migraine, neurological disorders, nausea, vomiting, respiratory disorders and hypertension (Ladero et al., 2010).

The objective of this study was to isolate and to identify LAB present during a craft brewing process to assess their potential to produce BA. For this last purpose a qualitative assay, using a synthetic medium, and a quantitative assay, using wort and beer, were carried out. In addition, the presence in the LAB of the genes encoding some amino acid decarboxylases was also assayed by multiplex PCR.

2. Materials and methods

2.1. Sampling and LAB isolation

A total of 16 samples of a Pale Ale beer were taken in a small craft brewery located in Toledo (Spain) at different stages of the production process: after maceration, after boiling of the wort, after cooling, daily during fermentation and in the filling taps (Fig. 1). Fifty milliliters of each sample were taken under aseptic conditions, which were maintained refrigerated while transported to the laboratory and immediately analyzed.

The samples were serially diluted in sterile saline solution and plated in duplicate on MRS agar (De Man, Rogosa, & Sharpe, 1960) (Scharlab, Barcelona, Spain) supplemented with 100 mg/mL cycloheximide (Sigma, USA) for growth of LAB, and Plate Count Agar (PCA, Scharlab) for total counts of viable bacteria. MRS agar plates were incubated anaerobically (Gas Pack System, Oxoid Ltd., Basingstoke, Hampshire, UK) at 30 °C for 5 days while PCA plates were incubated aerobically at 30 °C for 48 h. Counts were expressed as colony forming units per mL of sample (CFU/mL).

From each countable MRS agar plate, between five and seven isolated colonies were picked at random and purified by successive streaking on the same medium. Pure cultures were stored at −80 °C with 20% (v/v) glycerol (Panreac, Barcelona, Spain).

2.2. Genotyping of isolates by randomly amplified polymorphic DNA-PCR (RAPD-PCR) analysis

Genomic DNA was obtained from well-developed single colonies on MRSA and the RAPD-PCR analysis using the M13 primer (5′-GAGGGTGGCGGTTCT-3'; Integrated DNA Technologies, Inc., Coralville, USA) was performed (Pérez-Martín et al., 2014). A reproducibility study to determine the minimum percentage of similarity (r) necessary for strain discrimination was carried out as described by Pérez-Martín et al. (2014).

RAPD-PCR gels were photographed with a KODAK DC290 Zoom Digital Camera (Eastman Kodak Company, USA). The patterns were normalised and further processed with the GelCompar version 4.0 analysis software (Applied Maths, Kortrijk, Belgium). Isolates were grouped using the Pearson product-moment correlation coefficient and cluster analysis by UPGMA (Unweighted Pair Group Method with Arithmetic Average).

2.3. Identification of isolates

Isolates were identified by matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry in a Vitek-MS Instrument (Bio-Merieux, Marcy-l'Etoile, France) in the facilities of Probisearch (Tres Cantos, Madrid, Spain). External calibration of mass spectra was performed using *Escherichia coli* ATCC 8739 standard peaks. Mass spectra were processed using VITEK-MS Acquisition™ software (Vitek MS, Biomerieux, France). For each isolate, a mean spectrum was constructed with at least 50 m/z spectra profiles and used for the identification by comparison with the spectra database Myla™ (Biomerieux, France). Identification was defined as a 99–100% match to the species-specific m/z values in the database.

2.4. Qualitative assay of biogenic amine production using synthetic medium

The potential to produce tyramine, histamine, putrescine (via ornithine decarboxylase) and cadaverine was assessed by using both liquid and solid decarboxylase medium (DM) (pH 5.3) (Bover-Cid & Holzapfel, 1999).

Before analysis, and to promote enzyme induction, strains were passaged six times in MRS broth added with 0.1% (w/v) of each precursor amino acid (L-histidine monohydrochloride, L-ornithine monohydrochloride, tyrosine disodium salt and L-lysine monohydrochloride) purchased from Sigma (St. Louis, USA) and 0.005% (w/v) of pyridoxal-5-phosphate, and incubated at 30 °C for 48 h.

For assays in DM broth, sterile 96-well polystyrene microtiter plates were fulfilled with 200 μL of DM broth and inoculated with 10% (v/v) of induced overnight culture of the strain in MRS broth (described above). For assays in DM agar plates, 5 μL of the induced overnight culture were spotted on the agar. Both DM broth and DM agar plates without amino acids were used as controls. All assays