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Prevalent lactic acid bacteria in cider cellars and efficiency of *Oenococcus oeni* strains

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ABSTRACT

Malolactic fermentation (MLF) is an important step in cider production in order to allowing for improvement of microbiological stability and organoleptic characteristics of cider. Induction of this fermentation by using starter cultures enables a better control over this bioprocess, but although it is a common practice in winemaking, starters specifically focussed for cider MLF are not yet commercially available. Proper starter cultures need to present the ability to degrade L-malic acid conferring pleasing sensory characteristics while avoiding toxicological risks. In this work, lactic acid bacteria (LAB) were first isolated from MLF industrial cider samples, obtained in a cellar in the main cider-producing region of Spain, Asturias. Isolates, identified by molecular tools, belonged to the *Lactobacillus brevis* and *Oenococcus oeni* species. After a phylogenetic analysis, representative strains of both identified species were evaluated in order to determine their fermentation capacity, showing *O. oeni* the best behaviour in this cider fermentation, as previously demonstrated for wine in the literature. Consequently, and with the aim to test the influence at strain level, selection of *O. oeni* isolates as starters for cider fermentation has been undergone. In order to check the influence of geography over biodiversity, *O. oeni* strains from six different industrial cellars representing the distinct producing areas in the region (located in a ratio of 30 km) were analyzed by using a specific RAPD method. In this way, isolates were typed in five distinct groups, mainly corresponding to each producing area. All strains isolated from the same cellar showed the same RAPD profile revealing the significance of geographical origin in the indigenous cider LAB. Molecular tools were applied to reject those isolates exhibiting presence of genes related to organoleptic spoilage (exopolysaccharides and acrolein production) or food safety (biogenic amine production), as key selection criteria. Representative strains of each of the five *O. oeni* RAPD groups were tested as pure cultures to evaluate their technological utility for cider production. Experimental data of malic acid degradation and cell concentration obtained were fitted to previously selected kinetic models aimed to optimization and prediction of bioprocess performance. Four strains revealed as suitable potential starter cultures for conducting MLF in cider production.

1. Introduction

The food industry is expected to produce safe, healthy and nutritious products of high quality. For many food products, fermentation with starter cultures containing LAB is an essential part of the production process (Smid et al., 2005). Cider production in Asturias (a north-western region in Spain) is made via a spontaneous fermentation, interactions between microorganisms are not controlled during the process, and completing MLF by indigenous microbiota may take several months or it may occur in some tanks but not in others. Induction of the MLF using selected bacterial starter cultures is becoming the best option at industrial level. However, the application of starter cultures for the MLF has not been employed yet in cider production, due to the lack of adapted cultures (Xu et al., 2005) commercially available to conduct this fermentation. Asturias manufactures 80% of the total cider production in the country, which meant around 50,000 tons in 2009.

In winemaking, as a result of natural selection, *Oenococcus oeni* becomes the dominant species among those triggering MLF (Bon et al., 2009). Consequently, winemaking practices involve inoculation of wine with commercially prepared strains of *O. oeni*

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(Delaquis et al., 2000). Nevertheless, it is important to realize that not all strains of this species are good candidates to be used as starters. It has been reported that the lack of the mismatch repair (MMR) genes (*mutS* and *mutL*) in the genus *Oenococcus* (Marcobal et al., 2008) facilitates the generation of isolates with beneficial mutations, resulting in increased fitness for the wine environment (Bon et al., 2009). Thus, differentiation at strain level becomes a major concern, since their adaptation to wine and their influence on the organoleptic quality of the product are strain-specific (Britz and Tracey, 1990). There is also evidence that, in wine, the intra-specific biodiversity of *O. oeni* can be related to the geographical origin of the strains (Granchi et al., 2007).

Traditionally, the selection criteria for malolactic starters have been based only on classic tests of survival in wine and on the ability of L-malic acid consumption (Henick-Kling et al., 1989). Nowadays, desirable sensory attributes and reduction of toxicological risks are considered important criteria along with an efficient malolactic activity. The main spoilage compounds in cider and wine are exo-polysaccharides (EPS) and acrolein (from the organoleptic perspective), as well as biogenic amines (BA) (from the food safety perspective). Their production must be prevented in order to assure a high quality final product, because they can cause food poisoning if large amounts are ingested, or if the natural human mechanisms for their catabolism are inhibited or genetically deficient (Silla-Santos, 1996). Within the same species, the presence, the activity and the specificity of decarboxylases are strain-specific (De las Rivas et al., 2008). Since some *O. oeni* strains had been described showing the ability to produce BA, a lack of amino acid decarboxylase activity has been included in the starter cultures selection criteria (Rosi et al., 2009). Based on the increasing interest on public health concerns, nowadays the selection criteria related to safety should be located in the first place, along with the lack of spoilage related genes when seeking for industrial implementations.

In contrast to wine, little is known about microbial ecology and physiology of MLF in cider production. Although similarities with wine have been well established since long, a previous work (Sánchez et al., 2010) on population dynamics of MLF sets forth differences in cider at species level, without showing a clear predominance of a particular species during the process but rather an alternation in their ecological succession.

In this work, indigenous lactic acid bacteria (LAB) were isolated from MLF industrial cider samples, and identified by molecular tools. After a phylogenetic analysis, representative strains of identified species were evaluated in order to determine their fermentation capacity. Secondly, further work was focused on characterization at strain level, considering geographical biodiversity, in order to identify their biotechnological potential for efficiently conducting MLF in cider fulfilling the requirements previously exposed.

2. Materials and methods

2.1. Sampling and LAB isolation

At the beginning of the MLF samples were collected in duplicates from a local industrial cellar. Two oak vats and one refrigerated stainless steel vat were selected for sampling. The selected cider-making cellar had never used commercial starters neither for the MLF nor for the alcoholic fermentation. Samples were taken under aseptic conditions, from the sampler located one third in the way from the bottom of the stainless steel tank or alternatively, located at the bottom of the oak tank, after getting a homogeneous stream by rejecting the initial flow.

In order to isolate LAB, each sample was independently processed by two different techniques: by direct plating on MRS (Biokar Diagnostics), supplemented with 5% (v/v) ethanol (SIGMA) and 100 ppm of pimaricine (VGP Pharmachem) to inhibit yeast growth, and alternatively, plating on the same medium just after sample reactivation in liquid MRS (Biokar Diagnostics). In the second technique, 1 mL of the cider sample was inoculated in 9 mL of MRS and incubated at 30 °C for 4 h. Enumeration of LAB, in both cases, was performed by the standard plate-counting method. Before plating, cells were sonicated for 5 min followed by vortexing for 30 s (times optimized to assure no viability reduction) in order to avoid aggregates. Plates were incubated under anaerobic conditions (CO_2 gen, OXOID) to inhibit acetic acid bacteria, at 30 °C for 15 days.

2.2. Species identification

For molecular identification, colonies were randomly picked from Petri dishes containing about 50 colonies. Preliminary identification of LAB was carried out on the basis of Gram staining, morphological microscopic observation and catalase activity. Gram-negative or catalase-positive isolated colonies were rejected. Selected colonies were inoculated independently into MRS broth and incubated overnight at 30 °C. For LAB identification the protocol described by Sánchez et al. (2010) was followed.

In order to carry out phylogenetic analyses, sequences were aligned using Clustal W (Thompson et al., 1994) as implemented in BioEdit Sequence Alignment Editor (Hall, 1999). The results were manually adjusted to maximize the number of homologous characters and minimize the number of insertions and deletions. After alignment, COLLAPSE 1.2 (D. Posada, http://darwin.uvigo.es) was used to reduce the data set to unique genotypes for phylogenetic analyses. For genetic distance analyses, PHYLIP version 3.5 (Felsenstein, 1993) was used; distance matrices were prepared using the Kimura 2-parameter model and trees constructed using the neighbour-joining method.

2.3. Fermentation process

Alcoholic fermentation was performed as described in Sánchez et al. (2010). The fermentative capacity essays were carried out as described in Sánchez et al. (2010), with the only exception that the yeast fermented medium (green cider) was inoculated at a final concentration of 10^7 CFU/mL of each preculture. Software Micro-Math Scientist for Windows Version 2.0 was used to fit experimental data.

2.4. Analytical methods

To follow MLF evolution, samples were collected approximately each 12 h. For each sampling time, duplicates were collected and submitted to microbiological and chemical analyses. Microbiological counts of viable cells were performed by the standard plate-counting method. For each sampling time, total bacterial counts were performed in triplicates, in statistically significant dilutions (25–250 colonies per plate) in MRS plates supplemented as described in 2.1. Plates were incubated under aerobic conditions at 30 °C. Total biomass was also determined by following the optical density (OD_{660}) of the fermentation medium (UV 1203 model spectrophotometer; Shimazdu) *vs* a blank (the same medium without cells) and converted to dry weight by means of the corresponding calibration curve (D.O *vs* g/L).

For chemical analysis, samples were immediately filtered by membrane (0.45 μm pore size) and 2 mL-samples were frozen (−20 °C) until chromatography analysis. A pH meter (Crison micropH 2001) was used to perform three measurements on each