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Population dynamics of lactic acid bacteria during spontaneous malolactic fermentation in industrial cider

Ainoa Sánchez [a], Raquel Rodríguez [a], Monika Coton [b], Emmanuel Coton [b], Mónica Herrero [a,*], Luis A. García [a], Mario Díaz [a]

[a] Department of Chemical Engineering and Environmental Technology, Faculty of Chemistry. University of Oviedo, C/Julián Clavería s/n. 33071, Oviedo, Spain
[b] ADRIA Normandie, Bd du 13 juin 1944, 14310 Villers-Bocage, France

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ABSTRACT

A deeper knowledge on the complex microbial ecology of malolactic fermentation (MLF) in spontaneous cider production is essential to understand the cider making process, thus, applicable to develop adequate malolactic starters. Trends in food fermentation focussed on the isolation of proper wild-type strains from traditional products to be used as starter cultures, with the aim of conducting industrial production processes without losing their unique flavour and product characteristics. A mixed inoculum consisted of samples taken from the four main areas of the most traditional cider making region in Spain was used to carry out MLF under controlled laboratory conditions. A culture-based approach was used to investigate the diversity of lactic acid bacteria (LAB) by molecular tools. By monitoring MLF, population dynamics involved in the fermentation process were assessed. Interactions of different microorganisms adapted to a common environment, competition and resistance during the process, give rise to dominant species showing selective advantages. The main aim of this work was the identification of dominant species during the process in order to obtain potential starter cultures with a clear industrial utility. The fermentative capacity of a representative strain belonging to each isolated species, after analysis of interspecific polymorphisms in 16S rRNA sequencing of 84 isolates, was compared. Molecular characterization for the presence of gene coding for exopolysaccharide production (*dps* gene amplification), acrolein production (*pdu* gene amplification) and biogenic amine production (*tyrdc*, *hdc* and *odc* genes amplification) was carried out to verify the suitability of the selected strains.

1. Introduction

Isolation of wild-type strains from traditional products is a classical method to obtain starter cultures for food fermentations (Leroy & De Vuyst, 2004). By using selected wild-type strains, the large-scale production of fermented foods can be developed without losing their unique flavour and particular characteristics (Caplice & Fitzgerald, 1999). An adequate malolactic fermentation (MLF) process is fundamental to guarantee a high standard quality cider. Still now starter cultures of lactic acid bacteria (LAB) are not employed to ensure its correct development in cider, as occurs in the wine industry. Due to this fact, it is important to develop suitable malolactic starter cultures, to carry out the process in a controlled way. Starter cultures should be well adapted indigenous LAB involved in the traditional process (Herrero, de la Roza, Garcia, & Diaz, 1999). Deeper knowledge on the complex microbial ecology during spontaneous MLF in traditional cider results is therefore vital. Microbial succession is a universal phenomenon observed in spontaneous fermentation processes. This phenomenon is a reflection of microbial interactions, competition for intrinsic growth factors such as nutrients, and resistance to inhibitory environmental conditions such as high acidity. Consequently, microorganisms showing a selective advantage emerge in a given period as the dominant populations during fermentation. Knowledge of such microbial interactions in spontaneous fermentations provides a fundamental understanding of the process (Singh & Ramesh, 2008). Over the last years, several approaches have been made to investigate the microbial diversity of complex ecosystems, like food ecosystems (Aquilanti et al., 2007).

Several ecological studies have been done in wine in order to identify microorganisms involved in MLF, nevertheless little is known about cider MLF ecology. As Zhang and Lovitt (2006) reported, cider making is very similar to wine making, using fruits containing many of the same constituents and employing similar processing techniques. In fact, Morrissey, Davenport, Querol, and Dobson (2004) claimed that there is great similarity between yeast and population dynamics involved in both processes, as previously reported (Beech, 1972, 1973; Davenport, 1974; Williams, 1974; Salih & Drilleau, 1988; Fleet, 1990; Vaughan-Martini & Martini, 1995; Le Quere & Drilleau, 1996; Laplace, Apery, Frere, & Auffray, 1998; Mortimer & Polsinelli, 1999). Wine studies worldwide reported *Lactobacillus*, *Oenococcus*, *Pediococcus* and

* Corresponding author. Tel.: +34 985 103439; fax: +34 985 103434.
E-mail address: herreromonica@uniovi.es (M. Herrero).

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Leuconostoc as the genera involved in MLF (Izuagbe, Dohman, Sandine, & Heatherbell, 1985; Sieiro, Cansado, Agrelo, Velazquez, & Villa, 1990; Edwards, 1992; Patarata, Serpa-Pimentel, Pot, Kersters, & Mendes, 1994; Juhasz, 1994; Kosseva, Beschkov, Kennedy, & Lloyd, 1998).

LAB taxonomy is particularly striking. Similar nutritional requirements of different species belonging to this group, due to the adaptation to a particular environment, hamper identification by traditional methods. Due to this fact, LAB identification, when exclusively based on physiological and biochemical characteristics, is intrinsically ambiguous (Settanni, van Sinderen, Rossi, & Corsetti, 2005), and results can change drastically when based on genotypic data (Sohier, Berthier, & Reitz, 2008). The use of molecular methods for the identification and characterization of microorganisms in foods and beverages is currently considered as the most reliable tool for the accurate description of the microbial ecology (De Vuyst & Vancanneyt, 2007).

Ribosomal DNA genes are the most frequently used targets (Coenye, Gevers, Van de Peer, Vandamme, & Swings, 2005). In particular, analysis of 16S rRNA genes, aided by PCR to amplify target sequences in environmental samples, has enabled molecular ecologists to provide better estimates of bacterial diversity (Amann, Ludwig, & Schleifer, 1995).

In cider, as in wine, *Lactobacillus* and *Oenococcus* were described as the predominant genera during MLF, finding *Pediococcus* in low proportion (Cabranes, Moreno, & Mangas, 1991; Dueñas, Irastorza, Fernandez, Bilbao, & Huerta, 1994). It must be kept in mind that these studies were only based on phenotypic and morphological criteria. In the first work, samples were taken from three different cellars over two consecutive years. In the second one, the evolution of LAB was examined following two cider making methods: the traditional one, employing unwashed apples of different varieties, and a modified method using a sole washed acidic apple variety with temperature control during the cider making process.

De Vuyst and Neysens (2005) described the influence of geography on LAB taxa distribution in sourdoughs (a fermentation process based on traditional practices), this distribution being highly variable from one ecosystem to another. As cider production is also the result of traditional practices in specific regions, it can be considered that geography may also affect the process. Similarly, in wine there is evidence that the intraspecific biodiversity of *Oenococcus oeni* can be related to the geographical origin of the strains (Granchi, Guerrini, Mangani, & Vincenzini, 2007). Due to these facts, in this study, the four main areas of traditional cider making in the region, the first producer in Spain, were considered for the preparation of a representative inoculum which collects the geographical microbial biodiversity of the region.

The identification of bacteria species involved in MLF during cider production is necessary in order to select strains which could be employed as an autochthonous starter culture. So, a culture-based approach was used to investigate the diversity of LAB by molecular tools. A total of 84 Gram-positive and catalase-negative isolates were identified during the process. LAB population dynamics during the MLF could be assessed. Additionally, the fermentative capacity of representative strains belonging to each isolated species was compared. A molecular characterization for exopolysaccharide production, acrolein production and biogenic amines production was also performed.

2. Materials and methods

2.1. Sampling and inoculum preparation

In order to prepare a representative inoculum from different independent spontaneous fermentations, the four main areas of traditional cider making in Asturias (locally distant approximately 30 km) have been considered for sampling. Samples at the beginning of MLF were collected from refrigerated stainless steel vats from six industrial cellars previously selected. The selected cider making cellars had never used commercial starters (neither for MLF nor for alcoholic fermentation). Samples were taken under aseptic conditions and immediately transported to the laboratory. Firstly, 25 mL for each sample were mixed and centrifuged (4000 g, 10 min). The pellet was resuspended in 5 mL of sterile green cider (apple must just after the alcoholic fermentation was completed). This volume was used as inoculum for the MLF, which was carried out under controlled laboratory conditions. Enumeration of LAB was performed by the standard plate-counting method in MRS (Biokar Diagnostics) supplemented with 5%(v/v) ethanol (SIGMA) and 100 ppm of pimaricine (VGP Pharmachem) to inhibit yeast growth. Plates were incubated under anaerobic conditions (CO_2 gen, OXOID) to inhibit acetic acid bacteria, at 30 °C for 15 days. Additionally, yeasts were enumerated in YPD agar. At the moment of inoculation, LAB population was 3.7 10^4 CFU/mL, and yeast population was 4.2 10^5 CFU/mL, in the starter culture.

2.2. Fermentation process

2.2.1. Alcoholic fermentation

Concentrated apple must (supplied by an industrial cider factory) was reconstituted with distilled water (1:5) and sterilized in a tangential flow filtration device (Pellicon 2, Millipore) using polyethersulfone membranes (0.22 μm pore diameter). An active dried commercial preparation of *Saccharomyces cerevisiae* var. *bayanus* (strain Pasteur Institute, Paris, 1969, supplied by Novo ferment, Switzerland) was rehydrated in sterile apple juice and grown under aerobic conditions (250 rpm, 28 °C for 18 h). Apple must was then inoculated with yeast at a final concentration of 10^6 CFU/mL. Alcoholic fermentation under controlled laboratory conditions was performed in static at 15 °C and it was considered ended when specific gravity reached 1006 g/L. After that, green cider was filtrated using a cellulose nitrate membrane (0.45 μm pore diameter) to remove yeasts. Final green cider characteristics were: pH 3.68, 5.6% v/v ethanol, reducing sugars 4.3 g/L and 4.8 g/L of malic acid.

2.2.2. Malolactic fermentation

Filtered green cider (200 mL) was inoculated with the inoculum prepared as previously described. Fermentations were carried out in two pre-sterilized 100 mL bottles filled to capacity, as independent experiments, at 15 °C, in static. MLF was considered completed when residual malic acid was approx. 0.5 g/L.

2.2.3. Fermentative capacity

Frozen stock cultures were first grown in MRS. Then, the selected LAB precultures were grown in apple juice, prepared as previously described, supplemented with yeast extract 0.5% (w/v) and sterilized by tangential flow filtration (0.22 μm pore diameter). LAB precultures were incubated in static 96 h at 30 °C. To start MLF, the green cider obtained as described (as in 2.2.1) was inoculated with 10^6 UFC/mL of each preculture, as duplicate independent fermentation experiments. Malolactic bioconversion was carried out as previously described and its evolution was controlled by pH and organic acids (malic and lactic acids) monitoring.

2.3. Analytical methods

To control MLF evolution, samples were collected alternatively from the two fermentation flasks, approximately each 12 h or 24 h, until the final of the process. For each sampling time, duplicates were collected and submitted to chemical, microbiological and molecular analyses.

To carry out chemical analysis, samples were immediately filtered by membrane (0.45 μm pore size) and frozen (−20 °C, in 2 mL-vials) until chromatographic analysis.

A pH meter was employed (Crison micropH 2001), performing three pH measurements on each sample. Malic and lactic acids were