LWT - Food Science and Technology 92 (2018) 312–317

Contents lists available at ScienceDirect

LWT - Food Science and Technology

journal homepage: www.elsevier.com/locate/lwt

Control of *Hanseniaspora osmophila* and *Starmerella bacillaris* in strawberry juice using blueberry polyphenols

Claudia Verónica Vallejo[a], Osvaldo Daniel Delgado[b], Graciela Celestina Rollán[c], María José Rodríguez-Vaquero[a,*]

[a] Faculty of Biochemistry, Chemistry and Pharmacy at the National University of Tucumán, UNT-CONICET, Ayacucho 471, Tucumán, Argentina
[b] CITCA, CONICET-UNCA, Faculty of Exact and Natural Sciences, UNCA, Prado 366, Catamarca, Argentina
[c] Cerela, Chacabuco 145, Tucumán, Argentina

ARTICLE INFO

Keywords:
Polyphenols
Antifungal activity
Berries
Spoiled juice
Yeasts

ABSTRACT

The aims of the present work were to isolate and identify the principal yeasts present in spoiled Argentine strawberry juice, identify polyphenols present in four blueberry cultivars and use these blueberry extracts in the control of yeasts using strawberry juice as food system model. *Hanseniaspora osmophila* and *Starmerella bacillaris* were identified for the first time in Argentine strawberry juice. The blueberry extracts assayed showed antifungal activity against *H. osmophila* and *S. bacillaris* through individual phenolic compounds such as quercetin, kaempferol and chlorogenic, ρ-coumaric and ellagic acid. The cytotoxicity assay demonstrated that the blueberries were not toxic to humans and that they did not modify the sensorial qualities of strawberry juice. No viable *S. bacillaris* and *H. osmophila* cells were detected after 7 days in strawberry juice supplemented with 150 μg/ml *Blue Crisp* or *Millennium* extract, inoculated with the isolated spoilage yeasts and conserved at 4 °C. This is the first evidence of *S. bacillaris* and *H. osmophila* in spoiled Argentine strawberry juice and blueberry extracts could be a good natural and non-toxic alternative to prevent growth of these yeasts. Blueberry extracts could be feasible alternatives to improve the microbiological quality without impact on the organoleptic properties of polyphenol-enriched strawberry juice.

1. Introduction

The growing importance of yeasts regarding food spoilage is partly because of the use of modern technologies in traditional food processing, but also as a result of numerous new food and beverage formulations and the tendency to reduce the use of preservatives to avoid yeast spoilage (Loureiro & Malfeito-Ferreira, 2003).

Tucumán is the leading producer of strawberries and blueberries in northern Argentina and consumption of strawberry juice is high. The contaminating microbiota that can be present in strawberry juice is mainly constituted of yeasts, as they can tolerate and grow at high osmotic pressure, low pH and low temperature. Some yeasts are extraordinarily resistant to chemical preservatives such as sodium benzoate and potassium sorbate (ICMSF, 1998) commonly used in the juice industry, which reduces the shelf-life of commercial natural strawberry juice if they are spoilage yeasts. Nevertheless, currently there are no reports available about identification of yeasts present in spoiled Argentine strawberry juices.

Actually, besides the use of chemical preservatives such as benzoate or sorbate, pasteurization is probably the most common preservation method applied in the juice industry to eliminate pathogens from juices, but this process may cause a loss of vitamins, minerals, fresh color or flavor (Burt, 2004). During the past decade, there has been an increasing interest in the use of biologically active compounds from natural sources, because consumers are looking for safer and healthier food without addition of chemicals and without the use of thermal treatment. In nature there are many different types of antimicrobial compounds; natural products of higher plants provide a variety of antimicrobial agents, probably demonstrating novel mechanisms of action (Barbour et al., 2004; Rodríguez-Vaquero & Manca de Nadra, 2008). Berries are rich in phenolic compounds, and several researchers have reported the content and antimicrobial activity of phenolic compounds in berries (Vallejo, Aredes-Fernández, Farías, & Rodríguez-Vaquero, 2013). Blueberry (*Vaccinium* spp.) contains relatively high amounts of acids and phenolic compounds (Kalt et al., 2008) that display potential health benefits such as protection against cancer and cardiovascular diseases (Almeida, Farah, Silva, Nunam, & Glória, 2006; Santos, Almeida, Lopes, & Souza, 2006; Serafini et al., 2009). However, at

* Corresponding author. Tel.: +54 381 4247752x7067.
E-mail address: mariajo@fbqf.unt.edu.ar (M.J. Rodríguez-Vaquero).

https://doi.org/10.1016/j.lwt.2018.02.006
Received 1 November 2017; Received in revised form 27 January 2018; Accepted 5 February 2018
Available online 09 February 2018
0023-6438/ © 2018 Published by Elsevier Ltd.

present there is no evidence of antifungal activity of phenolic blueberry extracts against yeasts isolated from commercial deteriorated natural juice in Argentina.

The aims of this study were to 1) isolate and identify yeasts present in deteriorated Argentine strawberry juice, 2) extract and characterize antifungal activity of four phenolic extracts from blueberries widely grown in Argentina and 3) control isolated yeasts and carry out sensorial assays in strawberry juices supplemented with blueberry extracts.

2. Materials and methods

2.1. Isolation and identification of yeasts from deteriorated strawberry juice

Yeasts were isolated from spoiled commercial strawberry juice (Tucumán, Argentina) with a pH of 2.9, and with off-odor, a brown color and presence of lumps and precipitate at the bottom. Samples of deteriorated juices were plated onto YMPG agar medium (Oxoid Ltd., London, England), pH 5.5, and 1.0% chloramphenicol (YMPG-C) was added in order to suppress bacterial growth. YMPG-C plates were aerobically incubated at 28 °C for 48 h. Presumptive identification of isolates was performed based on morpho-physiological criteria (Yarrow, Kurtzman, & Fell, 1998). Genotypic identification of yeasts was carried out using chromosomal DNA isolated according to the protocol described by Hoffman and Winston (1987). Molecular identification of the selected isolated yeasts was carried out by amplification and sequence analysis of the fragment containing the genes encoding ribosomal RNA (rRNA): 18S, 5.8S and 26S. Universal primers ITS1 and ITS4 were used to amplify the internal transcribed spacer 1 (ITS1), 5.8S rRNA, and internal transcribed spacer 2 (ITS2) sequences, while primers NL-1 and NL-4 were used to amplify the 26S rRNA D1/D2 domain. PCR amplifications of a fragment containing ITS1, 5.8S rRNA, and ITS2 sequences were performed according to Lott, Kuykendall, and Reiss (1993) while that of 26S rRNA D1/D2 domain were carried out according to Kurtzman and Robnett (1998). DNA sequencing of both strands was performed using the dideoxy chain termination method with an ABI Prism 3730 DNA analyzer, at the DNA sequencing facility at Macrogen Inc., Korea, using ABI Prism BigDye® terminator cycle sequencing Ready Reaction Mix (PE Biosystems) according to the manufacturer's protocol. Sequence comparisons were performed using the BLAST tool available within the GenBank database. ClustalW software (Thompson, Higgins, & Gibson, 1994) was used for local alignment of multiple sequences. Phylogenetic and molecular evolutionary analyses were carried out with MEGA 5.2 (Tamura et al., 2012) by using neighbor-joining analysis (Saitou & Nei, 1987). For construction of phylogenetic trees, only sequences belonging to type strains of closely related species, whose names have been validly published in public databases, were considered. Sequences obtained from isolates of selected yeasts were submitted to the public Genbank database.

2.2. Extraction and characterization of the low molecular weight phenolic fraction (LMPF) in different blueberry cultivars

The 4 cultivars selected, *Misty*, *Blue Crisp*, *O'Neal* and *Millennium*, are widely cultivated in Tucumán. The extraction method used was described by Ghiselli, Nardini, Baldi, and Scaccini (1998). Total phenolic compounds in blueberry cultivars were measured using the Folin Ciocalteu method (Singleton & Rossi, 1965) adapted by Cicco, Lanorte, Paraggio, Viggiano, and Lattanzio (2009) for microtechnics. Phenolic compounds in the 4 blueberry extracts were identified and quantified by HPLC analysis coupled to a diode array detector according to the technique by Fanzone et al. (2011). Toxicological assessment was carried out with fresh human blood according to the technique by Shubha et al. (2016).

2.3. Antifungal activity of blueberry LMPF and individual phenolic compounds against isolated yeasts

The agar diffusion test was used to assay antifungal activity of blueberry LMPF and individual phenolic compounds, and the assay was carried out according to Rodríguez-Vaquero, Alberto, and Manca de Nadra (2007). Cycloheximide (500 μg/ml) was used as a positive control and sterile water or ethanol was used as negative control. After 48 h of incubation the inhibition zones were measured to an accuracy of 0.5 mm and the antifungal effect was calculated as the mean of three replication assays. For each blueberry LMPF the IC_{50} and IC_{90} (inhibitory concentration of 50 and 90% of yeast viability, respectively) were determined following the CLSI guidelines (Clinical and laboratory Standards Institute, 2006); tubes with Mueller-Hinton broth supplemented with serial dilutions of blueberry LMPF were inoculated individually with isolated yeasts. Cultures were incubated at 28 °C during 24–48 h, and the number of viable cells was determined using successive dilutions.

2.4. Yeast viability in strawberry juice supplemented with blueberry LMPF and sensorial quality

Commercial pasteurized strawberry juice (pH 3.12, viscosity 1.357, 16 °Brix, bright red color) in 1-L tetra bricks was purchased at a supermarket in Tucumán, Argentina. One hundred ml of juice were poured in 200 ml flasks and supplemented with 50 μg, 100 μg or 150 μg of sterile blueberry LMPF per ml of juice under aseptic conditions immediately after opening. Then, yeast cells were washed and inoculated individually to obtain a concentration of approximately 4 log cfu/ml and samples were incubated at 4 °C protected from light, to simulate initial contamination. Inoculated juice without additives was used as yeast growth control. Samples were taken after 0, 7 and 14 days of incubation at 4 °C. Yeast viability was determined using successive dilutions on YMPG-C agar medium.

A qualitative descriptive assay was used to assess sensory attributes of strawberry juice supplemented with 50, 100 and 150 μg/ml blueberries LMPF at day 0 and 7. A panel of ten members with sensory evaluation experience conducted the evaluation of strawberry juice. Four attributes were evaluated: odor, flavor, color and turbidity on a scale from 1 to 4. Odor and flavor scored from 1 (deteriorated juice) to 4 (fresh juice), color scored from 1 (roseate juice) to 4 (red juice) and turbidity scored from 1 (clean juice) to 4 (high turbidity). The limit of acceptance for each attribute was 2.5; lower values for any of the attributes were considered to have passed the shelf-life.

2.5. Statistical analysis

All experiments were repeated three times with duplicate samples and viable plate counts from three replications. Data were analyzed by ANOVA using Minitab (Minitab Inc., PA, USA). Multiple means comparison was carried out by Duncan's multiple range tests ($p < 0.05$).

3. Results

Table 1 shows the morpho-phenotypic characterization of 100 yeasts isolated from deteriorated juice based on morpho-physiological criteria. The results demonstrate that 49 of the 100 isolates seem to be the same yeast (Group I) and 51 isolates share the same/similar characteristics. Isolates of group I and II were presumptively identified as *Hanseniaspora* and *Starmerella* genus, respectively. Partial sequence analysis of rRNA of the isolates showed that Group I bore a remarkably close relationship, sharing 100% similarity with strains of the genus *Hanseniaspora* and Group II shared 95% identity with the genus *Starmerella* (Fig. 1a and b). Partial rRNA sequences of *H. osmophila* and *S. bacillaris* were submitted to the public Genbank database under access numbers KJ880968 and KJ880972, respectively (Vallejo, Delgado,

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