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Susceptibility and resistance of lactic acid bacteria and yeasts against preservatives with potential application in table olives

Verónica Romero-Gil, Pedro García-García, Antonio Garrido-Fernández, Francisco Noé Arroyo-López*

Food Biotechnology Department, Instituto de la Grasa (CSIC), Campus Universitario Pablo de Olavide, Building 46, Ctra, Utrera, Km 1, 41013, Seville, Spain

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ABSTRACT

In the present study, a dose-response model was used to investigate the susceptibility (NIC) and resistance (MIC) of the lactic acid bacteria and yeast populations with respect to five chemical preservatives (fumaric and pyruvic acids, cinnamaldehyde, sodium metabisulphite and natamycin) with potential application in table olives. Results were compared with respect to potassium sorbate, a well-known preservative habitually used in olive packaging. Sodium metabisulphite was the most efficient preservative to control lactic acid bacteria growth (MIC, 50 ppm), followed by cinnamaldehyde (1060 ppm) while pyruvic acid required higher concentrations (3211 ppm). Natamycin (25 ppm) was highly efficient against yeasts, followed by cinnamaldehyde (125 ppm), potassium sorbate (553 ppm), sodium metabisulphite (772 ppm) and pyruvic acid (3038 ppm). Fumaric acid, in the range assayed (0–2000 ppm), did not show any inhibitory effect against these two microbial groups. This survey presents for the first time a comparative study of the efficiency of potential preservatives to control the growth of table olive related microorganisms. Further studies should be performed to validate their effects and interactions in the food matrix.

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1. Introduction

Worldwide table olive production reached 2,595,500 tons in 2014/2015 season (IOC, 2015). The elaboration of this fermented food is mainly related to the Mediterranean basin, but there are also important production regions in Australia, South-America and USA. The most popular processing styles are: i) green Spanish-style (olives debittered by alkaline treatment), ii) natural (directly brined) olives, and iii) Californian style (olives darkened by oxidation in an alkaline medium) (Garrido-Fernández et al., 1997).

Yeasts (mainly from *Saccharomyces*, *Candida*, *Debaryomyces* and *Pichia* genera), and lactic acid bacteria (LAB) (belonging especially to *Lactobacillus* genera) have an essential role during processing of table olives determining quality, flavour and safety of final products. Both microbial groups can coexist during fermentation and they are responsible for diverse favourable effects such as sugar consumption, production of lactic acid, bacteriocins, killer factors and desirable volatile compounds, among others (Arroyo-López et al., 2012a; Hurtado et al., 2012). However, their uncontrolled presence during packaging may cause product spoilage due to the production of CO_2, swollen containers, softening of fruits, and clouding of brines. Hence, the microbiological stabilization of the final products during the commercialization period is critical.

Due to its high pH (close to neutrality), ripe olives require sterilization while Spanish-style and natural olives are fermented products that may be preserved by different methods (physico-chemical characteristics, modified atmosphere, vacuum or pasteurization) (Garrido-Fernández et al., 1997). However, the thermal treatments may cause undesirable changes in the traditional flavour of several presentations, particularly seasoned (alkali treated or natural) olives which, thus, should be stabilized by the use of preservatives (Arroyo-López et al., 2009). Currently, the only two preservatives permitted in table olives, according to the Table Standard Applying to Table Olives (IOC, 2004) are benzoic and sorbic acids (or their respective salts) at maximum doses of 1000 ppm (wt/wt flesh) for benzoic and 500 ppm for sorbic acid, or 1000 ppm for their combination. However, these chemical compounds have some drawbacks such as i) accumulation in the olive (flesh) fat, with the subsequent limitation of their effects in the brines, ii) development of undesirable sensorial notes for

* Corresponding author.
E-mail address: fnarroyo@cica.es (F.N. Arroyo-López).

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consumers, iii) browning of fruits, and iv) degradation by microorganisms (Garrido-Fernández et al., 1997; Arroyo-López et al., 2005). As a result, the table olive sector is demanding research for obtaining more appropriate preservatives.

Predictive microbiology uses mathematical models to describe quantitatively the response of microorganisms as a function of environmental variables or preservatives (McMeekin et al., 1993). One of the most common methods used for the estimation of the effect of an inhibitory compound is the calculus of its NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values with a progressive inhibitory effect as the concentrations move from the NIC to the MIC. As shorter is the range between both points, the stronger is the inhibitory effect (Lambert, 2001; Chorianopoulos et al., 2006). The method developed can be easily automatized using optical density (OD) measurements. This technique has been used for testing the growth response of *Salmonella typhimurium* in the presence of natural and synthetic antimicrobials (Guillier et al., 2007), the effect of lemon extract on foodborne microorganisms (Conte et al., 2007) or the antifungal activity of fatty acids and their monoglycerides against *Fusarium* spp. in a laboratory medium (Altieri et al., 2009). In table olives, the same methodology has been used to study the effects of diverse chloride salts on *Lactobacillus pentosus* and *Saccharomyces cerevisiae* growth (Bautista-Gallego et al., 2008), modelling the inhibitory effect of $ZnCl_2$ on table olive related yeasts (Bautista-Gallego et al., 2012), or testing the effect of salt (NaCl) on table olive related microorganisms (Romero Gil et al., 2013; Bonatsou et al., 2015). Hence, this technique has been widely used and validated to investigate the efficiency of diverse compounds for controlling the microorganisms involved in table olive packaging.

In the present survey, we use statistical modelling techniques (dose-response model) to quantify the individual effects of five chemical compounds (fumaric and pyruvic acids, sodium metabisulphite, natamycin and cinnamaldehyde) to prevent the growth of yeasts and LAB species related to table olive packaging. Results were compared with those obtained for potassium sorbate, a preservative habitually used for the stabilization of packaged olives. Data obtained could provide clues for producing safer and more stable olive presentations when thermal treatments are non-viable. Also, it may also be helpful for supporting possible changes in their legal status in table olives.

2. Material and methods

2.1. Microorganisms and cocktail preparation

A total of 10 LAB and 8 yeast strains, representing the yeast and LAB species usually found in table olive processing, were used in the present study (Table 1). All of them were previously identified by molecular methods (data not shown) and belong to the Table Olive Microorganisms Collection (TOMC) of Instituto de la Grasa (CSIC, Seville). The use of a microbial cocktail instead individual species is a convenient and faster way of checking the overall susceptibility/sensibility that a particular compound could have against a specific microbial group. This way, the NIC and MIC values will be obtained for the most resistant species or strain of the cocktail. This strategy has been successfully used in food microbiology to estimate the overall response of the yeast and bacteria populations as a function of storage conditions or preservatives (Arroyo-López et al., 2012b; Leong et al., 2014). Inoculum were prepared by inoculating one single colony of each strain into 5 mL of a YM broth medium (Difco™, Becton and Dickinson Company, Sparks, USA) for yeasts; or 5 mL of a MRS broth medium (de Man, Rogosa and Sharpe) (Oxoid, Cambridge, UK) for LAB. After 48 h of incubation at 30 °C, 1 mL from each tube was centrifuged at 9000× *g* for 10 min, the pellets were washed with sterile saline solution (9 g/L), centrifuged and re-suspended again in 0.5 mL of a sterile saline solution to obtain a concentration of about 7 $\log_{10}$ CFU/mL for yeasts and 8 $\log_{10}$ CFU/mL in the case of LAB, which was confirmed by surface spread on appropriate media. These microorganism suspensions were mixed and the same proportions, obtaining one cocktail for yeasts and other for LAB, and then used to inoculate the different experiments as described below.

Table 1
Yeasts and lactic acid bacteria species and strains used to prepare the microbial cocktails.

Microbial cocktail	Strains
LAB	*Lactobacillus pentosus TOMC-LAB2*
	Lactobacillus pentosus TOMC-LAB3
	Lactobacillus pentosus TOMC-LAB4
	Lactobacillus pentosus TOMC-LAB5
	Lactobacillus pentosus TOMC-LAB6
	Lactobacillus plantarum TOMC-LAB8
	Lactobacillus plantarum TOMC-LAB9
	Lactobacillus paraplantarum 271
	Pediococcus pentosaceus E11
	Pediococcus pentosaceus P56
Yeasts	*Candida diddensiae TOMC-Y1*
	Issatchenkia occidentalis TOMC-Y3
	Saccharomyces cerevisiae TOMC-Y4
	Debaryomyces hansenii TOMC-Y25
	Pichia membranifaciens TOMC-Y31
	Candida boidinii TOMC-Y47
	Candida tropicalis TOMC-Y72
	Lodderomyces elonsgisporus TOMC-Y73

2.2. Modelling the inhibitory effects of preservatives

Growth was monitored in a Bioscreen C automated spectrophotometer (Labsystem, Helsinki, Finland) with a wideband filter (420–580 nm). Measurements were taken every 2 h after a pre-shaking of 5 s for 7 days. The wells of the microplate were filled with 20 μL of inoculum and 330 μL of medium (according to treatment as described below), always reaching an initial OD of approximately 0.2 (inoculum level above 6 $\log_{10}$ CFU/mL). The inocula were always above the detection limit of the apparatus, which was determined by comparison with a previously established calibration curve. Uninoculated wells for each experimental series were also included in the microplate to determine, and consequently subtract, the noise signal.

Sterilized YM or MRS broth were modified with 5% NaCl and adjusted to pH 4.0 by citric acid addition (mother stock solution 30%) to mimic industrial packaging conditions. Based in our experience and bibliography, this pH level is usually found in real table olive packaging (Arroyo-López et al., 2009; Blana et al., 2016), and therefore, appropriate for a first selection of the preservatives with the highest inhibitory effects. The basal media were supplemented with the different chemical compounds and concentrations shown in Table 2. The use of a well-known, standardized synthetic laboratory medium to carry out the experiments was preferred because, in the olive matrix, the presence of diverse components released by fruits such as polyphenols, organic acids, etc., may mask the real inhibitory effect of preservatives.

The basis of the technique used for estimating the NIC and MIC values of the assayed microbial cocktails for preservatives was the comparison of the area under the OD/time curve of a positive control (absence of preservative, optimal conditions) with the areas of the tests (presence of preservative, increasing inhibitory conditions). As the amount of inhibitor in the well increases, the effect on

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