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

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Running title: Testing new preservatives in table olives

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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.

Keywords: Table olives; Preservatives; Dose-response model; Lactic acid bacteria; Yeasts; MIC; NIC.

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 (physicochemical 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

43 seasoned (alkali treated or natural) olives which, thus, should be stabilized by the use of
44 preservatives (Arroyo-López et al., 2009). Currently, the only two preservatives
45 permitted in table olives, according to the Table Standard Applying to Table Olives
46 (IOC, 2004) are benzoic and sorbic acids (or their respective salts) at maximum doses of
47 1000 ppm (wt/wt flesh) for benzoic and 500 ppm for sorbic acid, or 1000 ppm for their
48 combination. However, these chemical compounds have some drawbacks such as i)
49 accumulation in the olive (flesh) fat, with the subsequent limitation of their effects in
50 the brines, ii) development of undesirable sensorial notes for consumers, iii) browning
51 of fruits, and iv) degradation by microorganisms (Garrido-Fernández et al., 1997;
52 Arroyo-López et al., 2005). As a result, the table olive sector is demanding research for
53 obtaining more appropriate preservatives.

54 Predictive microbiology uses mathematical models to describe quantitatively the
55 response of microorganisms as a function of environmental variables or preservatives
56 (McMeekin et al., 1993). One of the most common methods used for the estimation of
57 the effect of an inhibitory compound is the calculus of its NIC (non-inhibitory
58 concentration) and MIC (minimum inhibitory concentration) values with a progressive
59 inhibitory effect as the concentrations move from the NIC to the MIC. As shorter is the
60 range between both points, the stronger is the inhibitory effect (Lambert, 2001;
61 Chorianopoulos et al., 2006). The method developed can be easily automatized using
62 optical density (OD) measurements. This technique has been used for testing the growth
63 response of *Salmonella typhimurium* in the presence of natural and synthetic
64 antimicrobials (Guillier et al., 2007), the effect of lemon extract on foodborne
65 microorganisms (Conte et al., 2007) or the antifungal activity of fatty acids and their
66 monoglycerides against *Fusarium* spp. in a laboratory medium (Altieri et al., 2009). In
67 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 30ºC, 1 mL from each tube was centrifuged at 9000 x *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.

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

115 calibration curve. Uninoculated wells for each experimental series were also included in
116 the microplate to determine, and consequently subtract, the noise signal.

117 Sterilized YM or MRS broth were modified with 5% NaCl and adjusted to pH
118 4.0 by citric acid addition (mother stock solution 30%) to mimic industrial packaging
119 conditions. Based in our experience and bibliography, this pH level is usually found in
120 real table olive packaging (Arroyo-López et al., 2009; Blana et al., 2016), and therefore,
121 appropriate for a first selection of the preservatives with the highest inhibitory effects.
122 The basal media were supplemented with the different chemical compounds and
123 concentrations shown in Table 2. The use of a well-known, standardized synthetic
124 laboratory medium to carry out the experiments was preferred because, in the olive
125 matrix, the presence of diverse components released by fruits such as polyphenols,
126 organic acids, etc., may mask the real inhibitory effect of preservatives.

127 The basis of the technique used for estimating the NIC and MIC values of the
128 assayed microbial cocktails for preservatives was the comparison of the area under the
129 OD/time curve of a positive control (absence of preservative, optimal conditions) with
130 the areas of the tests (presence of preservative, increasing inhibitory conditions). As the
131 amount of inhibitor in the well increases, the effect on the growth of the organism also
132 increases. This effect on growth is manifested by a reduction in the area under the
133 OD/time curve relative to the positive control at any specified time. The areas under the
134 OD/time curves were calculated by integration using OriginPro 7.5 software (OriginLab
135 Corporation, Northampton, USA). The relative amount of growth for each preservative
136 concentration, denoted as the fractional area (*Fa*), was obtained using the ratios of the
137 test area ($area_{test}$) to that of the positive control of the microbial cocktails ($area_{cont}$),
138 according to the following formula:

$$Fa = (area_{test})/(area_{cont})$$

The plot of the *Fa* versus the natural logarithm (ln) of the preservative concentration produced a sigmoid-shape curve that could be well-fitted with a reparameterized modified Gompertz function for decay (Bonatsou et al., 2015), which had the following expression:

$$y=exp(-(x/(ln(MIC)/exp(-(ln(ln(NIC)/ln(MIC))/2.71828))))^(-2.71828/(ln(ln(NIC)/ln(MIC)))))$$

where y is the dependent variable (*Fa*), x is the independent variable (ln preservative concentration, ppm), MIC is the minimum preservative concentration (ppm) above which growth is not observed, and NIC is the preservative concentration (ppm) above which an inhibitory effect begin to be observed. These parameters were obtained by non-linear regression procedure, minimizing the sum of squares of the difference between the experimental data and the fitted model, i.e., loss function $(observed-predicted)^2$. This task was accomplished using the non-linear module of the Statistica 7.1 software package (StatSoft Inc, Tulsa, OK, USA) and its Quasi-Newton option. Fit adequacy was checked by the proportion of variance explained by the model (R^2) with respect to the experimental data.

2.3. Statistical data analysis

Significant differences among NIC and MIC values for preservatives were checked by one-way ANOVA using Statistica 7.1 software (Statsoft Inc., Tulsa, USA). Post-hoc comparisons were performed using the least significant difference (LSD) test. Data were obtained from four independent experiments.

3. Results

161 To determine the individual effect of five different chemical preservatives with
162 potential application in table olive processing (pyruvic and fumaric acids, sodium
163 metabisulphite, natamycin and cinnamaldehyde) and comparison with another currently
164 used by the industrial sector (potassium sorbate), a total 47,040 raw data belonging to
165 560 OD growth curves (280 for the LAB and other 280 for the yeasts) were obtained in
166 an automated spectrophotometer and then modelled. The addition of fumaric acid did
167 not show any inhibitory effect within the concentration range tested (0-2000 ppm) for
168 either LAB or yeast. Potassium sorbate and natamycin did not affect LAB growth, and
169 *Fa* was kept constant around 1.0 value, regardless of their concentrations. However, for
170 the rest of chemical compounds, there was a clear *Fa* decrease as concentrations were
171 greater. Thereby, a dose-response model was properly fitted in the case of inhibition,
172 with an R^2 usually above 0.922 (data not shown).

173 Figure 1 shows two examples of the reparameterized Gompertz equation for
174 decay fitted to the experimental data, for both yeast (upper panel) and LAB (lower
175 panel) as a function of the ln sodium metabisulphite and cinnamaldehyde concentrations
176 (ppm), respectively. The fit followed a typical sigmoid decay function, which could be
177 divided into three sections: i) a first section corresponding to preservative
178 concentrations below the NIC (concentrations at which no effect of the inhibitor was
179 observed and *Fa* was around 1), ii) concentrations between NIC and MIC values (within
180 which growth inhibition progressively occurred and the *Fa* decreased), and iii) a third
181 section above MIC (where no growth relative to the control was recorded, and *Fa* was
182 close to 0).

183 Table 3 shows the NIC and MIC values individually obtained for the
184 preservatives with an inhibitory effect on the growth of the yeast and LAB cocktails.

185 Values are the average of four experiments for each microbial cocktail and preservative,
186 performed and fitted independently. The NIC value, related to susceptibility of
187 microorganism to the specific chemical compound, was widespread among
188 preservatives and ranged from 6 ppm (natamycin in the case of yeasts) to 2713 ppm
189 (pyruvic acid in the case of LAB), while the MIC value, related to the resistance of the
190 microorganism to the preservative, ranged from 25 ppm (natamycin in the case of
191 yeasts) to 3211 ppm (pyruvic acid in the case of LAB). According to values shown in
192 Table 3, only pyruvic acid, sodium metabisulphite and cinnamaldehyde showed
193 inhibitory effects on both LAB and yeast populations. Among them, pyruvic acid was
194 the preservative with the lowest inhibitory effects (the highest NIC and MIC values),
195 whilst sodium metabisulphite and cinnamaldehyde were the compounds with the
196 highest inhibitory effects for both LAB and yeasts, respectively. Statistically significant
197 differences were found among preservatives within the same microbial cocktail (LAB
198 or yeast) according to the LSD posthoc comparison test.

199 According to Figure 2, which shows the concentration range where the
200 progressive inhibitory effect of preservatives (from NIC to MIC) was noticed for
201 microorganisms, the microbial behaviour depended on the preservative assayed. As
202 shorter is the range between both values, the stronger is the inhibitory effect for the
203 chemical compound. Sodium metabisulphite for LAB, and cinnamaldehyde and
204 natamycin for yeasts, were extremely toxic for cells, with a very narrow inhibitory
205 range, while this range was wider for the rest of preservatives, especially for pyruvic
206 acid in the case of yeasts.

207 The ANOVA analysis carried out with the NIC, and MIC values obtained for the
208 LAB and yeast populations (Figure 3) showed that, effectively for both microbial

groups, pyruvic acid showed the lowest inhibitory effect without significant differences between yeasts and LAB. The preservative with the highest inhibitory effect on LAB was sodium metabisulphite followed by cinnamaldehyde (with significant differences between them), whilst the preservative with the highest inhibitory effect on yeasts was natamycin, followed by cinnamaldehyde (without significant differences between them). Sodium metabisulphite had a very similar effect than potassium sorbate on yeasts while this later preservative did not show any inhibitory effect against bacteria.

4. Discussion

The control of spoilage microorganisms is one of the most important aspects in food preservation. Many of the food preservatives habitually used by industry for this purpose are weak acids, such as sorbic, benzoic, propionic, acetic and sulphite (Piper, 2011). Weak acids are widely used in low-pH foods, where its inhibitory power increases. Therefore, they could have direct application in table olive packaging, albeit the experience on their effects on table olive related microorganisms is scarce. This work attempts to determine, using a dose-response model, the influence of different preservatives to control the growth of LAB and yeasts isolated from table olive processing. This type of modelling has proved to be appropriated to obtain the NIC and MIC values of diverse chemical compounds against table olive related microorganisms (Bautista-Gallego et al., 2008, 2012; Romero-Gil et al., 2013; Bonatsou et al., 2015).

The effect of potassium sorbate on the main olive yeast species has already been studied in several occasions, using a probabilistic model for the determination of the growth/no growth interfaces in combination with other additives (Arroyo-López et al., 2007a, 2007b, 2008b). Its use in table olive packaging is accepted regardless of legislation (CODEX, EU or Spanish Government), provided the maximum dose

allowed (500 ppm of sorbic acid in pulp) is not exceeded. In previous works, a concentration of 300 ppm of potassium sorbate together with 5-6% NaCl at pH 4.0 was enough to inhibit *S. cerevisiae* and *Issatchenkia occidentalis* growth (Arroyo-López et al., 2007a, 2007b). However, scarce information is available for bacteria. Arroyo-López et al. (2005) showed that a concentration of 175 ppm of potassium sorbate was not enough to inhibit LAB growth in real olive packaging. The effect of the influence of this weak acid on microorganisms is strongly related to the pH of the medium. Data obtained in this study show that sorbate in the range assayed (0-2000 ppm), did not have any inhibitory effect against LAB at pH 4.0 but, on the contrary, exerted a clear inhibitory effect against a cocktail formed by a considerable number of yeast species, with a MIC value of 553 ppm (413 ppm expressed as sorbic acid). The comparison of its efficiency with respect to other new potential preservatives could be of interest for the proper selection of an adequate alternative.

Fumaric acid is an unsaturated dicarboxylic acid with low water solubility and a strong acid taste; however, its combination with flavouring compounds may intensify the aftertaste of a flavour. The use of this acid in food as either acidifying agent or microbial inhibitor is rather usual (Davidson et al., 2005). Particularly, it has been efficient against LAB for the preservation of acidified cucumbers (Pérez-Díaz, 2011). Due to the rather similarity between cucumbers and table olives, fumaric acid could have application for preventing spoilage by LAB in vegetable products. However, due to the lack of effectiveness noticed in the present study for this compound, which did not exert inhibitory effect in the range tested for either LAB or yeasts, no further discussion on its role as preservative in table olives is pertinent.

According to the General Standard for Food Additives (Codex Alimentarius, 2015) the use of metabisulphite is permitted for the products included in the Food

Category num. 04.2.2.3 (which includes table olives). The recently issued Codex Standard for Table Olives (Codex Stan 66-1891, rev 2013) also refers to this Standard in the section related to food additives. However, according to Directive (CE) Nº 1333/2008 (European Parliament & Council, 2014), which follows a similar scheme and criterion that the Food Additive Standards issued by the Codex, the metabisulphite, although allowed for products in the food category num. 04.2.2 (which include olives), is explicitly excluded for table olives and yellow peppers in brine. Apparently, the re-introduction of this additive in the Standard issued by the Codex (trv. 2006) has not implied the subsequent rectification in the European Directive, in spite of the diverse modifications suffered in the last years. The Spanish legislation (Ministerio de la Presidencia, 2001) does not permit either the use of metabisulphite due to its submission in this aspect to the EU regulation on additives. However, metabisulphite was traditionally used in table olives until its temporary prohibition in the Food Additive Standards issued by the Codex, which also caused its elimination from the Directive (CE) Nº 1333/2008 (European Parliament & Council, 2014) and from the Trade Standard for Table Olive (COI, 2004). However, after the re-inclusion of the metabisulphite use in Codex Stan 192-1995, rev 2006) neither of these legislative organisms has modified the metabisulphite status accordingly. Nowadays, the discrepancies between the EU legislation and Codex may lead to disputes and insecurity in the international table olive trade. Thus, studies on the inhibitory effects on table olive related microorganisms are necessary to help legislators on the homogenization of standards. Besides, its use in table olives would be convenient due to its antioxidant (browning prevention) and inhibitory effects on the microbial populations (Arroyo-Lopez et al., 2008a; Echevarría et al., 2010). Furthermore, sodium metabisulphite may also remain as a result of its use as antioxidant during postharvest treatments (Segovia-

283 Bravo et al., 2010) and this carry over effect should also be considered. In the present
284 study, this compound has shown to have a moderate inhibitory effect in laboratory
285 medium against yeast (MIC value 772 ppm) and especially against LAB (MIC value 50
286 ppm) cocktails. However, a concentration of 1500 ppm was not enough to inhibit LAB
287 and yeast populations in real olive fermentations for two months, albeit showed a higher
288 inhibitory effect than ascorbic acid (Echevarría et al., 2010). Taking into consideration
289 these results, probably the metabisulphite levels necessary to inhibit LAB growth could
290 be compatible with olive packaging. On the contrary, the higher doses necessary to
291 control yeast growth may cause allergic reactions and headache in sensitive persons to
292 this preservative. In the specific case of table olives, its residue would be below the 100
293 mg/kg flesh (expressed as sulphur dioxide) as established in the Codex Stan 192-1995
294 rev. 2014 (Codex Alimentarius, 2015). At this level, any possible health effect would be
295 markedly reduced for most consumers.

296 Natamycin is a preservative used in diverse dairy products (Thomas & Delves-
297 Broughton, 2003; Gallo et al., 2006). The first tentative of use in table olives was
298 reported by Mahjoub & Bullerman (1986) to control the mould growth and the
299 production of aflatoxin. Natamycin has also shown good behaviour for the prevention of
300 mould growth on the surface of natural black Greek-style fermenting olives at 100 ppm
301 (Hondrodimou et al., 2011). Recently, Arroyo-López et al. (2012b) found natamycin
302 very efficient (12-30 ppm) against table olive related yeasts at NaCl concentrations
303 around 4.5%, albeit the presence of citric acid (and low pH levels in general) decreased
304 its effect. Hence, the use of natamycin in table olives could be promising to control
305 yeast and mould growth. However, according to data obtained in this work, this
306 preservative did not exert any inhibitory effect against LAB at levels assayed. The
307 EFSA Panel for Food Additives and Nutrient Sources for Foods has revised its

application as preservative and has concluded that natamycin is very poorly absorbed in the gastrointestinal tract. Hence, its intake hardly can induce antimicrobial resistance and there is an appropriate margin of safety for its current application (EFSA, 2009). This position opens the possibility of natamycin utilization in other foods, provided its use could be adequately supported. In this context, this work has showed its usefulness in controlling the yeast population.

Pyruvic acid was first patented for its preservative properties by Ernst et al. (1979) to stabilize high moisture food products without refrigeration. The use of pyruvic acid with natural colorants may improve their stabilities at acidic pH and presence of ascorbic acid (Ojwang & Awika, 2008). Pyruvic (and acetaldehyde)-bound sulphur dioxide produced inhibition against wine LAB at concentration of 5 ppm, albeit the LAB finally degraded such compounds, suggesting that sulphur dioxide -bound pyruvic acid could have a bacteriostatic effect rather than bactericidal action (Wells & Osborne, 2011). Pyruvate was effective for lowering lipid oxidation in high-oxygen meat packages; then, its use in table olive might also have an favourable antioxidant effect on olive fat due to the high proportion of oil in the processed fruits and the adverse environmental condition (e.g. high storage temperature) during transportation or shelf live (Ramathan et al., 2011). Moreover, pyruvic acid has a low pK_a value (2.39), a circumstance that also validates its use for acidification purposes in table olives. The inhibitory effect was very similar for both LAB and yeasts populations, although the concentrations required were relatively high; its MIC values were 3211 and 3037 ppm, respectively.

Recently, cinnamaldehyde was applied to stabilize acidified cucumbers that were adequately preserved free of yeasts (Pérez-Díaz, 2011). The presence of essential oils is common in seasoned table olives due to the usual addition to them of garlic,

rosemary, or extracts. However, one of the leading causes of instability in these products is the yeast growth (Arroyo-López et al., 2012a). Considering the efficient inhibition of yeast in cucumbers, testing cinnamaldehyde against the microorganisms (mainly yeasts) present in table olives may be interesting, especially for the development of table olives with other flavours. This compound is obtained from the cinnamon bark. The mechanism of the bactericidal action of cinnamaldehyde against *Listeria monocytogenes*, possible inhibition of glucose uptake and utilization and effects on membrane permeability, was suggested by Gill & Holley (2004). This compound had both antimicrobial and antioxidant activities when applied to meat, thus preventing microbial spoilage and lipid oxidation (Naveena et al., 2013). Cinnamaldehyde has been reported to show a potential inhibitory effect on methicillin-resistant *Staphylococcus aureus* biofilm-related to infections (Jia et al., 2011). Recently, cinnamaldehyde has been suggested as a useful compound for the control of *Escherichia coli* at refrigeration temperature (Visvalingam & Holley, 2012). Data obtained in this work show that this organic compound was effective to control microorganisms, but its effect was microbial group dependent, with a higher inhibitory effect on yeast (125 ppm) than for LAB (1060 ppm).

5. Conclusions

In summary, the results obtained in this work show that three preservatives (sodium metabisulphite, pyruvic acid and cinnamaldehyde) had a broad inhibitory effect against the growth of both LAB and/or yeasts and may have application in table olive packaging, whilst traditional preservative (potassium sorbate) only showed inhibitory effect against yeasts. Further studies should be performed to determine the possible interaction of these compounds with food matrixes and their influence on the

organoleptic profile of final products, which could be especially relevant in the case of the essential oils (cinnamaldehyde).

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Figure Legends

Figure 1. Fit of the reparameterized Gompertz equation for decay (see Materials and methods) to the fractional areas (*Fa*) of the yeast (upper panel) and lactic acid bacteria (lower panel) populations as a function of ln (ppm) of sodium metabisulphite and cinnamaldehyde, respectively, for the estimation of NIC (non-inhibitory concentration) and MIC (minimum inhibitory concentration) values. Parameters for each preservative were the average of four independent experiments.

Figure 2. NIC to MIC interval for the LAB and yeast cocktails as a function of the preservative concentrations. CIN, MET, SOR, NAT and PYR stand for cinnamaldehyde, sodium metabisulphite, potassium sorbate, natamycin and pyruvic acid, respectively. Parameters for each compound were the average of four independent experiments.

Figure 3. Graphical representation of the one-way ANOVA for the NIC and MIC (ppm) parameters as a function of the different preservatives (categorical variable) and microbial cocktail. CIN, MET, SOR, NAT and PYR stand for cinnamaldehyde, sodium metabisulphite, potassium sorbate, natamycin and pyruvic acid, respectively. Parameters for each chemical compound were the average of four independent experiments.

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 tropicalisTOMC-Y72
	Lodderomyces elonsgisporusTOMC-Y73

Table 2. Type of preservatives and concentrations (ppm) assayed in the present study for the modification of basal YM (yeasts) and MRS (lactic acid bacteria) broth laboratory medium.

Preservatives	**Concentrations (ppm)**
Pyruvic acid	0, 500, 1000, 1500, 2000, 2500, 3000, 3500, 4000, 5000
Fumaric acid	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Sodium metabisulphite	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Potassium sorbate	0, 10, 25, 50, 75, 100, 150, 250, 500, 1000, 1500, 2000
Natamycin	0, 2, 4, 6, 8, 10, 12, 16, 18, 20, 25, 30
Cinnamaldehyde	0, 20, 50, 100, 150, 200, 250, 500, 750, 1000, 1250, 1500

Table 3. NIC and MIC (ppm) values obtained for the preservatives assayed in this work against the lactic acid bacteria and yeasts cocktails. Mean and standard deviation (in parentheses) values were obtained from four independent experiments (n=4).

	LAB		Yeasts	
Preservative	NIC	MIC	NIC	MIC
Pyruvic acid	2713.97 (54.50) [a]	3210.99 (42.52) [a]	2050.81 (134.25) [d]	3037.63 (105.16) [d]
Fumaric acid	*	*	*	*
Sodium metabisulphite	49.00 (0.00) [b]	50.07 (0.09) [b]	296.08 (85.16) [c]	771.89 (172.77) [c]
Potassium sorbate	*	*	150.41 (15.58) [a]	552.98 (58.15) [b]
Natamycin	*	*	6.49 (0.99) [b]	24.59 (2.76) [a]
Cinnamaldehyde	382.85 (23.62) [c]	1060.18 (66.77) [c]	124.00 (0.00) [a]	125.00 (0.00) [a]

(*) It was not observed a reduction of the *Fa* (value close to 1) within the range of concentrations assayed. Values followed by different superscript letters, within the same column, are significantly different according to the LSD posthoc comparison test.

Figure 1

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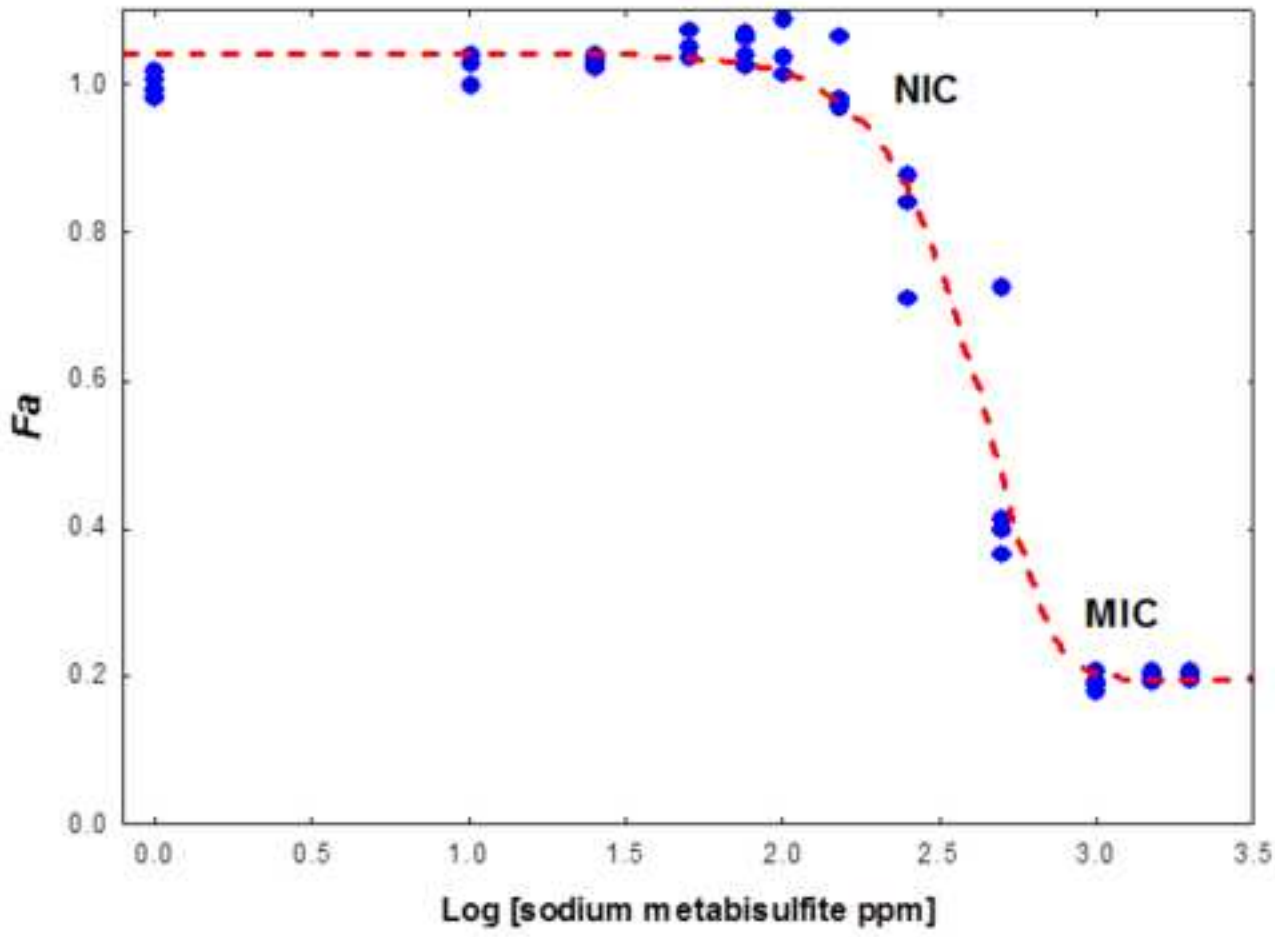

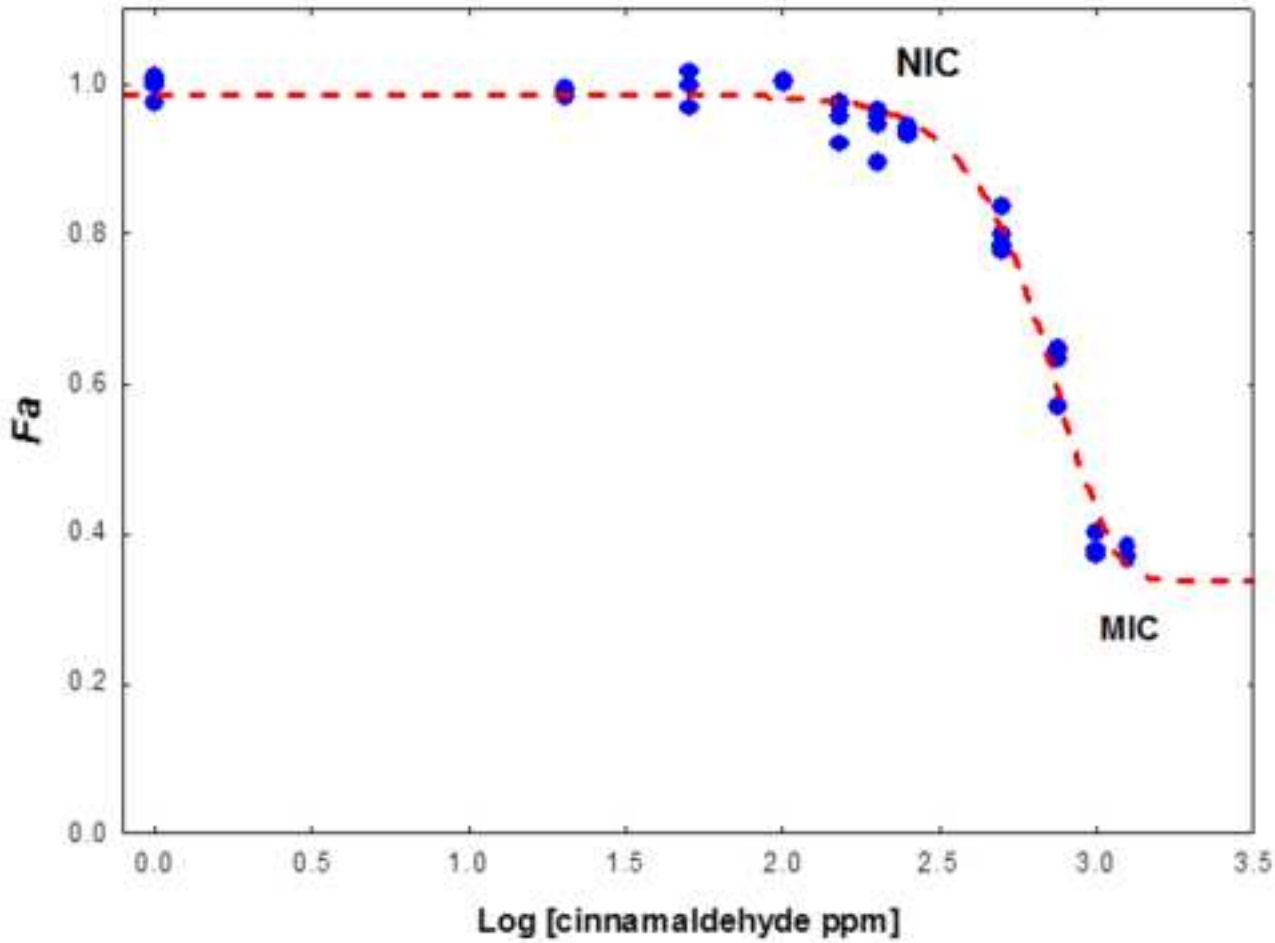

Figure 2

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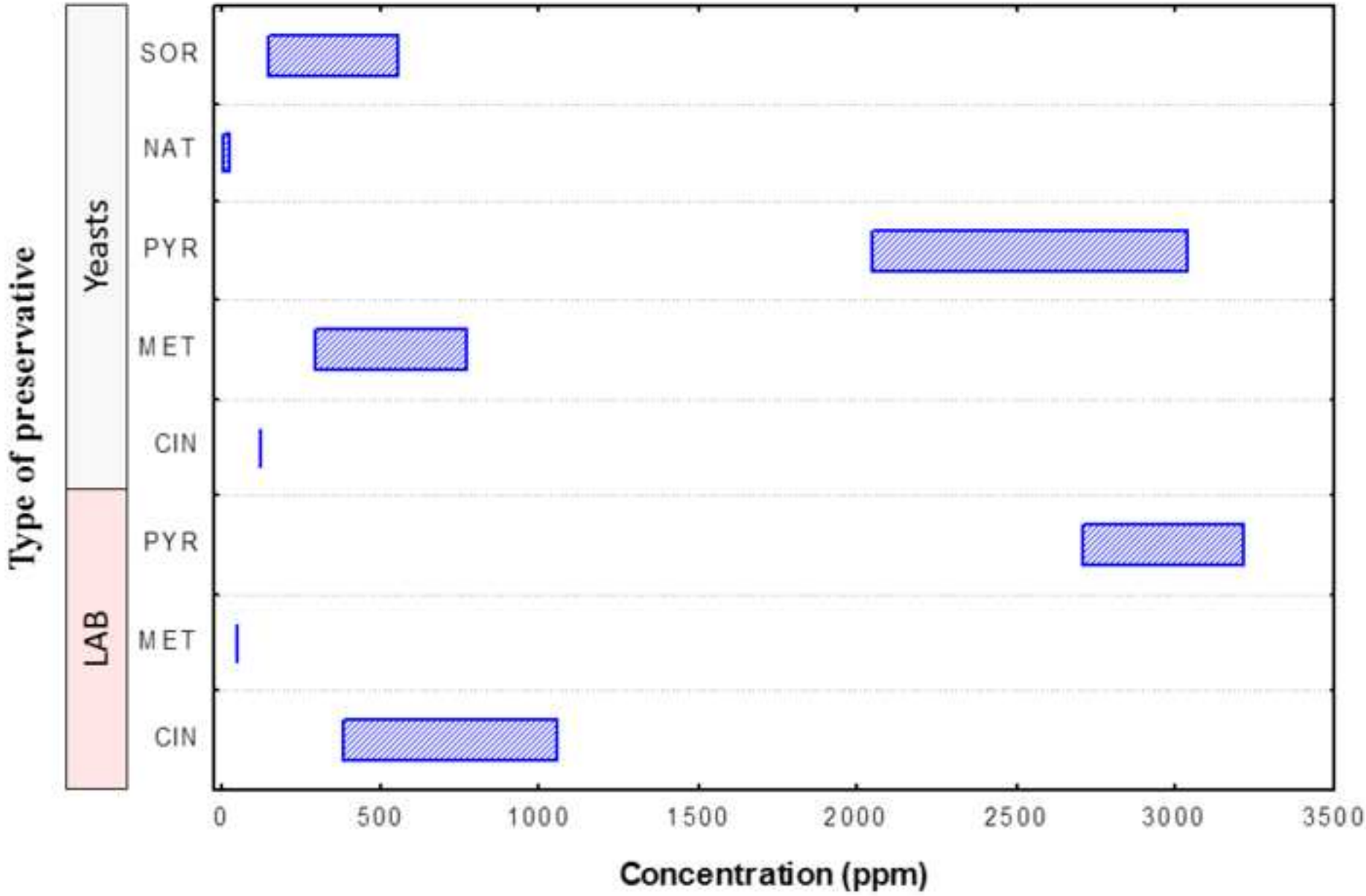

Figure 3

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