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Antimicrobial Activity of Binary and Ternary Mixtures of Vanillin, Citral, and Potassium Sorbate in Laboratory Media and Fruit Purées

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Abstract The susceptibility of *Escherichia coli*, *Salmonella enteritidis*, *Saccharomyces cerevisiae*, and *Zygosaccharomyces bailii* to binary and ternary mixtures of potassium sorbate (KS), vanillin (V), and citral (C) was evaluated according to the Berenbaum experimental design, in laboratory media. For some V/C combinations, KS inhibitory concentrations were determined in agarized melon and mango purées by the spiral gradient endpoint (SGE) method. In laboratory media, inhibitory antimicrobial combinations were generally additives. For the yeasts, some synergistic effects were observed. All Berenbaum mixtures which resulted inhibitory in laboratory media were confirmed in the fruit purées. When the SGE method was used, several inhibitory ternary mixtures were found. The lowest inhibitory KS concentrations, estimated for a given V/C combination, corresponded to the bacteria assayed in melon purée. *Z. bailii* was not inhibited at any condition. Some synergistic antimicrobial combinations (595 ppm V + 251 ppm C + 8 ppm KS in melon and 280 ppm V + 123 ppm C + 8 ppm KS in mango purées) could be useful to achieve a desired inhibitory effect in fruit purées while reducing their concentrations.

Keywords Natural antimicrobial · Spiral gradient endpoint · Berenbaum design · Fruit purées

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Introduction

In the last 20 years, there has been a considerable pressure by consumers to reduce or eliminate chemically synthesized food additives. Although traditionally only one chemical antimicrobial agent was used to preserve a food product, the use of combined agents in a single food system has become more usual (Davidson et al. 2012). Moreover, the interest in the use of alternatives to prevent microbial growth has notably increased (Lanciotti et al. 2004). In particular, among emerging hurdles, naturally occurring antimicrobial compounds have been used in replacement of traditional ones for some years now. Their potential as natural agents for food preservation has been widely recognized and their antibacterial, antifungal, and antioxidant activities, as well as their flavoring properties, have found applications in pharmaceutical, food, and cosmetic industries (Adorjan and Buchbauer 2010; Char et al. 2010; Rivera et al. 2010). Notwithstanding, many of them have a limited activity spectrum and are effective only at very high concentrations which may adversely modify the sensory characteristics of food products, making them unacceptable for consumption. Therefore, proper combinations of antimicrobials may allow to obtain safe products with appreciable sensory properties.

Vanillin is the major phenolic constituent of vanilla beans. At low concentrations, phenols affect the activity of enzymes associated with energy production and, while at high concentrations, provoke protein denaturalization. The effect of phenolic compounds on microbial growth could be the result of their ability to alter the microbial cell permeability, allowing loss of macromolecules. They could also interact with membrane proteins, causing a deformation in their structure and functionality (Yemiş et al. 2011).

Citral (3,7-dimethyl-2,6-octadienal) is an acyclic unsaturated monoterpene aldehyde found naturally in the leaves and

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fruits of several plant species such as lemons, oranges, and bergamots. This aroma compound is widely used due to its citrus notes and as a flavoring agent in foods and beverages. Citral has shown to inactivate *Cronobacter sakazakii* by inducing changes in ATP concentration, cell membrane hyperpolarization, and cytoplasmic pH reduction (Shi et al. 2016). Moreover, citral had the ability to destroy cell membrane integrity of *Geotrichum citri-aurantii* (Zhou et al. 2014) and dramatically inhibited the mycelial growth of *Penicillium italicum* through a mechanism of cell membrane damage, compromising its integrity and permeability (Tao et al. 2014). In addition, Lanciotti et al. (2004) demonstrated that citrus essential oil was able to increase the shelf life of minimally processed fruit mixtures (apple, pear, grape, peach, and kiwifruit) packed under ordinary or modified atmosphere and abuse temperature conditions. These authors also reported that the addition of these substances increased the death rate of *Escherichia coli* inoculated at levels of 10^6 CFU/g.

Potassium sorbate is an effective inhibiting agent of many microorganisms, including yeasts, molds, and bacteria. It is used in the preservation of a wide variety of products (Khanipour et al. 2014). Its mechanism for microbial inactivation has been attributed to the inhibition of various enzymatic systems (Sofos and Busta 1981; Smoot and Pierson 1981). Nevertheless, the use of high concentrations of sorbate as a conventional preservative has some side effects as its consumption may be related to urticarial and contact dermatitis (Lee and Paik 2016). Therefore, it is of high importance the need of reducing its use by a total or partial replacement with natural antimicrobials.

Microbial contamination of whole or minimally processed fruits and vegetables can occur at different stages of processing such as harvest, trimming, washing, slicing, soaking, dehydrating, blending, and/or packaging of fruit products. Fruit products could be easily spoiled by yeasts, being *Saccharomyces cerevisiae* the most responsible strain, due to its ethanol-tolerant condition (Fleet 1992). In addition, *Salmonella* spp., *Listeria* spp., and *Escherichia coli* O157:H7 are the most commonly identified as etiological agents associated with fresh produce-related infections (Heaton and Jones 2008; Mihajlovic et al. 2013). Food preservation is achieved by disturbing the homeostasis of microorganisms. The best way to do this, it is to deliberately disturb the homeostasis of microbial cells by a combination of sublethal antimicrobial factors at a number of sites or in a cooperative manner, thus increasing the effectiveness of food preservation (Guerrero et al. 2017). A promising alternative could be the combination of two or more antimicrobials with different action mechanisms, under hurdle approach, thus reducing their individual concentrations. This approach has been successfully applied for the development of a large variety of foods. In particular, Char et al. (2008) and Ferrante et al. (2007) observed that combination of sub-minimal inhibitory concentration (MIC) levels of vanillin (up to 2000 ppm) and citral (up to 100 ppm) with mild heat (45 to 61 °C) or ultrasound (20 kHz, 95.6 μm), respectively, increased orange juice safety. Similarly, mixtures of 1500 ppm potassium sorbate and 1500 ppm sodium benzoate added to pasteurized tomato juice increased its shelf life as it provoked a decrease in yeast and mold counts along storage at 5 °C (Pina-Pérez et al. 2015).

In vitro or explanatory (endpoint and descriptive methods) and applied (inhibition curves and endpoint methods) tests are the most used strategies for efficacy evaluation of essential oils from plant and spices (Panda 2012; Sethi and Gupta, 2016). Unfortunately, a great number of these studies have been accomplished in vitro, and only few of them have been conducted in real foods (López-Malo et al. 2006). Generalized industrial use of approved naturally occurring antimicrobials encompasses validating their use in real food systems, as their activity may be reduced due to interactions with food components. Thus, the concentrations required for inhibitory or inactivation effects on microorganisms in real foods are considerably higher in comparison with laboratory media and frequently above tolerable taste thresholds (Gutierrez et al. 2009). Therefore, the use of combinations of natural antimicrobials, with different action sites, could be a promising alternative for fruit derivative preservation, thus reducing the individual required amounts and, at the same time, avoiding sensorial rejection. The objective of this research was to study the effect of binary and ternary mixtures of natural (vanillin and citral) and chemical (potassium sorbate) antimicrobial agents on *Escherichia coli* ATCC 35218, *Salmonella enteritidis* MA44, *Saccharomyces cerevisiae* KE 162, and *Zygosaccharomyces bailii* NRRL7256 growth and non-growth responses in laboratory media and agarized melon and mango purées during 10 days of storage at abuse temperature (30 or 37 °C). Moreover, antimicrobial ternary mixtures were validated by the spiral gradient endpoint technique.

Materials and Methods

Antimicrobials

Vanillin (V) and citral (C) alcoholic stock solutions (5.0% *w*/*w*, Firmenich SAICYF, Buenos Aires, Argentina) and potassium sorbate (KS) aqueous stock solution (5.0% *w*/*w*, Química Oeste, SAICYF, Buenos Aires, Argentina) were used in this study. Once prepared, these solutions were sterilized by filtration using a cellulose acetate membrane (pore diameter 0.45 μm, Bellows SRL, Buenos Aires, Argentina) and kept under refrigeration (5 ± 1 °C) in amber glass bottles until use no more than a month. Microbial control assays using ethanol stock solution (96%, Anedra SRL, Buenos Aires, Argentina) without antimicrobial addition were performed to determine any possible solvent effect on microbial growth.

Preparation of Agarized Fruit Systems

Ripe melon (*Cucumis melo*, var. Rocío de miel, pH 6.0 ± 0.2, 12.5–13.8 °Brix) and mango (*Mangifera indica* var. Tommy Atkins, pH 3.5 ± 0.2, 13.5–14.6 °Brix) fruits used in this study were purchased in a local market and immediately refrigerated until use (maximum elapsed time 1 day). Whole fruits were disinfected by immersion in a 1% *v/v* sodium hypochlorite solution during 5 min. They were carefully rinsed with sterilized water and then gently dried with a sterile cloth. Fruits were hand peeled and pit or seeds were removed. Subsequently, it was firstly cut into cubes and pureed using a conveniently sanitized household processor (Moulinex, Thurles, Ireland). Citric acid or sodium hydroxide (5% *v/v*) solutions were used to adjust the pH of both purées to 4.5. Purée samples (250 g) were then packed under vacuum conditions in moisture-proof pouches (Cryovac CN530® film). Pouches containing fruit purées were thermally treated in boiling water for 3 min and rapidly cooled in ice water for the main purpose of inactivating the indigenous flora that could interfere with the microbial challenge study. Finally, each pouch content was mixed with 150 g of recently sterilized and tempered agar-agar (~ 60 °C) and subsequently fractioned into 25-g aliquots. Antimicrobials agents were incorporated and after mixing well, pouch content was poured into 150-mm Petri dishes, hermetically sealed, and stored at 5 ± 0.5 °C until use (maximum elapsed time 24 h).

Inocula

Experiments were performed using *Escherichia coli* ATCC 35218, *Salmonella enteritidis* MA44, *Saccharomyces cerevisiae* KE 162, and *Zygosaccharomyces bailii* NRRL 7256 (all strains were provided by Medica-Tec SRL, Buenos Aires, Argentina). All bacterial inocula were prepared by transferring a loopful of Trypticase Soy Agar (TSA) plus 0.6 g/100 g Yeast Extract (TSAYE) slant stock culture to a 20-mL Erlenmeyer flask of Trypticase Soy Broth supplemented with 0.6 g/100 g Yeast Extract (TSBYE). They were incubated at 37 ± 1 °C under agitation for 18 h until they reached a stationary phase. A similar procedure was performed for the yeasts, for which the initial inocula were prepared by transferring a loopful of a fresh stock culture maintained in Potato Dextrose Agar (PDA) (Britania, CABA, Argentina) to an Erlenmeyer flask containing 20 mL of Sabouraud Dextrose Broth. Incubation was performed at 27 ± 1 °C for 24 h. All inocula were harvested by centrifugation (1475*g*, 5 min) (Labnet International Inc., Edison, NJ, USA), washed twice with saline solution, and resuspended in peptone water (0.1% *w/v*) to give cell densities between 10^7 and 10^9 CFU/mL. High concentrations of inocula are commonly used in this type of study to facilitate the visualization of inhibition of radial streaks.

Determination of Minimal Inhibitory Concentrations of Antimicrobial Agents

Individual MICs corresponding to the three antimicrobials were determined in 100-mm Petri dishes for each microorganism using the agar dilution method. Presence (G) or absence of growth (NG) was determined after 48 h incubation in TSA (pH 4.5, 37 ± 1 °C, bacteria) or PDA (pH 4.5, 30 ± 1 °C, yeast), mixed with a given concentration of one antimicrobial agent and pour plated into Petri dishes. Once solidified the agar, three streaks per plate were evaluated. Plates were stored in hermetical plastic containers to avoid media dehydration. Experiments were performed in triplicate. Enough headspace was left in the containers to avoid anoxic conditions. Based on previous studies (Ferrante et al. 2007; Char et al. 2010), concentration ranges for the three antimicrobial agents used to determine the corresponding MIC values were established as follows: 0–4000 ppm for V, 0–750 ppm for C, and 0–5000 ppm for KS. The MIC value was registered as the concentration corresponding to the NG condition. Growth control plates without antimicrobial addition were prepared and inoculated as above, for testing normal microbial growth.

Efficacy of Antimicrobial Mixtures

Antimicrobial binary and ternary mixtures of V, C, and KS were evaluated by the agar dilution method in agarized culture media and fruit purées and by the spiral gradient endpoint (SGE) method in agarized fruit purées.

Agar Dilution Method

Antimicrobial mixtures were evaluated in TSA (bacteria) or PDA (yeasts) using the Berenbaum experimental design (Berenbaum et al. 1983). It is based on an arrangement of 14 antimicrobial mixtures (Table 1), for which the maximum concentration of each antimicrobial in culture media (featured as 1) corresponded to the determined MIC value (Table 2), while the concentration of each agent in the mixture was estimated as a fraction of its MIC value (Table 1). Once calculated and properly prepared, each system was poured into Petri dishes as explained above and incubated up to 5 days at 30 ± 1 °C (yeasts) or 37 ± 1 °C (bacteria) examining daily for G/NG response. To analyze antimicrobial combinations, MIC values were transformed to fractional inhibitory concentrations (FICs). FICs and FIC indexes were calculated according to the following equations (Davidson and Parish 1989; López-Malo et al. 2006) illustrated, as a mode of example, for a binary combination of V/C

$$FIC_V = (MIC_{V\ with\ C})/MIC_V \quad (1)$$

Table 1 Experimental design utilized to evaluate binary and ternary mixtures of antimicrobial agents (Berenbaum et al. 1983)

Assay*	Vanillin (V)	Citral (C)	Potassium sorbate (KS)
1	1	0	0
2	0	1	0
3	0	0	1
4	0	1/2	1/2
5	1/2	0	1/2
6	1/2	1/2	0
7	1/3	1/3	1/3
8	1/6	1/6	1/6
9	1/6	1/6	2/3
10	2/3	1/6	1/6
11	1/6	2/3	1/6
12	1/12	1/12	1/12
13	1/3	1/12	1/12
14	1/12	1/3	1/12

*Assays 1, 2, and 3 correspond to individual MIC values

$$FIC_C = (MIC_{C\ with\ V})/MIC_C \quad (2)$$

and a ternary combination of V/C/KS

$$FIC_V = (MIC_{V\ with\ C\ and\ KS})/MIC_V \quad (3)$$

$$FIC_C = (MIC_{C\ with\ V\ and\ KS})/MIC_C \quad (4)$$

$$FIC_{KS} = (MIC_{KS\ with\ V\ and\ C})/MIC_{KS} \quad (5)$$

FIC_{Index} was calculated based on these FIC values as follows:

$$FIC_{Index} = FIC_V + FIC_C + FIC_{KS} \quad (6)$$

Several antimicrobial mixtures with NG response (Table 3, marked with *) in culture media were evaluated in melon and mango purées, which were prepared according to the procedure described in "Preparation of Agarized Fruit Systems." Different inocula were subsequently swabbed as described above.

Table 2 Individual MIC values (ppm) determined in laboratory media (pH 4.5) for different microorganisms after 2 days of incubation at 30 °C (yeasts) or 37 °C (bacteria)

Strain	Vanillin (V)	Citral (C)	Potassium sorbate (KS)
E. coli ATCC 35218	1000	750	150
S. enteritidis MA 44	1400	550	200
Z. bailii NRRL7256	1400	400	5000
S. cerevisiae KE 162	3500	375	4500

Spiral Gradient Endpoint Test

SGE test allows to determine the gradient minimal inhibitory concentration (GMIC) for a given antimicrobial agent, which is comparable to its MIC value. Firstly, a gradient of agent concentrations in a spiral pattern is generated by plating a minimal volume of 50 μL of the antimicrobial stock solution (5%). Plates were left for 1 h until antimicrobial diffusion through the agar or agarized fruit purées was completed. Secondly, radial inocula streaks were deposited on the surface of the agar. After plate incubation, visible growth could be observed only on the radial streaks (Fig. 1).

According to this procedure, GMIC values for KS ($GMIC_{KS}$) were determined in fruit purées previously added with proper V and C amounts corresponding to Berenbaum V/C antimicrobial mixtures that previously had accounted for growth response (G) in culture media. Because of the continuous profile of the KS gradient, the growth endpoint could be more accurately determined. A continuous exponential KS concentration gradient was obtained by spiral plating (Autoplate 4000, Spiral Biotech) onto the surface of a 150-mm Petri dish, generating a concentration gradient ranging from 0 to 512 ppm from the outer (64 mm) to the inner of the plate (20 mm) (Fig. 1). Seven radial streaks of each culture (10^6–10^8 CFU/mL) were swabbed onto each Petri dish containing agarized fruit purée with a given V/C mixture. Two microorganisms were tested per plate in septuplicate. Two Petri dishes were used for each condition. Growth control plates without antimicrobial addition were prepared and evaluated as it was described above. It is worthy to note that when stock solutions of V and C were spiral-plated, non-reproducible results were obtained, probably due to their non-aqueous solubility condition. This fact drove the experiment to spiral plate KS (a water-soluble antimicrobial), while proper amounts of V and C were incorporated directly into the agar, which was subsequently poured into the Petri dish, as was previously described. Inoculated systems were incubated at 30 ± 1 or 37 ± 1 °C for the yeasts and bacteria, respectively, and examined at 2, 5, and 10 days of storage. During incubation, biocidal interactions among V, C, and KS were characterized by measuring the distance from the center of the plate to the endpoint of confluent growth. The corresponding antibiotic concentration was calculated using these data and the SGE software. The radial location at which that event occurred was called the endpoint radius (ER), which assumed reading values from 20 (G) to 64 (NG), corresponding to the absence of any inhibition to complete inhibition, respectively (Fig. 1).

Principal Component Analysis

Principal component analysis (PCA) was used to illustrate the relationship between all the assayed ternary antimicrobials'

Table 3 Growth/non-growth (G/NG) response, fractional inhibitory concentration (FIC), and index (FIC_{Index}) corresponding to *E. coli* ATCC 35218 (A), *S. enteritidis* MA44 (B), *S. cerevisiae* KE 162 (C), and *Z. bailii* NRRL 7256 (D) when using binary and ternary mixtures of potassium sorbate (KS), vanillin (V), and citral (C) in laboratory media (pH 4.5) at 5 days, according to the Berenbaum design. Assays 1, 2, and 3 correspond to individual MIC values, (*) combinations of antimicrobials with NG response selected to be validated in agarized melon and mango purées, (+) combinations of antimicrobials with G response selected to be validated by SGE method, (—) inhibitory antimicrobial mixtures

A)

Assay	V (ppm)	C (ppm)	KS (ppm)	Response	FIC_V	FIC_C	FIC_{KS}	FIC_{Index}
1	1000	0	0	NG	1	0	0	1
2	0	750	0	NG	0	1	0	1
3	0	0	150	NG	0	0	1	1
4	0	375	75	NG	0	0.50	0.50	1
5*	500	0	75	NG	0.50	0	0.50	1
6*	500	375	0	NG	0.50	0.50	0	1
7*	330	247	49	NG	0.33	0.33	0.33	1
8+	170	127	25	G				
9	170	127	100	G				
10*	670	127	25	NG	0.67	0.17	0.17	1
11	170	502	25	NG	0.17	0.67	0.17	1
12+	80	60	49	G				
13+	330	60	12	G				
14+	80	247	12	G				

B)

Assay	V (ppm)	C (ppm)	KS (ppm)	Response	FIC_V	FIC_C	FIC_{KS}	FIC_{Index}
1	1400	0	0	NG	1	0	0	1
2	0	550	0	NG	0	1	0	1
3	0	0	200	NG	0	0	1	1
4	0	275	100	G	0	0.50	0.50	1
5	700	0	100	NG	0.50	0	0.50	1
6	700	275	0	NG	0.50	0.50	0	1
7+	462	182	66	G				
8+	238	94	34	G				
9*	238	94	134	NG	0.17	0.17	0.67	1
10*	938	94	34	NG	0.67	0.17	0.17	1
11+	238	369	34	G				
12+	112	44	66	G				
13+	462	44	16	G				
14+	112	182	16	G				

C)

Assay	V (ppm)	C (ppm)	KS (ppm)	Response	FIC_V	FIC_C	FIC_{KS}	FIC_{Index}
1	3500	0	0	NG	1	0	0	1
2	0	375	0	NG	0	1	0	1
3	0	0	4500	NG	0	0	1	1
4	0	187	2250	NG	0	0.50	0.50	1
5*	1750	0	2250	NG	0.50	0	0.50	1
6*	1750	187	0	NG	0.50	0.50	0	1
7*	1155	123	1485	NG	0.33	0.33	0.33	1
8*	595	63	765	NG	0.17	0.17	0.17	0.50
9*	595	63	3015	NG	0.17	0.17	0.67	1
10*	2345	63	765	NG	0.67	0.17	0.17	1
11+	595	251	765	NG	0.17	0.67	0.17	1
12	280	30	1485	NG	0.08	0.08	0.33	0.50
13+	1155	30	360	NG	0.33	0.08	0.08	0.50
14+	280	123	360	NG	0.08	0.33	0.08	0.50

D)

Assay	V (ppm)	C (ppm)	KS (ppm)	Response	FIC_V	FIC_C	FIC_{KS}	FIC_{Index}
1	1400	0	0	NG	1	0	0	1
2	0	400	0	NG	0	1	0	1
3	0	0	5000	NG	0	0	1	1
4	0	200	2500	NG	0	0.50	0.50	1
5*	700	0	2500	NG	0.50	0	0.50	1
6*	700	200	0	NG	0.50	0.50	0	1
7	462	132	1650	NG	0.33	0.33	0.33	1
8*	238	68	850	NG	0.17	0.17	0.17	0.50
9*	238	68	3350	NG	0.17	0.17	0.67	1
10	938	68	850	NG	0.67	0.17	0.17	1
11	238	268	850	NG	0.17	0.67	0.17	1
12	112	32	1650	G				
13+	462	32	400	G				
14+	112	132	400	G				

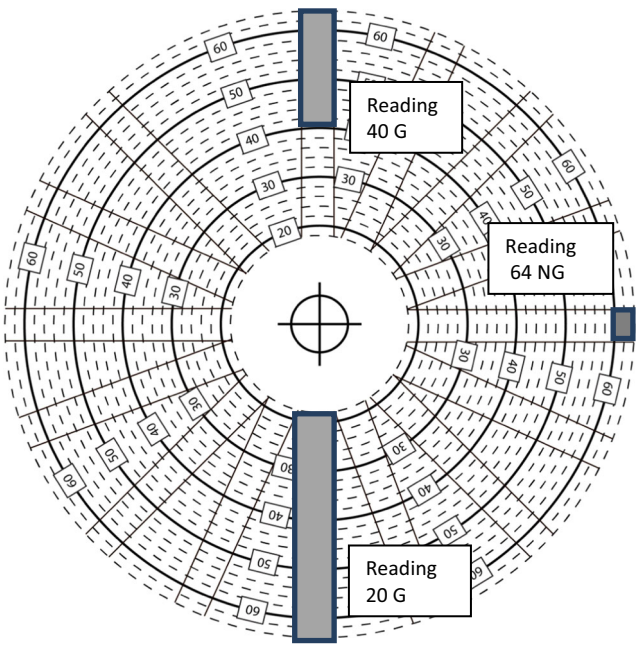

Fig. 1 Example of endpoint radius (ER) readings necessary to estimate MIC values by using the SGE software. Concentric lines describe the route of the antimicrobial (reading values 20–64) and the radial lines show microbial growth. *G*: growth; NG: no growth

combinations which yielded inhibition response in purée samples. The cophenetic correlation coefficient (CCC) was obtained as a measure of how faithfully the analysis preserved the original euclidean distances among data points. A good PCA analysis corresponds to a CCC value close to 1.0. Statistical analysis was performed using InfoStat 2009 (InfoStat Group, FCA-UNC, Córdoba, Argentina).

Results and Discussion

Determination of MICs

Table 2 shows MICs of the synthetic (KS) and naturally occurring (V and C) antimicrobials corresponding to *Escherichia coli* ATCC 35218, *Salmonella enteritidis* MA44, *Saccharomyces cerevisiae* KE 162, and *Zygosaccharomyces bailii* NRRL 7256 in laboratory media. No solvent effect was detected as regular microbial growth, without any inhibition, was observed in the case of using ethanol without antimicrobial addition. V concentrations ranging from 1000 to 1400 ppm inhibited *E. coli*, *S. enteritidis*, and *Z. bailii*, except for *S. cerevisiae* which was the most resistant strain (V 3500 ppm). Fitzgerald et al. (2003) determined vanillin MICs corresponding to three yeast strains, in yeast extracts

peptone dextrose broth (pH 4.0) at 25 °C during 4 days. Susceptibility testing showed that *S. cerevisiae* NCYC 956 and *Z. bailii* NCYC 1427 were more resistant than *Z. rouxii* NCYC 568 (vanillin MIC 1200 ppm) with 2000 and 1900 ppm of vanillin MIC values, respectively. In contrast to our results, lower vanillin MIC values were obtained. Nevertheless, it is important to highlight that different antimicrobial amounts are required to achieve microbial inhibition depending on whether the study is conducted in liquid or agarized media (Gutierrez et al. 2009). Similarly, Cava-Roda et al. (2012) examined the influence of different vanillin concentrations on *Listeria monocytogenes* Scott A and *E. coli* O157:H7 growth by the broth dilution method in TSB broth (pH 7.0), reporting MIC values of 3000 and 2800 ppm, respectively.

On the other hand, when C was used, the bacteria were more resistant than the yeasts. In particular, 750 and 550 ppm of C were needed to inhibit *E. coli* and *S. enteritidis*, respectively (Table 2). Somolinos et al. (2009) showed that 500 ppm of C was required to inhibit *E. coli* ATCC 33985 in a citrate-phosphate buffer. On the same fashion, Leite et al. (2014) reported that for citral, a MIC value of 64 μg/mL inhibited 10 strains of *Candida albicans* in culture media, whereas Lima et al. (2012) observed complete inhibition of *C. albicans* in culture media containing citral in a range from 256 to 512 μg/mL. The lower inhibitory concentrations observed by these authors could be attributed to a well-known better antimicrobial efficacy in liquid media compared to solid matrixes (Gutierrez et al. 2009).

A range of KS from 150 to 200 ppm was enough to inhibit the bacteria (Table 2). Nevertheless, the yeasts were highly resistant, being necessary between 4500 and 5000 ppm of KS to inhibit them (Table 2). These results are in agreement with those reported by Santiesteban-López et al. (2007), who found that 250 and 400 ppm KS were necessary to inhibit *E. coli* ATCC 35218 and *S. typhimurium* ATCC 14028, respectively, in casein-peptone soymeal-peptone agar.

Although some MIC values corresponding to the antimicrobials assayed were extremely high, and thus, inacceptable organoleptic speaking, this assay was considered useful to more precisely establish effective binary and ternary antimicrobial combinations.

Evaluation of Antimicrobial Mixtures in Laboratory Media

Combinations of the three antimicrobials along with the corresponding growth/no growth (G/NG) responses in laboratory media are displayed in Table 3. Fractional inhibitory concentrations (FIC) and FIC indexes are also included. The selected experimental design, proposed by Berenbaum et al. (1983), has been used for antibiotics efficacy. It is focused on determining synergistic mixtures and establishing if they are truly synergistic, regarding all the mixtures assayed. According to this design, individual, binary, and ternary combinations of the three antimicrobials are evaluated for inhibitory effects (Table 3). In particular, combinations in the Berenbaum design represent several fractions of each antimicrobial MIC, which altogether add up to 1 in the mixture. If NG response is determined for a given tested combination, the mixture will be synergic in a consistent way. In particular, a FIC index near 1 implies additivity, < 1 implies synergy, and > 1 implies antagonism (Alzamora et al. 2005).

Bacteria NG responses were obtained in laboratory media that included certain binary and ternary mixtures observing additive effects ($FIC_{Index} = 1$). This response was observed in trials 4, 5, 6, 7, 10, and 11, in the case of *E. coli* (Table 3), and trials 5, 6, 9, and 10 for *S. enteritidis* (Table 3) during 5 days of storage. No synergy was observed for these strains. *E. coli*, *S. enteritidis*, and *Z. bailii* were inhibited, in systems involving ternary mixtures, observing additive effects ($FIC_{Index} = 1$), by combinations which included 1/6, 1/6, and 2/3 MIC of the three antimicrobials (trials 10 and 11 for *E. coli*; 9 and 10 for *S. enteritidis*; and 9, 10, and 11 for *Z. bailii)* (Table 3) or 1/3 MIC of the three antimicrobials in the case of trial 7 (Table 3). For *Z. bailii*, synergistic effects ($FIC_{Index} < 1$) were observed for a combination of the three antimicrobials (1/6 MIC, trial 8). In the case of *S. cerevisiae*, inhibitory synergistic effects were observed at certain ternary combinations encompassing 1/6 MIC of the three antimicrobials (trial 8, Table 3); 1/12 MIC of the three antimicrobials (trial 12, Table 3); or 1/3 and 1/12 MIC (trials 13 and14, Table 3). *S. cerevisiae* was the most sensitive strain with regard to binary and ternary mixtures of KS, V, and C in laboratory media during 5 days of storage as was the only one which exhibited no growth all assayed binary and ternary combinations, followed by *Z. bailii*, *E. coli*, and *S. enteritidis* (Table 3). Despite the fact that *S. cerevisiae* exhibited the highest MIC values against individual antimicrobials (Table 2), it was the most sensitive strain when antimicrobial mixtures were used (Table 3). It is important to highlight that the responses of microorganisms to the action of different antimicrobial agents combined in a hurdle approach could be totally different from those corresponding to individual agents. Those responses may be influenced by several factors such as type, number, and intensity of stressors; the impact of the hurdles on quality; the shelf life required; and microbial sensitivity and matrix, as well as the order in which they are applied (Guerrero et al. 1996).

Efficacy of the Antimicrobial Mixtures in Fruit Purées

It is well known that, in general, antimicrobials could be more effective in culture media than in real foods (Fu et al. 2016). In most cases, inhibitory concentrations determined in model systems significantly increase when the same strains are tested

in real foods. This may be attributed to the fact that the direct use of antimicrobial compounds in food systems faces challenges such as poor water affinity, physical and chemical degradation, and impact on food organoleptic properties. Therefore, new methods of antimicrobial incorporation are being developed such us suitable delivery systems (Fu et al. 2016).

Some combinations of the studied antimicrobials in culture media which exhibited NG response (Table 3, marked with *) were selected to be evaluated in agarized melon and mango purées. These combinations inhibited all tested strains during 5 days of storage in fruit purées (data not shown). These results validated the effectiveness of the selected mixtures in real food systems. Burt (2004) suggested that the low fat content of fruits may contribute to the antimicrobial success of essential oils in fresh produce. This is in agreement with the results reported by Gutierrez et al. (2009), who observed that MIC values of oregano, thyme, and marjoram essential oils were higher in TSB than lettuce leaf model media and beef extract for *Listeria innocua* NCTC 11288, *L. monocytogenes* IL323, and *Enterobacter cloacae.*

Antimicrobial Response in Fruit Purées by SGE Method

Some combinations of the three antimicrobials which exhibited growth response according to the Berenbaum design (Table 3, marked with a plus symbol, +) were selected to be validated in mango and melon purées using the SGE method. It is worthy to note that *S. cerevisiae* inhibition was also evaluated by the SGE method despite the fact that all combinations in the Berenbaum design resulted in NG response, as these results needed to be confirmed by this versatile method. SGE allows the evaluation of a wide range of KS concentrations instead of using several fixed values of the antimicrobial agent. In addition, measuring G/NG microbial responses in agarized fruit purées provides the possibility of comparing them to the results obtained in agar. KS concentrations necessary to inactivate the assayed strains were estimated by spiral plating onto agarized purée systems containing V and C. Equal concentrations of V and C to those used in ternary mixtures were employed, following the procedure described in "Materials and Methods."

Lower KS levels were required to inhibit *E. coli* in melon (< 8 ppm) compared to mango purée (128 ppm) after 2 days of storage at 37 ± 1 °C (Table 4) when combined with 80–170 ppm V and 60–127 ppm C. Exceptionally, *E. coli* was inhibited in mango purée only at the highest V concentration assayed (330 ppm). Notwithstanding, in melon purée, *E. coli* was inhibited for 10 days when 8 ppm KS + 170 ppm V + 127 ppm C were used (Table 4, assay 8). Nevertheless, KS threshold values increased along storage up to 64 ppm in the systems 80 ppm V + 60 ppm C and 80 ppm V + 247 ppm C, by 5 days of storage, or up to 32 ppm in the case of the combination 330 ppm V + 60 ppm C by 10 days of storage (Table 4), whereas the following MIC ranges at 5 days of storage were obtained: 12 to 49 ppm of KS + 80 to 330 ppm of V + 60 to 247 ppm of C for *E. coli* in laboratory media (trials 8, 12, 13, and 14, Table 3). The discrepancy between MIC values obtained in mango and melon purées could be explained by their differences in fat and protein composition. According to McCance and Widdowson (1993), protein and fat content may bind and/or solubilize phenolic compounds, reducing their availability for antimicrobial activity. Fat and protein percentages in the edible portion of mango and melon were described as follows: mango, 0.5% fat and 0.6% protein; melon, 0.1% fat and 0.5% protein. Therefore, in the present study, the higher content of fat and protein present in mango might be responsible for the lower inhibitory effect observed for the natural antimicrobials V and C, thus requiring higher KS levels (64–128 ppm) to inhibit *E. coli* compared to melon purée (8 ppm). The lowest KS concentration (8 ppm) inhibited *E. coli* only in the case of using the highest V concentration was applied (330 ppm) in mango purée. Similarly, a lower inhibitory effect of vanillin was found in banana compared to apple purées, which could be explained to the higher fat and protein content commonly found in this fruit (Alzamora et al. 2005). Likewise, Cava-Roda et al. (2012) obtained vanillin MIC values in milk against *L. monocytogenes* and *E. coli* O157:H7 1.25–3.00 times higher than TSB broth and attributed the lower inhibition of vanillin observed in milk to the protective effect of fat molecules present in this matrix.

All ternary antimicrobial mixtures added to evaluate *S. enteritidis* response in laboratory media (trials 7, 8, 11, 12, 13, and 14, Table 3) involved concentrations in the range between 16 and 66 ppm KS +, between 112 and 462 ppm V, + between 44 and 369 ppm C. However, all these combinations required less than 8 ppm of KS to inhibit *S. enteritidis* in melon and mango purées, during the 10 days of storage (data not shown).

Overall, higher GMICs of KS were necessary (> 512 ppm) to inhibit *S. cerevisiae* in melon and mango purées added with V and C (Table 4) compared to the required KS levels in laboratory media (trials 13 and 14; 360 ppm KS). Exceptionally, lower KS levels (< 8 ppm) in combination with the binary 595-ppm V + 251-ppm C mixture (trial 11) were needed to yield *S. cerevisiae* inhibition in both fruit purées during 10 days of storage (Table 4). Similar results were obtained in trials 13 and 14, between laboratory media and in both fruit purées for *Z. bailii* during the 10 days of storage. This strain was not inhibited at any assayed condition requiring more than 512 ppm of KS, which was the threshold concentration used in the present study (data not shown). Therefore, *Z. bailii* resulted in the most resistant strain in all media assayed in this work. Under certain conditions, some yeasts and molds may exhibit resistance against sorbate. Yeast strains of *Zygosaccharomyces* and *Saccharomyces* genera,

Table 4 Inhibitory potassium sorbate (KS) concentration obtained by SGE method for (A) *E. coli* and (B) *S. cerevisiae* in melon and mango purées added with vanillin (V) and citral (C)

Experiment	V (ppm)	C (ppm)	Melon			Mango		
			KS stock 5% (ppm)					
			Storage time (day)			Storage time (day)		
			2	5	10	2	5	10
A								
8	170	127	< 8	< 8	< 8	128	128	128
12	80	60	< 8	64	64	128	128	128
13	330	60	< 8	< 8	32	< 8	> 512	> 512
14	80	247	< 8	64	64	64	64	64
B								
11	595	251	< 8	< 8	< 8	< 8	< 8	< 8
13	1155	30	> 512	> 512	> 512	< 8	> 512	> 512
14	280	123	> 512	> 512	> 512	< 8	> 512	> 512

among others, may be resistant to potassium sorbate. It is important to highlight that Sofos (2000) evaluated the resistance to this agent over more than 100 yeast strains, reporting that most of them tolerated 150 ppm KS, whereas two strains of *Z. bailii* tolerated 800 ppm KS (Sofos 2000) which is in agreement with the highest resistance observed in this study for this strain.

The SGE method resulted in a simple, accurate, and economic method to measure antimicrobial capacity since many replicates of one condition were evaluated in a single plate. Conversely, the estimation of a range of KS concentrations instead of a specific value represented a disadvantage for this method, in addition to the limitation that requires the use of aqueous stock solutions of antimicrobials.

Principal Component Analysis for Ternary Antimicrobial Combinations

Principal component analysis (PCA) showed spatial relationships among the assayed microorganisms in mango and melon purées with the antimicrobials' concentrations which yielded growth inhibition. A two-dimensional representation of principal components 1 and 2 is presented in Fig. 2. The CCC value was 0.95, indicating that an accurate reduction was achieved with the analysis, and only the first two principal components (PC_1 and PC_2) were retained as they explained 85.1% of the total variance. PC_1 and PC_2 explained 62.2 and 22.9% of the variance, respectively. *Z. bailii* was not included on the PCA analysis because growth was observed at any combination used in fruit purées (trials 13 and 14, Table 3). Bacteria were more sensitive to V and C than *S. cerevisiae* as they were inhibited at lower concentrations of both natural antimicrobials. Thus, *S. cerevisiae* required higher V and C levels at the same KS concentration than used for bacteria (8 ppm). For both fruit purées, all ternary GMICs resulted in *S. enteritidis* inhibition, and no differences were observed between matrices. Therefore, the lowest antimicrobial combination assayed (112 ppm V + 44 ppm C + 8 ppm KS) was effective inhibiting *S. enteritidis* in both purées. Similar GMICs were also obtained for *E. coli* in melon purée, as the lowest ternary combination which yielded inactivation was 80 ppm V + 60 ppm C + 8 ppm KS. Nevertheless, higher concentrations of KS were required for mango compared to melon

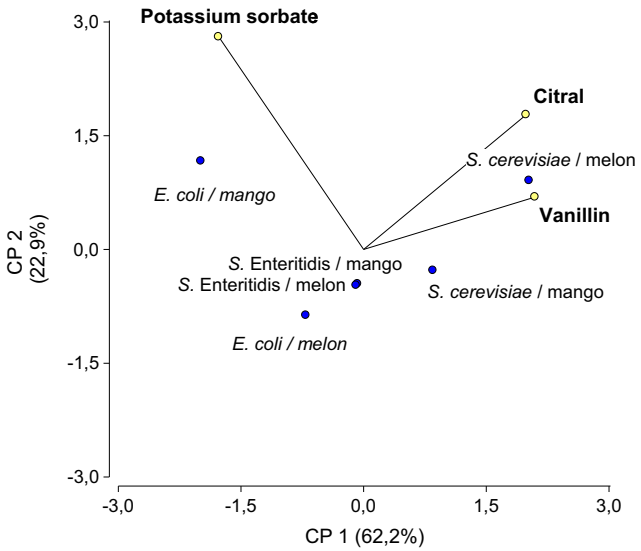

Fig. 2 Principal component analysis (PCA) corresponding to ternary antimicrobial combinations inhibiting *E. coli* ATCC 35218, *S. cerevisiae* KE 162, and *S. enteritidis* MA 44 in mango and melon purées

purée when *E. coli* inhibition was measured. These differences could be explained by higher fat and protein amounts present in the mango fruit, which may be responsible for the lower inhibitory effect observed when used V and C, thus requiring higher KS amount (64–128 ppm) to inhibit *E. coli*. Regarding *S. cerevisiae*, the lowest GMICs obtained in this study were as follows: 595 ppm V + 251 ppm C + 8 ppm KS or 280 ppm V + 123 ppm C + 8 ppm KS, in melon and mango purées, respectively. Therefore, *S. cerevisiae* was the most resistant yeast, as it required higher inhibitory concentrations of these agents. Hence, the antimicrobial response depended on the considered strain and media.

Finally, according to this study, the ternary antimicrobial combinations that were selected due to successfully inhibit *E. coli*, *S. cerevisiae*, and *S. enteritidis* growth in melon and mango purées during 10 days of storage were the following: 595 ppm V + 251 ppm C + 8 ppm KS and 280 ppm V + 123 ppm C + 8 ppm KS.

Conclusions

This study evidenced many factors influencing the antimicrobial efficacy of vanillin, citral, and potassium sorbate used in culture media and fruit purées under a hurdle approach. Antimicrobial response depended on the considered strain, matrix, and evaluation method. Berenbaum design allowed to find some binary and ternary mixtures of KS, V, and C which resulted in additive inhibitory effects. In addition, some antimicrobial mixtures with synergistic effect against yeasts were found, encompassing inhibitory concentrations between 1/12 MIC and 1/3 MIC. They were further successfully applied in fruit purées. Binary and ternary combinations of KS, V, and C that resulted in growth response in laboratory media achieved different inhibitory capacity according to the SGE method, depending on the considered fruit purée. Overall, for a given V/C combination, lower KS concentrations were necessary to inhibit the studied strains in melon compared to mango purée, being the bacteria more sensitive than the yeasts. The ternary antimicrobial mixtures found in this study, 595 ppm V + 251.25 ppm C + 8 ppm KS and 280 ppm V + 123.75 ppm C + 8 ppm KS, have a promising application in melon and mango purées preservation, respectively, as they inhibited for 10 days of storage several relevant microorganisms, even under optimal growth conditions (30 or 37 °C). Although further investigation using these recommended antimicrobial combinations is needed, regarding the study of native microflora response and sensory parameters, among other aspects, this investigation provides an interesting alternative to the use of single antimicrobials for the preservation of mango and melon purées.

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