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Antimicrobial activity of malic acid against *Listeria monocytogenes*, *Salmonella Enteritidis* and *Escherichia coli* O157:H7 in apple, pear and melon juices

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ABSTRACT

Minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of malic acid against *Listeria monocytogenes*, *Salmonella Enteritidis* and *Escherichia coli* O157:H7 inoculated in apple, pear and melon juices stored at 5, 20 and 35 °C were evaluated. MICs and MBCs against *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 were significantly affected by storage temperature, juice characteristics and type of microorganism. Malic acid was more effective at 35 and 20 °C than at 5 °C in all studied fruit juices. *E. coli* O157:H7 was more resistant to malic acid than *S. Enteritidis* and *L. monocytogenes*. Apple, pear and melon juices without malic acid were inhibitory to *E. coli* O157:H7, *S. Enteritidis* and *L. monocytogenes* at 5 °C, whereas, MBCs of 1.5% (v/v) of malic acid in apple and pear juices, and 2% (v/v) in melon juice at 5 °C were needed to reduce *E. coli* O157:H7, those concentrations being higher than those required to reduce *S. Enteritidis* and *L. monocytogenes* in those fruit juices. In addition, concentrations of 2%, 2.5% and 2.5% (v/v) of malic acid added to apple, pear and melon juices, respectively, were required to inactivate the three pathogens by more than 5 log cycles after 24 h of storage at 5 °C. Transmission electron microscopy showed that malic acid produced damage in the cell cytoplasm of pathogens without apparent changes in the cell membrane.

1. Introduction

The consumption of unpasteurized fruit juices defined as the product obtained by pressing or squeezing of the fruits (Harris et al., 2003) has increased in recent years presumably due, in part, to their characteristics of freshness, high vitamins content, low calorie contribution, and an active promotion of fruits and their derivatives as important components of a healthy diet. However, foodborne disease outbreaks caused by *Escherichia coli* O157:H7 and different serovars of *Salmonella* have been associated with unpasteurized fruit juices (CDC, 2007; Harris et al., 2003) demonstrating that those products can serve as a vehicle for pathogenic microorganisms. In addition, incidence or survival/growth of *Listeria monocytogenes*, *Listeria innocua*, *Salmonella* serovars and *Escherichia coli* O157:H7 in fruit juices and apple cider has been demonstrated (Ceylan, Fung, & Sabah, 2004; Harris et al., 2003; Ingham, Schoeller, & Engel, 2006; Miller & Kaspar, 1994; Raybaudi-Massilia, Mosqueda-Melgar, & Martín-Belloso, 2006). In response to the high number outbreaks caused by these pathogenic microorganisms following consumption of fresh products, the Regulatory Organizations have recommended the use of good cleaning and sanitation practices (Garcia, Henderson, Fabri, & Oke, 2006) as well as the application of a hazard analysis and critical control point program for juices production (McLellan & Splittstoesser, 1996). Likewise, the Food and Drug Administration has established regulations for juice manufacturing, indicating that treatments for commercial preparation of fresh juices should be capable of reducing pathogenic loads by a minimum of 5.0 log (Derrickson-Tharrington, Kendall, & Sofos, 2005; USFDA, 2002).

The use of organic acids is considered as a good alternative in the fruit processing industry because of their natural origin and preservative, antioxidant, flavoring and acidifying properties as well as their low cost. However, some important aspects such as kind of juice, characteristics of the spoilage or pathogenic flora and characteristics of the acid must be considered before selecting an acid as antimicrobial agent for fruit juices. Different studies in vitro about the pH effect on *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 have shown that the inhibitory or bactericidal effect depends on the characteristics of the acid used to adjust the medium pH (Buchanan & Klawitter, 1990; Chung & Goepfert, 1970; Glass, Loeffelholz, Ford, & Doyle, 1992; Parish & Higgins, 1989). Thus, variations in effectiveness among acids depend on their molecular structure, size and p*Ka* (Chung & Goepfert, 1970; Eswaranandam, Hettiarachchy, & Johnson, 2004; Parish & Higgins, 1989). In addition, the acid-tolerancy of microorganisms could also affect the effectiveness of organic acids as antimicrobial agents. Hence, studies that show the minimal inhibitory and bactericidal

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concentrations of specific organic acids against those pathogenic microorganisms in fruit juices may be of interest for the industry. Malic acid could be considered as not lipophilic according to its low partition coefficient −1.26 log octanol/water (Leo, Hansch, & Elkins, 1971), thus its mode of antimicrobial action was attributed mainly to reduction in lowering of the pH value (Beuchat & Golden, 1989). However, some authors have indicated that its low molecular size can permit a free diffusion across the cell membrane causing significant damage in the cell cytoplasm (Eswaranandam et al., 2004). Therefore, a better understanding about the mode of antimicrobial action of malic acid is still necessary.

The objective of the present study was to determine the minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations of malic acid against *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in apple, pear and melon juices stored at 5, 20 and 35 °C.

2. Materials and methods

2.1. Fruits and juices preparation

"Fuji" apples (*Malus domestica* Borkh), "Flor de invierno" pears (*Pyrus communis* L.) and "Piel de sapo" melons (*Cucumis melo* L.) at commercial ripeness were purchased in a supermarket of Lleida (Spain) for preparing fruit juices. Each fruit was washed, peeled, cut into pieces and blended using an Ufesa blender (Model BP 4512, Vitoria, Spain). Fruit juices obtained were then centrifuged at 12,500 rpm for 15 min at 4 °C in an Avanti™ J-25 Centrifuge (Beckman Instrument Inc., USA). Each supernatant juice was filtered, bottled and autoclaved in a Presoclave 75 (J.P. Selecta, S.A., Barcelona, Spain) at 121 °C for 15 min to obtain fruit juices free of microorganisms.

2.2. Addition of malic acid to fruit juices

From a sterile solution of D-L-malic acid (Scharlau Chemie S.A., Barcelona, Spain) at 30%, final concentrations to 0%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.5%, 2.0% and 2.5% (v/v) of this acid were added to 100 ml of sterile apple, pear and melon juices individually bottled into 150 ml sterilized polypropylene containers with polyethylene screw-cap (Deltalab, Barcelona, Spain) under a horizontal laminar air flow cabinet (Telstar, S.A., Barcelona, Spain) in aseptic conditions. A pair of containers of each fruit juice and malic acid concentration was prepared. Experiments were carried out twice.

2.3. Cultures and inoculation process

L. monocytogenes 1.131 (CECT 932) and *E. coli* O157:H7 (CECT 4267) from the Spanish Type Culture Collection of the University of Valencia, Valencia, Spain, and *S. Enteritidis* 1.82 (NCTC 9001) from the National Collection of Type Culture of the Central Public Health Laboratory, London, UK, were maintained in tryptone soy agar (TSA) (Biokar Diagnostics, Beauvais, France) slants at 5 °C until use. Stock cultures of *L. monocytogenes* and *E. coli* O157:H7 were grown on tryptone soy broth (TSB) (Biokar Diagnostics) with 0.6% (w/v) yeast extract (YE) (Biokar Diagnostics); whereas, *S. Enteritidis* was cultured in TSB. *E. coli* O157:H7 and *S. Enteritidis* were incubated at 37 °C with continuous agitation for 11 h at 120 rpm, while *L. monocytogenes* was incubated at 35 °C with continuous shaking for 15 h at 200 rpm to obtain cells in early stationary growth phase. The maximum growth for *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 was 10^9 colonies forming units/milliliter (CFU/ml). Concentrations were then adjusted to 10^8 CFU/ml using saline peptone water (0.1% (w/v) peptone plus 0.85% (w/v) NaCl, Scharlau Chemie, S.A., Barcelona, Spain). An aliquot of 1 ml of bacterial suspension (*L. monocytogenes*, *S. Enteritidis* or *E. coli* O157:H7) at approximately 10^8 CFU/ml was individually added to each fruit juice sample containing malic acid in different concentrations. A control of each juice (apple, pear and melon) without malic acid was also inoculated.

2.4. Determination of minimal inhibitory (MIC) and minimal bactericidal (MBC) concentrations

MICs and MBCs of malic acid against *L. monocytogenes*, *S. enteritidis* and *E. coli* O157:H7 were determined by the broth dilution method reported by Davidson and Parish (1989). For that, apple, pear and melon juices with or without malic acid added and individually inoculated with *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 were incubated at 5 °C (temperature normally used for their preservation) for 120 h and, at 20 and 35 °C during 24 h to simulate abuse temperatures. Afterwards, an aliquot of 1 ml of those incubated fruit juices and serial decimal dilutions prepared from their were added to sterile petri plates, and then molten and cooled TSA medium was added to check viable bacteria. In addition, an aliquot of 500 μl of those incubated fruit juices were added to tubes with TSB medium (4.5 ml) to reconfirm cellular death. Those plates and tubes were incubated at 35 °C for 24 h. The MIC was considered as the lowest concentration to maintain or reduce $\leqslant$1 log CFU/ml the inoculum level, whereas, the MBC was considered as the lowest concentration where a reduction >1 log CFU/ml of the inoculated population was observed. Likewise, the necessary minimum concentration to inactivate more than 5 log CFU/ml of each microorganism was also established after examination of the plates and tubes.

2.5. pH determination

The pH of apple, pear and melon juices with different concentrations of malic acid was determined (Table 1) using a Microprocessor pH meter Hanna Instruments PH210 (Vernon Hills, USA).

2.6. Transmission electron microscopy (TEM)

Cells of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 were cultured in TSB medium as in Section 2.3, fruit juices (melon, pear and apple) and fruit juices with malic acid. Afterwards, they were fixed in glutaraldehyde (2.5% in 0.1 M phosphate buffer, pH 7.4) for 1 h, rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and post-fixed with 1% osmium tetraoxide for 2 h a 4 °C. After fixation, the cells were rinsed three times for 10 min with 0.1 M phosphate buffer (pH 7.4) and then dehydrated using 30%, 50%, 70% and 95% acetone sequentially for 15 min each. Next, the cells were dehydrated three times for 30 min with 100% acetone. After dehydration, the cells were treated with propylene oxide twice for 10 min a 4 °C. The cells were sequentially infiltrated with a mixture of propylene oxide:Durcupan's ACM epoxy resin (3:1, 1:1 and 1:3)

Table 1
pH values of apple, pear and melon juices with different concentrations of malic acid

Acid concentration (%)	pH[a]		
	Apple	Pear	Melon
0	3.94 ± 0.01	4.60 ± 0.03	5.45 ± 0.21
0.2	3.57 ± 0.01	3.73 ± 0.01	4.31 ± 0.04
0.4	3.31 ± 0.02	3.45 ± 0.04	3.84 ± 0.03
0.6	3.13 ± 0.03	3.25 ± 0.02	3.62 ± 0.02
0.8	3.06 ± 0.01	3.20 ± 0.15	3.47 ± 0.01
1.0	2.97 ± 0.02	2.99 ± 0.01	3.32 ± 0.04
1.5	2.79 ± 0.04	2.81 ± 0.03	3.13 ± 0.03
2.0	2.68 ± 0.04	2.65 ± 0.01	3.03 ± 0.01
2.5	2.61 ± 0.01	2.51 ± 0.02	2.91 ± 0.01

[a] Means ± standard deviation obtained in two determinations, each one in duplicated (n = 4).