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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.

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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).

for 45 min. Polymerization of the resin to form specimen blocks was performed in an oven at 60 °C for 72 h. The specimen blocks were hand trimmed with a razor blade and sectioned with a diamond knife in a Reichert Ultracut R ultramicrotome (Leica, Wetzler, Germany). Thin sections (70–80 nm) were placed on 300-mesh copper grids. The sections were stained for 15–20 min in uranyl:ethyl alcohol (1:1), after washed three times for 2 min and then incubated in a drop of Reynold's lead citrate and examined using an EM 910 Zeiss transmission electron microscope, Germany.

2.7. Statistical analysis

Statistical analysis of the microbial counts was performed individually for each microorganism using the Statgraphics plus v.5.1 software. A multifactor analysis of variance with posterior multiple range test was used to find significant differences ($p < 0.05$) within kinds of juice (apple, pear and melon), range of concentrations of malic acid (levels: 0%, 0.2%, 0.4%, 0.6%, 0.8%, 1.0%, 1.5%, 2.0% and 2.5%, v/v) and storage temperatures (5, 20 and 35 °C) evaluated.

3. Results and discussion

3.1. Minimal inhibitory concentration (MIC)

Growth of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in apple (pH 3.94), pear (pH 4.60) and melon (pH 5.45) juices without addition of malic acid stored at 5 °C for 120 h was not observed. Therefore, the establishment of MIC of malic acid in these cases was not necessary. Similar results were reported by Ceylan et al. (2004) who demonstrated that *E. coli* O157:H7 population showed negligible changes in apple juice (pH 3.75) stored at 8 °C for 14 days. Likewise, Miller and Kaspar (1994) indicated that *E. coli* O157:H7 population was unchanged in apple cider (3.7–4.1) stored at 4 °C for 14 days. Yuste and Fung (2002) also reported survival but not growth of *L. monocytogenes* in apple juice (pH 3.7) at 5 °C.

Apple and pear juices stored at 20 and 35 °C did not show growth of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 populations either after 24 h (Tables 5–7). A similar behavior was reported by Raybaudi-Massilia et al. (2006) on *L. innocua*, *S. Enteritidis* and *E. coli* inoculated in apple and pear juices stored at 35 °C. Likewise, Yuste and Fung (2002) did not observe growth of *L. monocytogenes* in apple juice (pH 3.7) stored at 20 °C. In addition, survival but not growth of *E. coli* O157:H7 in apple cider (pH 3.6–4.0) from 2 to 3 days at 25 °C as well as in apple juice (pH 3.75) by more than 3 days at 25 °C were reported by Zhao, Doyle, and Besser (1993) and Ceylan et al. (2004), respectively. On the contrary, melon juice stored at 20 and 35 °C did not inhibit the growth of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, since those populations increased significantly after 24 h (Tables 5–7). The microbial growth in melon juice is a direct consequence of its initial pH (Table 1), which is higher than the minimum pH for growth reported for *L. monocytogenes* (4.4), *S. Enteritidis* (3.99) and *E. coli*

Table 2
Effect of malic acid concentration and storage time on *Listeria monocytogenes* inoculated in apple, pear and melon juices stored at 5 °C

Storage time (h)	Malic acid (%)	Survival population in juice (log_{10}CFU/ml)[a]		
		Apple	Pear	Melon
0	0	7.03 ± 0.04 Aaα	6.75 ± 0.24 Aaβ	6.88 ± 0.01 Aaδ
	0.2	6.96 ± 0.05 Aabα	6.68 ± 0.28 Aaβ	6.54 ± 0.09 Abδ
	0.4	6.92 ± 0.03 Abα	6.6 ± 0.4 Aaβ	6.45 ± 0.08 Abcδ
	0.6	6.88 ± 0.01 Abα	6.44 ± 0.06 Aaβ	6.42 ± 0.07 Abcδ
	0.8	6.11 ± 0.02 Acα	6.41 ± 0.01 Aaβ	6.27 ± 0.09 Acdδ
	1.0	5.42 ± 0.08 Adα	5.50 ± 0.17 Abβ	6.12 ± 0.06 Adδ
	1.5	ND Aeα	ND Acβ	2.59 ± 0.16 Aeδ
	2.0	ND Aeα	ND Acβ	ND Afδ
	2.5	ND Aeα	ND Acβ	ND Afδ
24	0	6.99 ± 0.03 Baα	6.6 ± 0.4 Baβ	6.79 ± 0.08 Baδ
	0.2	5.57 ± 0.17 Baα	6.5 ± 0.3 Baβ	6.74 ± 0.06 Baδ
	0.4	3.4 ± 0.3 Baα	4.81 ± 0.05 Bbβ	6.60 ± 0.22 Babδ
	0.6	ND Bbα	1.89 ± 0.16 Bcβ	6.59 ± 0.16 Babcδ
	0.8	ND Bbα	ND Bdβ	2.40 ± 0.11 Bbcδ
	1.0	ND Bbα	ND Bdβ	ND Bcδ
	1.5	ND Bbα	ND Bdβ	ND Bcδ
	2.0	ND Bbα	ND Bdβ	ND Bcδ
	2.5	ND Bbα	ND Bdβ	ND Bcδ
48	0	6.95 ± 0.04 Caα	6.37 ± 0.01 Caβ	6.76 ± 0.08 Caδ
	0.2	1.78 ± 0.21 Cbα	4.4 ± 0.3 Cbβ	6.75 ± 0.04 Caδ
	0.4	ND Ccα	1.27 ± 0.22 Ccβ	6.4 ± 0.5 Caδ
	0.6	ND Ccα	ND Cdβ	5.47 ± 0.11 Cbδ
	0.8	ND Ccα	ND Cdβ	ND Ccδ
	1.0	ND Ccα	ND Cdβ	ND Ccδ
	1.5	ND Ccα	ND Cdβ	ND Ccδ
	2.0	ND Ccα	ND Cdβ	ND Ccδ
	2.5	ND Ccα	ND Cdβ	ND Ccδ
120	0	6.89 ± 0.09 Daα	6.32 ± 0.01 Daβ	6.68 ± 0.02 Daδ
	0.2	ND Dbα	3.2 ± 0.3 Dbβ	6.08 ± 0.26 Dbδ
	0.4	ND Dbα	1.56 ± 0.13 Dcβ	4.3 ± 0.5 Dcδ
	0.6	ND Dbα	ND Ddβ	1.98 ± 0.19 Ddδ
	0.8	ND Dbα	ND Ddβ	ND Deδ
	1.0	ND Dbα	ND Ddβ	ND Deδ
	1.5	ND Dbα	ND Ddβ	ND Deδ
	2.0	ND Dbα	ND Ddβ	ND Deδ
	2.5	ND Dbα	ND Ddβ	ND Deδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

O157:H7 (4.0–4.5) in food (D'Aoust, Maurer, & Bailey, 2001; Lou & Yousef, 1999; Meng, Doyle, Zhao, & Zhao, 2001). Growth of *L. innocua*, *S. Enteritidis* and *E. coli* in melon juice at pH 5.91 stored at 35 °C was also observed by Raybaudi-Massilia et al. (2006).

In this study, MICs of malic acid against *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in melon juice at 20 and 35 °C were different; *S. Enteritidis* and *L. monocytogenes* were more sensitive than *E. coli* O157:H7, since a concentration of 0.2% of malic acid was sufficient to inhibit these first two pathogens after 24 h of storage, whereas, a concentration of 0.4% was necessary to inhibit the *E. coli* O157:H7 growth under the same conditions (Tables 5–7).

3.2. Minimal bactericidal concentration (MBC)

Malic acid was shown to be effective for reducing and inactivating to undetectable levels of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 populations in apple, pear and melon juices. The bactericidal activity of the acid depended upon storage temperature, kind of juice, malic acid concentration, microorganism type (Gram positive or Gram negative) and acid-tolerance of each microorganism. Bactericidal action of malic acid in fruit juices, where growth of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 was not detected, was confirmed after a cells-injured repair step in TSB medium at 35 °C for 24 h. Statistical analyses were made independently for each microorganism at 5, 20 and 35 °C, showing significant differences ($p < 0.05$) among microorganism counts depending on the malic acid concentration, kind of juice and storage time.

Higher concentrations of malic acid were generally required to reduce by more than 1 log cycle (MBCs) populations of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in melon and pear juices than in apple juice (Tables 2–7), indicating that the initial pH of the growth medium is an important factor to consider when organic acids are used to control pathogenic microorganisms in fruit juices. After 24 h of incubation at 20 and 35 °C MBCs of 0.2%, 0.4% and 0.6% (v/v) in apple, pear and melon juices, respectively, were required for *L. monocytogenes* and *S. Enteritidis*, whereas, MBCs of 0.6%, 1% and 2% (v/v) at 20 °C and 0.4%, 0.6% and 0.8% (v/v) at 35 °C were needed for *E. coli* O157:H7 in those juices (Tables 5–7). However, at 5 °C apple and pear juices required the same MBCs for each pathogen after 24 h of storage, in contrast with melon juice where higher MBCs were needed (Tables 2–4).

On the other hand, storage time had significant influence ($p < 0.05$) over the MBCs required to reduce or inactivate *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7, thus decreasing the MBCs and minimal concentration for totally inactivating those microorganisms when storage time increased (Tables 2–7). Hence, concentrations of 1.5%, 1.5% and 2% v/v were required to immediately inactivate ($t = 0$ h) *L. monocytogenes* in apple, pear and melon juices, whereas, concentrations over 2% and 2.5% (v/v) were needed

Table 3
Effect of malic acid concentration and storage time on *Salmonella Enteritidis* inoculated in apple, pear and melon juices stored at 5 °C

Storage time (h)	Malic acid (%)	Survival population in juice ($\log_{10}$CFU/ml)[a]		
		Apple	Pear	Melon
0	0	7.53 ± 0.03 Aaα	6.51 ± 0.05 Aaβ	6.56 ± 0.06 Aaδ
	0.2	7.44 ± 0.09 Aaα	6.54 ± 0.09 Aaβ	6.55 ± 0.10 Aaδ
	0.4	6.97 ± 0.10 Abα	6.51 ± 0.10 Aaβ	6.40 ± 0.14 Aaδ
	0.6	6.79 ± 0.12 Acα	6.34 ± 0.10 Abcβ	6.38 ± 0.03 Aaδ
	0.8	6.62 ± 0.03 Acα	6.25 ± 0.07 Aabβ	6.41 ± 0.09 Aaδ
	1.0	6.32 ± 0.12 Adα	6.22 ± 0.10 Acβ	6.38 ± 0.13 Aaδ
	1.5	5.90 ± 0.05 Aeα	5.09 ± 0.02 Adβ	5.98 ± 0.03 Abδ
	2.0	ND Afα	ND Aeβ	ND Acδ
	2.5	ND Afα	ND Aeβ	ND Acδ
24	0	7.52 ± 0.05 Baα	6.29 ± 0.10 Baβ	6.55 ± 0.05 Babδ
	0.2	7.21 ± 0.13 Bbα	6.19 ± 0.21 Baβ	6.53 ± 0.02 Baδ
	0.4	6.98 ± 0.03 Bcα	6.18 ± 0.26 Baβ	6.50 ± 0.06 Babδ
	0.6	6.92 ± 0.11 Bcα	5.98 ± 0.03 Baβ	6.39 ± 0.02 Bbδ
	0.8	4.33 ± 0.21 Bdα	5.2 ± 0.3 Bbβ	6.15 ± 0.21 Bcδ
	1.0	ND Beα	2.86 ± 0.07 Bcβ	4.63 ± 0.10 Bdδ
	1.5	ND Beα	ND Bdβ	ND Beδ
	2.0	ND Beα	ND Bdβ	ND Beδ
	2.5	ND Beα	ND Bdβ	ND Beδ
48	0	7.40 ± 0.03 Caα	6.28 ± 0.08 Caβ	6.55 ± 0.01 Caδ
	0.2	7.38 ± 0.17 Caα	6.23 ± 0.16 Caβ	6.54 ± 0.03 Caδ
	0.4	7.2 ± 0.3 Caα	6.15 ± 0.21 Cbβ	6.53 ± 0.03 Caδ
	0.6	5.29 ± 0.01 Cbα	5.66 ± 0.00 Ccβ	6.51 ± 0.16 Caδ
	0.8	ND Ccα	2.86 ± 0.06 Cdβ	5.36 ± 0.14 Cbδ
	1.0	ND Ccα	ND Ceβ	3.30 ± 0.05 Ccδ
	1.5	ND Ccα	ND Ceβ	ND Cdδ
	2.0	ND Ccα	ND Ceβ	ND Cdδ
	2.5	ND Ccα	ND Ceβ	ND Cdδ
120	0	7.15 ± 0.21 Daα	6.22 ± 0.01 Daβ	6.46 ± 0.11 Daδ
	0.2	5.60 ± 0.09 Dbα	6. 2 ± 0.4 Dbβ	6.45 ± 0.01 Daδ
	0.4	3.43 ± 0.16 Dcα	6.09 ± 0.25 Dbβ	6.41 ± 0.06 Dabδ
	0.6	ND Ddα	3.87 ± 0.08 Dcβ	6.29 0.10 Dbδ
	0.8	ND Ddα	ND D dβ	4.76 ± 0.08 Dcδ
	1.0	ND Ddα	ND Ddβ	2.59 ± 0.09 Ddδ
	1.5	ND Ddα	ND Ddβ	ND Deδ
	2.0	ND D dα	ND Ddβ	ND Deδ
	2.5	ND Ddα	ND Ddβ	ND Deδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

to achieve that same effect in *S. Enteritidis* and *E. coli* O157:H7 populations, respectively, in those juices. However, after 24 h of storage at 5 °C, lower concentrations of malic acid (0.6%, 0.8% and 1%, v/v) caused that same inactivation in *L. monocytogenes* in those fruit juices, whereas, concentrations of 1%, 1.5% and 1.5% (v/v) for *S. Enteritidis* and 2%, 2.5% and 2.5% (v/v) for *E. coli* O157:H7 were enough to inactivate them (Tables 2–4). At 20 and 35 °C, a similar effect of the storage time over the inactivation of those microorganisms was observed (Tables 5–7).

Significant influence of the storage temperature on the reductions of pathogenic microorganisms in fruit juices was observed. In general, higher concentrations of malic acid were required to reduce *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 in those fruit juices stored at 5 °C than at 20 and 35 °C (Tables 2–7). Thus, MBCs of 0.4% (v/v) of malic acid in apple and pear juices and 0.8% (v/v) in melon juice stored at 5 °C for 24 h were required for reducing *L. monocytogenes*, whereas, for *S. Enteritidis* (0.8% and 1%) and *E. coli* O157:H7 (1.5% and 2%) higher MBCs in those juices were needed. According to those results Conner and Kotrola (1995) reported that malic acid at 0.6% (v/v) added to TSB medium with YE was inhibitory but not bactericidal for *E. coli* O157:H7 during approximately 35 days at 4 °C. Nevertheless, at 20 and 35 °C MBCs of 0.2%, 0.4% and 0.6% (v/v) for *L. monocytogenes* and *S. Enteritidis* were found in apple, pear and melon juices, respectively, whereas, for *E. coli* O157:H7 0.6%, 1% and 2% (v/v) at 20 °C and 0.4%, 0.6% and 0.8% (v/v) at 35 °C were needed in those juices. Likewise, minimal concentrations to reduce by more than five cycles those pathogens in fruit juices were also higher at 5 °C than 20 and 35 °C. In general, concentrations of 2% (v/v) of malic acid in apple juice and 2.5% (v/v) in pear and melon juices were necessary to totally inactivate *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 after 24 h of incubation at 5 °C (Tables 2–4), whereas, lower concentrations of that acid in apple, pear and melon juices were enough to reach that same effect over those pathogens at 20 °C (0.8%, 1.5% and 2.5%, v/v) and 35 °C (0.8%, 1.5% and 1.5%, v/v) after 24 h of storage (Tables 5–7). A greater fluidity of the cellular membrane of the microorganisms at high temperatures (> 20 °C) could favor the entry of malic acid to the cell interior. Aronsson and Rönner (2001) indicated that the temperature of the medium in which cells are suspended has a significant influence on the membrane fluidity properties. At low temperatures, the phospholipids are closely packed into a rigid gel structure, while at high temperatures they are less ordered and membrane has a liquid-crystalline structure.

According to the results obtained in this study, temperature of 5 °C should be selected as an indicator temperature when organic acids are used as antimicrobial agents in fruit juices, since pathogenic microorganisms were less affected by malic acid at this temperature in comparison with 20 and 35 °C.

The bactericidal action of the organic acid can be also influenced by membrane structure and acid-tolerance of each micro-

Table 4
Effect of malic acid concentration and storage time on *Escherichia coli* O157:H7 inoculated in apple, pear and melon juices stored at 5 °C

Storage time (h)	Malic acid (%)	Survival population in juice (log_{10}CFU/ml)[a]		
		Apple	Pear	Melon
0	0	6.90 ± 0.06 Aaα	6.88 ± 0.03 Aabβ	6.89 ± 0.07 Aaδ
	0.2	6.89 ± 0.02 Aaα	6.87 ± 0.03 Abβ	6.85 ± 0.09 Aaδ
	0.4	6.89 ± 0.02 Aaα	6.80 ± 0.05 Abβ	6.79 ± 0.20 Aaδ
	0.6	6.88 ± 0.03 Aaα	6.83 ± 0.02 Aabβ	6.73 ± 0.24 Aaδ
	0.8	6.91 ± 0.02 Aaα	6.81 ± 0.03 Aabβ	6.74 ± 0.01 Aabδ
	1.0	6.91 ± 0.01 Aaα	6.83 ± 0.02 Aabβ	6.50 ± 0.01 Aabδ
	1.5	6.96 ± 0.11 Aaα	6.85 ± 0.01 Aabβ	5.97 ± 0.17 Aabδ
	2.0	6.3 ± 0.5 Abα	6.69 ± 0.02 Acβ	5.83 ± 0.19 Aabδ
	2.5	3.02 ± 0.21 Acα	5.83 ± 0.03 Adβ	5.70 ± 0.02 Abδ
24	0	6.75 ± 0.08 Baα	6.63 ± 0.03 Baβ	6.85 ± 0.08 Baδ
	0.2	6.74 ± 0.06 Baα	6.59 ± 0.16 Baβ	6.82 ± 0.06 Baδ
	0.4	6.68 ± 0.04 Baα	6.52 ± 0.11 Baβ	6.79 ± 0.08 Baδ
	0.6	6.71 ± 0.02 Baα	6.39 ± 0.12 Baβ	6.70 ± 0.06 Baδ
	0.8	6.45 ± 0.21 Babα	6.2 ± 0.3 Babβ	6.72 ± 0.17 Baδ
	1.0	6.3 ± 0.4 Bbα	5.95 ± 0.07 Bbβ	6.4 ± 0.6 Baδ
	1.5	3.54 ± 0.09 Bcα	3.64 ± 0.23 Bcβ	5.4 ± 0.4 Bbδ
	2.0	ND Bdα	1.2 ± 0.3 Bdβ	3.2 ± 0.4 Bcδ
	2.5	ND Bdα	ND Beβ	ND Bdδ
48	0	6.72 ± 0.02 Caα	6.56 ± 0.12 Caβ	6.80 ± 0.08 Caδ
	0.2	6.62 ± 0.22 Caα	6.53 ± 0.10 Caβ	6.75 ± 0.04 Caδ
	0.4	6.1 ± 0.4 Cbα	6.53 ± 0.11 Caβ	6.77 ± 0.04 Caδ
	0.6	5.7 ± 0.3 Cbα	6.41 ± 0.15 Caβ	6.74 ± 0.02 Caδ
	0.8	5.1 ± 0.3 Ccα	6.60 ± 0.08 Caβ	6.73 ± 0.03 Caδ
	1.0	3.64 ± 0.02 dCα	3.68 ± 0.14 Cbβ	6.31 ± 0.05 Cbδ
	1.5	1.38 ± 0.03 Ceα	1.44 ± 0.19 Ccβ	4.72 ± 0.11 Ccδ
	2.0	ND Cfα	ND Cdβ	ND Cdδ
	2.5	ND Cfα	ND Cdβ	ND Cdδ
120	0	6.54 ± 0.09 Daα	6.20 ± 0.20 Daβ	6.78 ± 0.05 Daδ
	0.2	6.51 ± 0.20 Daα	6.10 ± 0.07 Dabβ	6.74 ± 0.01 Dabδ
	0.4	6.25 ± 0.18 Daα	6.11 ± 0.22 Dabβ	6.72 ± 0.17 Dabδ
	0.6	5.3 ± 0.4 Dbα	5.92 ± 0.08 Dbβ	6.61 ± 0.10 Dbδ
	0.8	2.84 ± 0.05 Dcα	4.82 ± 0.04 Dcβ	6.04 ± 0.03 Dcδ
	1.0	2.16 ± 0.16 Ddα	2.77 ± 0.11 Ddβ	2.80 ± 0.28 Ddδ
	1.5	ND Deα	ND Deβ	ND Deδ
	2.0	ND Deα	ND Deβ	ND Deδ
	2.5	ND Deα	ND Deβ	ND Deδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

Table 5
Effect of malic acid concentration and storage time on *Listeria monocytogenes* inoculated in apple, pear and melon juices stored at 20 and 35 °C

Storage time (h)	Malic acid (%)	Survival population in juice (log_{10}CFU/ml)[a]					
		Apple		Pear		Melon	
		20 °C	35 °C	20 °C	35 °C	20 °C	35 °C
0	0	7.36 ± 0.05 Aaα		6.61 ± 0.12 Aaβ		6.63 ± 0.02 Aaδ	
	0.2	7.07 ± 0.04 Aaα		6.36 ± 0.18 Aaβ		6.57 ± 0.07 Aaδ	
	0.4	7.00 ± 0.05 Aaα		6.6 ± 0.3 Aaβ		6.46 ± 0.28 Aabδ	
	0.6	6.67 ± 0.04 Abα		6.43 ± 0.26 Aaβ		6.32 ± 0.11 Abcδ	
	0.8	6.37 ± 0.10 Acα		5.75 ± 0.09 Abβ		6.17 ± 0.10 Acδ	
	1.0	4.91 ± 0.15 Adα		5.06 ± 0.15 Acβ		6.08 ± 0.09 Adδ	
	1.5	ND Aeα		ND Adβ		2.81 ± 0.23 Aeδ	
	2.0	ND Aeα		ND Adβ		ND Afδ	
	2.5	ND Aeα		ND Adβ		ND Afδ	
24	0	6.44 ± 0.08 Baα	6.78 ± 0.01 Baα	6.44 ± 0.18 Baβ	6.61 ± 0.05 Baβ	8.54 ± 0.03 Baδ	8.7 ± 0.1 Baδ
	0.2	2.60 ± 0.18 Bbα	1.34 ± 0.06 Bbα	6.2 ± 0.3 Baβ	6.20 ± 0.28 Bbβ	6.44 ± 0.08 Bbδ	6.48 ± 0.20 Bbδ
	0.4	ND Bcα	ND Bcα	2.13 ± 0.07 Bbβ	1.87 ± 0.13 Bcβ	6.30 ± 0.03 Bcδ	6.30 ± 0.06 Bbδ
	0.6	ND Bcα	ND Bcα	ND Bcβ	ND Bdβ	5.30 ± 0.04 Bdδ	ND Bcδ
	0.8	ND Bcα	ND Bcα	ND Bcβ	ND Bdβ	ND Beδ	ND Bcδ
	1.0	ND Bcα	ND Bcα	ND Bcβ	ND Bdβ	ND Beδ	ND Bcδ
	1.5	ND Bcα	ND Bcα	ND Bcβ	ND Bdβ	ND Beδ	ND Bcδ
	2.0	ND Bcα	ND Bcα	ND Bcβ	ND Bdβ	ND Beδ	ND Bcδ
	2.5	ND Bcα	ND Bαc	ND Bcβ	ND Bdβ	ND Beδ	ND Bcδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

Table 6
Effect of malic acid concentration and storage time on *Salmonella Enteritidis* inoculated in apple, pear and melon juices stored at 20 and 35 °C

Storage time (h)	Malic acid (%)	Survival population in juice (log_{10}CFU/ml)[a]					
		Apple		Pear		Melon	
		20°C	35 °C	20 °C	35 °C	20 °C	35 °C
0	0	7.55 ± 0.01 Aaα		6.25 ± 0.05 Aaβ		6.22 ± 0.02 Aaδ	
	0.2	7.37 ± 0.19 Aabα		6.12 ± 0.11 Aaβ		6.21 ± 0.02 Aaδ	
	0.4	7.50 ± 0.03 Aaα		6.01 ± 0.06 Abβ		6.00 ± 0.08 Abδ	
	0.6	7.42 ± 0.01 Abα		5.95 ± 0.03 Abβ		6.08 ± 0.05 Abδ	
	0.8	7.22 ± 0.05 Acα		5.81 ± 0.20 Abcβ		5.91 ± 0.12 Abcδ	
	1.0	6.79 ± 0.02 Adα		5.72 ± 0.16 Acβ		5.88 ± 0.09 Acδ	
	1.5	3.90 ± 0.03 Aeα		4.5 ± 0.3 Adβ		5.58 ± 0.03 Adδ	
	2.0	ND Afα		ND Aeβ		ND Aeδ	
	2.5	ND Afα		ND Aeβ		ND Aeδ	
24	0	6.98 ± 0.17 Baα	6.74 ± 0.02 Baα	5.53 ± 0.10 Baβ	5.65 ± 0.03 Baβ	8.18 ± 0.10 Baδ	8.74 ± 0.01 Baδ
	0.2	6.10 ± 0.03 Bbα	5.67 ± 0.01 Bbα	5.39 ± 0.17 Baβ	5.71 ± 0.06 Baβ	6.00 ± 0.11 Bbδ	6.17 ± 0.01 Bbδ
	0.4	5.3 ± 0.3 Bcα	3.29 ± 0.02 Bcα	5.15 ± 0.07 Bbβ	4.5 ± 0.1 Bbβ	5.26 ± 0.12 Bcδ	5.71 ± 0.05 Bcδ
	0.6	2.92 ± 0.09 Bdα	2.04 ± 0.06 Bdα	3.37 ± 0.22 Bcβ	1.00 ± 0.10 Bcβ	3.68 ± 0.14 Bdδ	2.40 ± 0.09 Bdδ
	0.8	ND Beα	ND Beα	ND Bdβ	ND Bdβ	ND Beδ	ND Beδ
	1.0	ND Beα	ND Beα	ND Bdβ	ND Bdβ	ND Beδ	ND Beδ
	1.5	ND Beα	ND Beα	ND Bdβ	ND Bdβ	ND Beδ	ND Beδ
	2.0	ND Beα	ND Beα	ND Bdβ	ND Bdβ	ND Beδ	ND Beδ
	2.5	ND Beα	ND Beα	ND Bdβ	ND Bdβ	ND Beδ	ND Beδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

organism. In this sense, Nikaido (1996, 2003) has reported that the resistance mechanisms in Gram negative bacteria are more complicated than those present in Gram positive bacteria, since the former are surrounded by outer membranes or membrane-like structures of hydrophobic nature that may block the entry of hydrophilic molecules of low molecular mass such as monosaccharides, amino acids, nucleosides and alkali or alkaline ions, which only can pass through water-filled channels formed by transmembrane proteins (porins) embedded into the lipid bilayer that permit hydrophilic transport. On the other hand, the Gram positive cell wall contains only a thick peptidoglycan layer and a lipid bilayer. That fact would explain the greater sensitivity of *L. monocytogenes* (Gram positive) to malic acid than *S. Enteritidis* and *E. coli* O157:H7 (Gram negatives) in fruit juices. On the other hand, *E. coli* O157:H7 was more resistant to malic acid than *S. Enteritidis*, since higher concentrations of malic acid were needed to reduce more than 5 log cycles of this microorganism in apple, pear and melon juices in comparison with *S. Enteritidis*. That result is according to those reported by others authors (Arnold & Kaspar, 1995; Benjamin & Datta, 1995; Lin et al., 1996; Miller & Kaspar, 1994) who indicated that acid-resistance is a typical characteristic of this microorganism. Therefore, *E. coli* O157:H7 should be considered as a target microorganism to evaluate the effectiveness of organic acids in fruit juices.

Table 7
Effect of malic acid concentration and storage time on *Escherichia coli* O157:H7 inoculated in apple, pear and melon juices stored at 20 and 35 °C

Storage time (h)	Malic acid (%)	Survival population in juice (log_{10}CFU/ml)[a]					
		Apple		Pear		Melon	
		20 °C	35 °C	20 °C	35 °C	20 °C	35 °C
0	0	6.57 ± 0.02 Aaα		6.47 ± 0.04 Aaβ		6.55 ± 0.02 Aaδ	
	0.2	6.57 ± 0.16 Aaα		6.47 ± 009 Aaβ		6.55 ± 0.08 Aaδ	
	0.4	6.57 ± 0.18 Aaα		6.40 ± 0.03 Aaβ		6.55 ± 0.14 Aaδ	
	0.6	6.67 ± 0.08 Aaα		6.43 ± 0.10 Aaβ		6.51 ± 0.16 Aaδ	
	0.8	6.68 ± 0.04 Aaα		6.41 ± 0.08 Aaβ		6.50 ± 0.07 Aaδ	
	1.0	6.52 ± 0.12 Aaα		6.43 ± 0.02 Aaβ		6.50 ± 0.02 Aaδ	
	1.5	6.61 ± 0.00 Aaα		6.45 ± 0.05 Aaβ		6.50 ± 0.03 Aaδ	
	2.0	6.62 ± 0.05 Aaα		6.29 ± 0.10 Abβ		6.47 ± 0.19 Aaδ	
	2.5	2.94 ± 0.14 Abα		5.83 ± 0.21 Acβ		6.3 ± 0.3 Abδ	
24	0	6.57 ± 0.03 Baα	6.74 ± 0.01 Baα	6.21 ± 0.03 Baβ	5.96 ± 0.00 Baβ	8.59 ± 0.24 Baδ	8.65 ± 0.01 Baδ
	0.2	6.59 ± 0.01 Baα	5.72 ± 0.03 Bbα	6.15 ± 0.21 Babβ	5.91 ± 0.01 Baβ	6.73 ± 0.07 Bbδ	6.89 ± 0.06 Bbδ
	0.4	6.59 ± 0.01 Baα	5.06 ± 0.03 Bcα	6.07 ± 0.10 Bbβ	5.52 ± 0.07 Bbβ	6.54 ± 0.03 Bcδ	5.56 ± 0.01 Bcδ
	0.6	3.19 ± 0.02 Bbα	3.09 ± 0.02 Bdα	6.30 ± 0.05 Bcβ	4.35 ± 0.07 Bcβ	6.52 ± 0.06 Bcδ	5.11 ± 0.16 Bdδ
	0.8	ND Bcα	ND Beα	6.30 ± 0.03 Bcβ	2.62 ± 0.02 Bdβ	6.23 ± 0.09 Bdδ	1.30 ± 0.00 Beδ
	1.0	ND Bcα	ND Beα	3.83 ± 0.07 Bdβ	2.52 ± 0.11 Bdβ	6.36 ± 0.08 Beδ	1.36 ± 0.11 Beδ
	1.5	ND Bcα	ND Beα	ND Beβ	ND Beβ	5.97 ± 0.14 Bfδ	ND Bfδ
	2.0	ND Bcα	ND Beα	ND Beβ	ND Beβ	2.13 ± 0.21 Bgδ	ND Bfδ
	2.5	ND Bcα	ND Beα	ND Beβ	ND Beβ	ND Bhδ	ND Bfδ

ND = not detected.
Different capital letters indicate significant differences ($p < 0.05$) among storage times for each juice; different lower-case letters show significant differences ($p < 0.05$) among malic acid concentrations for each storage time and juice; different Greek letters demonstrate significant differences ($p < 0.05$) among fruit juices for each storage time.
[a] Means ± standard deviation obtained in two experiments, each one in duplicated ($n = 4$).

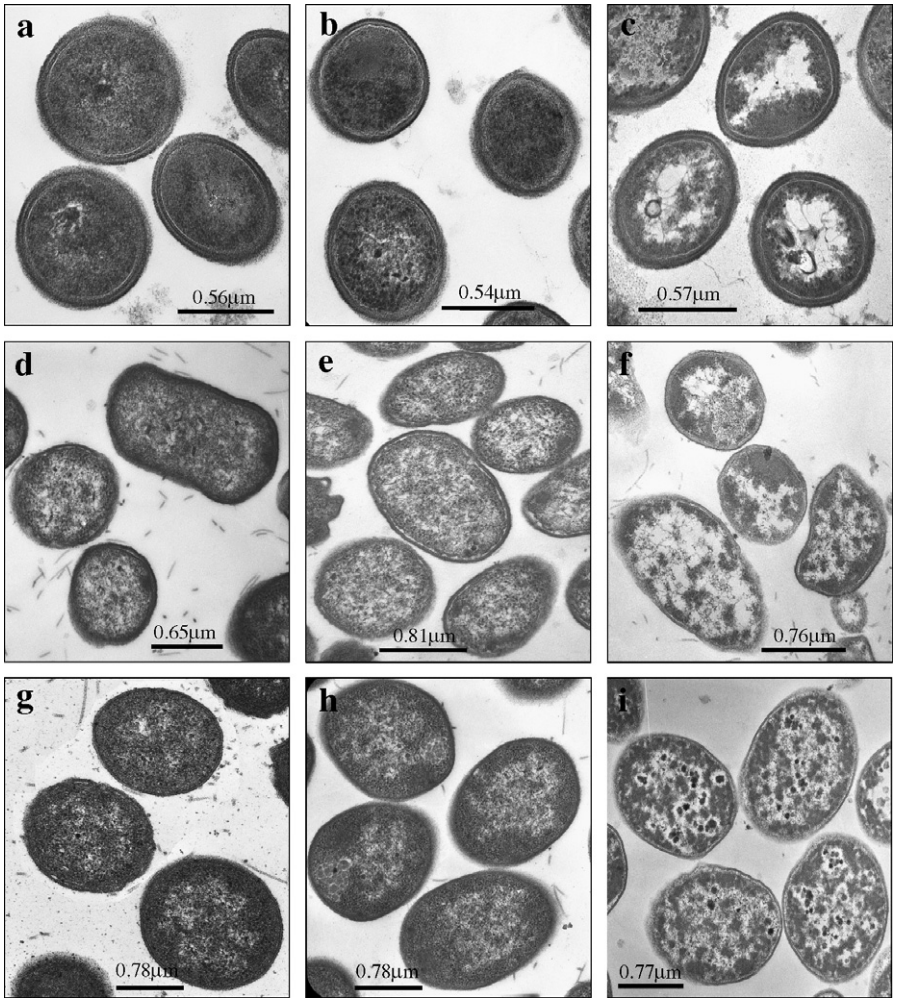

Fig. 1. Transmission electron microscopy (TEM) micrographs of *L. monocytogenes* (a–c; 50,000×), *S. Enteritidis* (d–f; 31,500×) and *E. coli* O157:H7 (g–i; 25,000×) cells from pure culture (a, d, g), melon (b), pear (e) and apple (h) juices, and melon juice with 0.6% (v/v) malic acid (c), pear juice with 0.6% (v/v) malic acid (f) and apple juice with 0.8% (v/v) malic acid (i). Fruit juices with or without malic acid were incubated at 37 °C for 24 h.

In general, the antimicrobial activity of organic acids have been attributed to pH reduction, depression of internal pH of the microbial cell by ionization of undissociated acid molecules, disruption of substrate transport by altering cell membrane permeability or reduction of proton motive force and chelation of metal ions essential for microbial growth (Eswaranandam et al., 2004; Stratford & Eklund, 2003).

Micrographs of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 cells by transmission electron microscopy (TEM) showed meaningful damages in cell cytoplasm without apparent changes on the cytoplasm membrane when malic acid was added to the fruit juices (Fig. 1). This fact could be due to a decrease in the intracellular pH, since, undissociated malic acid molecules might pass across the cell membrane and then dissociate as a consequence of the neutral pH of the cell cytoplasm. It is known that the dissociation of the organic acids in media depends directly of their pKa values and that their antimicrobial action is dependent upon undissociated molecules (Davidson, 2001).

Malic acid is an organic acid of low lipid solubility (Leo et al., 1971) and consequently its entry into the cell could be limited, since the cell membrane is generally impermeable to polar compounds (Lücke, 2003). However, some authors have found that effectiveness of the organic acids can vary depending on its molecular weight. Eswaranandam et al. (2004) indicated that undissociated smaller molecules of malic (134.09 Da) and lactic (90.08 Da) acids may entry into the bacterial cells easily and change the internal pH of the microorganism, thus showing higher antimicrobial activity than undissociated larger molecules of citric (192.13 Da) and tartaric (150.09 Da) acids, which may not gain entry to the cell interior effectively.

4. Conclusions

Apple, pear and melon juices inhibit the growth of *L. monocytogenes*, *S. Enteritidis* and *E. coli* O157:H7 at 5 °C by at least 5 days. However, at 20 and 35 °C concentrations of 0.2% (v/v) of malic acid were necessary to inhibit the growth of *L. monocytogenes* and *S. Enteritidis* and 0.4% (v/v) for *E. coli* O157:H7 in melon juice. On the other hand, higher concentrations of malic acid were shown to be efficacious in reducing and inactivating by more than 5-log cycles of those microorganisms in apple, pear and melon juices. That inactivation depended on the concentration of the malic acid, kind of juice, storage temperature and time as well as the microorganism type. According to our results, *E. coli* O157:H7 and refrigeration temperature (5 °C) should be considered as target microorganism and temperature, respectively, in the fruit juices preservation when organic acids are used.

MICs and MBCs found in this study may serve as a base to future studies where combination of malic acid with other preservation methods could be considered. In addition, studies of shelf-life and sensory evaluation are recommended to evaluate how the different concentrations of malic acid could affect the fruit juices quality.

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