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# EFFECTS OF ORGANIC ACIDS, NISIN, LYZOZYME AND EDTA ON THE SURVIVAL OF *YERSINIA ENTEROCOLITICA* POPULATION IN INOCULATED ORANGE BEVERAGES

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## ABSTRACT

*Inactivation of a* Yersinia enterocolitica *strain by 3.4% ascorbic acid (AA), 0.5% citric acid (CA), 0.2% lactic acid, 100 IU/mL nisin, 2,400 IU/mL lysozyme and 20 mM ethylenediaminetetraacetic acid disodium salt (EDTA), alone and combined, was studied at different temperatures (4 and 25C) in commercial and laboratory-prepared orange beverages. In laboratory-prepared juice, highest reductions (between 3.06 and 4.07 log units) were obtained with acid mixtures at 25C after 20 min of incubation. At 24 h of incubation, EDTA was bactericidal and the mixtures with nisin or lysozyme resulted in count reductions of 6.03 and 5.98 log units, respectively. In commercial orange beverage, AA and CA gave reductions of 5.43 and 4.26 log units, respectively. The three acid mixtures were bactericidal within the first 10 min. EDTA alone or mixed completely inhibited* Y. enterocolitica *strain at 6 and 24 h of incubation. At 4C, all the results were significantly lower than at 25C.*

## PRACTICAL APPLICATIONS

*Yersinia enterocolitica* is a common cause of gastrointestinal disorders and may lead to sequelae. This pathogen can maintain its virulence in a wide temperature range and under adverse conditions such as pH lower than 4.

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There are reports describing the isolation of *Y. enterocolitica* from water and ice in Brazil and Latin America. The sale of unpasteurized drinks is a common practice in our region and their consumption has increased over the past years. In the present work, chemical compounds and biological inhibitors were studied. Some of these compounds showed to be efficient for reducing *Y. enterocolitica* populations. Furthermore, they did not modify the organoleptic characteristics of the beverages and they are not toxic for human beings.

## INTRODUCTION

*Yersinia enterocolitica* is an important human pathogen with a global distribution. Yersiniosis in humans is generally associated with gastrointestinal disorders such as enterocolitis, mesenteric lymphadenitis and terminal ileitis (Bottone 1997), which can mimic appendicitis. *Yersinia enterocolitica* may be expected to occur in foods that are raw, improperly heat treated or cross-contaminated after adequate heat treatment (Fredriksson-Ahomaa *et al.* 2001). This microorganism has been isolated from water and ice (Highsmith *et al.* 1977; Thompson and Gravel 1986; Falcão *et al.* 2002, 2004) and from beverages such as sugarcane juice (Ram *et al.* 1996). Furthermore, *Y. enterocolitica* can grow and maintain virulence under adverse conditions, such as extreme pH values (Bodnaruk and Golden 1996; Canganella *et al.* 1998).

In Argentina, relatively few studies have involved the detection of *Y. enterocolitica* or related species in foods and water. However, in San Luis, Argentina, de Guzmán *et al.* (1984) reported the first isolations of *Y. enterocolitica* from meat foods. In later studies, serovar O:9 and other serovars of this bacterium were isolated from different samples such as hake fillets, porcine and bovine tongues, porcine cheese and cooked ham (Velázquez *et al.* 1993; Escudero *et al.* 1996).

The increasing demand for high-quality, nonthermally processed and microbiologically safe foods requires new techniques for the reduction of microorganisms. Organic acids have been extensively studied due to their antimicrobial properties. Lactic acid (LA) and citric acid (CA) have been effective in reducing contamination by *Y. enterocolitica* and other bacteria on eggshell and animal carcasses (Favier *et al.* 2000; Mikolajczyk and Rabkowski 2002). Among naturally occurring preservatives, nisin, lysozyme and sodium ethylenediamine tetraacetate (EDTA) are considered important components in the prevention of bacterial growth in foods. It has been claimed that the antimicrobial spectrum and potency of these molecules can be increased when used in combination with one another (Boziaris and Adams 1999; Branen and Davidson 2004) or other antimicrobials (Cutter and Siragusa 1995).

The purpose of the present study was to compare the effectiveness of organic acids (ascorbic acid [AA], CA and LA), nisin, lysozyme and EDTA,

alone and combined, on the reduction of a pathogenic *Y. enterocolitica* strain in orange beverages at two different temperatures. *Yersinia enterocolitica* survival in untreated juice was also investigated. The study of this bacterium in orange beverages is of interest because they provide extreme conditions for *Y. enterocolitica* growth, for example, their pH being usually lower than 4. Furthermore, they can be cross-contaminated after pasteurization, and this bacterium has been isolated from water and other kinds of juices.

## MATERIALS AND METHODS

### Orange Beverages

Two types of orange beverages were used in this investigation: commercial pasteurized orange beverage without pulp purchased in 1.0 L-containers, stored at 24C, acquired in San Luis city, and freshly squeezed unpasteurized orange juice with pulp, prepared in our laboratory. Fresh oranges of the specie *Citrus sinensis* Valencia late were bought from retail stores. They were washed with abundant warm tap water, rinsed with cold deionized sterile water and wiped with paper towel. Immediately, they were squeezed and the juice was introduced into a sterile flask.

The pH of the juice was determined with an Orion 420a pH meter (Orion Research Inc., Boston, MA). The AA concentration was determined by $KIO_3$ titration and the titratable acidity (g/100 mL CA anhidre) by 0.1 N NaOH titration to a pH 8.1 end point.

### Inoculum Preparation

*Yersinia enterocolitica* W1024 O:9 pYV (+) was kindly provided by Dr. Guy Cornelis, Catholic Louvain University, Belgium. The bacterial stock was maintained in trypticase soy agar (TSA; Merck Laboratories, Darmstadt, Germany) at 4C and in Luria broth (Merck), added with 20% glycerol, at –20C. This strain was streaked onto MacConkey agar (MC; Merck) and incubated at 25C for 48 h. A single colony from the MC plate was inoculated into 150 mL of trypticase soy broth (TSB; Merck) and incubated at 25C for 24 h; at this moment, the bacterial population was approximately $10^9$ cfu/mL ($DO_{600}$:0.2). Five milliliters of TSB culture were then centrifuged at 6,310 × g for 10 min at 4C in a refrigerated Sigma 3K30 laboratory centrifuge (Sigma, Steinheim, Germany) and the supernatant was discarded. The pellet was washed twice with 5 mL of 0.1% peptone water pH 7 (PW; Merck), suspended in 1 mL of TSB and stored in refrigerator at 4C prior to use. The acid adaptation test was not performed.

### Inoculation of Orange Beverage

The suspension of *Y. enterocolitica* in TSB (1 mL) was added to 199 mL of orange beverage for a final concentration of approximately $10^7$ cfu/mL. Inoculated orange beverage samples were immediately treated with the different compounds.

### Treatments

The organic acids and the mixtures used in this work were: (1) 3.4% (w/v) AA (100.3% purity; Parafarm, Buenos Aires, Argentine); (2) 0.2% (v/v) LA (90% purity; Parafarm); (3) 0.5% (w/v) CA (89.62% purity; Merck); (4) 3.4% (w/v) AA plus 0.2% (v/v) LA; (5) 0.2% (v/v) LA plus 0.5% (w/v) CA; and (6) 3.4% (w/v) AA plus 0.5% (w/v) CA.

Antimicrobial compounds nisin (2.5% nisin with an activity of 1,020 IU/mg; Sigma-Aldrich), pure lysozyme from hen egg white (with an activity of 36,000 IU/mg; Fluka Chemie, Buchs, Switzerland) and EDTA (99% purity; Sigma-Aldrich) were also tested. Nisin and lysozyme were added to the sample to reach an estimated final activity of 100 and 2,400 IU/mL, respectively; the EDTA final concentration was 20 mM. The mixtures tested were 100 IU/mL nisin plus 2,400 IU/mL lysozyme, 100 IU/mL nisin plus 20 mM EDTA and 2,400 IU/mL lysozyme plus 20 mM EDTA.

Samples without addition of any compound were treated under the same time and temperature conditions and used as control.

The number of sublethally injured bacteria was taken as the difference between counts obtained on TSA with 0.6% yeast extract (TSAYE; Merck) and those obtained on a selective media (TSAYE + 5% w/v NaCl). Plates were inoculated at 25C for 48 h (Virto *et al.* 2005).

### Enumeration of *Y. enterocolitica*

Once the corresponding acid or acid combination was added, the orange juice was incubated at 4 and 25C and samples were taken at 0, 5, 10 and 20 min (20 min was considered final time). When the antimicrobial compounds were tested, additional samples were also taken at 6 and 24 h. In all cases, 1.0 mL of the orange juice was serially diluted with 0.1% PW pH 7, and 0.1 mL of an appropriate dilution was spread onto duplicated plates of MC agar. Plates were then incubated at 25C for 48 h. The counts were expressed as $\log_{10}$ cfu/mL. Characteristic colonies were confirmed by Gram staining and biochemical tests (Bercovier and Morallet 1984).

### Survival of *Y. enterocolitica*

To determine the survival of *Y. enterocolitica* in untreated orange juice, the inoculum was prepared under the same conditions as explained above and

added to 199 mL of the corresponding juice. Immediately, the orange juice was incubated at 4 and 25C and samples were taken at 0, 1, 2, 3, 4, 6, 8, 12, 16 and 20 days. Serial dilutions (1:10) were made in 0.1% PW pH 7 and 0.1 mL of an appropriate dilution was spread plated onto MC agar. Plates were then incubated at 25C for 48 h.

### Data Analysis

Survival was expressed as $\log_{10} N - \log_{10} N_0$, where $N_0$ is the initial count in orange juice and $N$ was the count after acid treatment. Mean values from three replications, with different lots of beverages or juices produced on different days, were subjected to analysis of variance and Student's $t$-test by Infostat 1.0 (Estadística y Biometría, Facultad de Ciencias Agropecuarias,Universidad Nacional de Córdoba, Córdoba, Argentina) software to determine if significant difference ($P < 0.05$) in populations of microorganisms existed between treatments. The mean and the standard deviation were calculated for the $\log_{10}$ reduction of microorganism for each set of experiments.

## RESULTS

The pH values of the commercial pasteurized and the laboratory-prepared orange juices, before and after different treatments, are shown in Table 1. The AA concentrations in the laboratory-prepared and in the commercial orange beverages were 50.65 ± 2.08 and 29.6 ± 0.75 mg/100 mL, respectively. The Argentina Alimentary Code (De la Canal 1994) requires a minimum of 30 mg/100 mL of AA in orange juice. The titratable acidity was 0.31 g/100 mL CA anhidre in the laboratory-prepared juice and 0.15 g/100 mL CA anhidre in the commercial beverage. The color and flavor of the juices studied did not change with the addition of the compounds tested.

### Survival of *Y. enterocolitica* in Untreated Orange Beverages

The results showed that *Y. enterocolitica* could survive at 25C through 16 days at detectable levels in the laboratory-prepared orange juice and 3 days in the commercial orange beverage. When the temperature tested was 4C, this strain survived more than 20 days and 4 days, respectively (Fig. 1). The lower detection limit was 20 cfu/mL. Sublethally injured cells were not detected in any of the beverages (data not shown).

### Effect of Organic Acids

Figure 2 shows the inhibitory activity of organic acids in the laboratory-prepared orange juice. At 25C, the inhibitory effect of LA was significantly

TABLE 1.
VALUES OF pH IN INOCULATED ORANGE DRINKS BEFORE AND AFTER TREATMENTS WITH DIFFERENT COMPOUNDS

| Treatment | Laboratory prepared juice | Commercial beverage |
|---|---|---|
| | pH ± SD | pH ± SD |
| Untreated | 3.68 ± 0.22 | 3.51 ± 0.07 |
| 3.4% AA | 3.22 ± 0.11 | 3.07 ± 0.03 |
| 0.2% LA | 3.34 ± 0.08 | 3.31 ± 0.20 |
| 0.5% CA | 3.35 ± 0.12 | 3.09 ± 0.13 |
| 3.4% AA + 0.2% LA | 3.10 ± 0.03 | 2.98 ± 0.09 |
| 3.4% AA + 0.5% CA | 3.08 ± 0.08 | 2.91 ± 0.02 |
| 0.2% LA + 0.5% CA | 3.30 ± 0.04 | 3.02 ± 0.10 |
| 100 IU/mL N | 3.52 ± 0.42 | 3.47 ± 0.12 |
| 2,400 IU/mL L | 3.47 ± 0.35 | 3.58 ± 0.06 |
| 20 mM EDTA | 3.58 ± 0.02 | 3.51 ± 0.12 |
| 100 IU/mL N + 2,400 IU/mL L | 3.53 ± 0.09 | 3.39 ± 0.18 |
| 100 IU/mL N + 20 mM EDTA | 3.51 ± 0.08 | 3.51 ± 0.09 |
| 2,400 IU/mL L + 20 mM EDTA | 3.53 ± 0.13 | 3.54 ± 0.16 |

AA, ascorbic acid; CA, citric acid; EDTA, ethylenediamine tetraacetate; LA, lactic acid; L, lysozyme; N, nisin; SD, standard deviation.

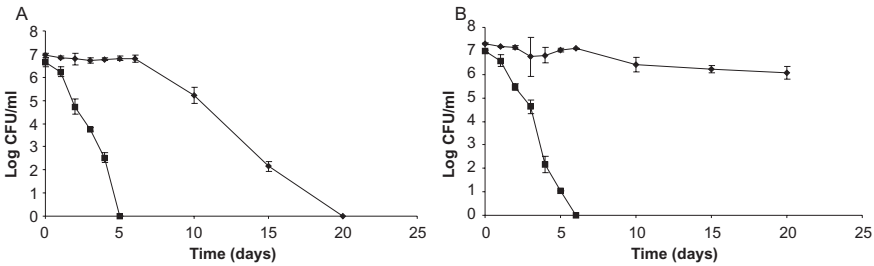


FIG. 1. SURVIVAL OF *YERSINIA ENTEROCOLITICA* IN UNTREATED ORANGE BEVERAGE DURING STORAGE AT DIFFERENT TEMPERATURES: (A) 25C AND (B) 4C IN (◆) COMMERCIAL ORANGE BEVERAGE, AND (■) LABORATORY-PREPARED ORANGE JUICE

higher than the effect of the other acids. Count reductions of 4.07 and 3.68 log units were obtained with the CA plus LA and AA plus CA mixtures, respectively. These results were significantly higher than those obtained with AA plus LA. The count reductions at 4C were insignificant for all tested acids.

When the commercial pasteurized orange beverage was tested, AA and CA gave reductions of 5.43 and 4.26 log units, respectively, whereas the

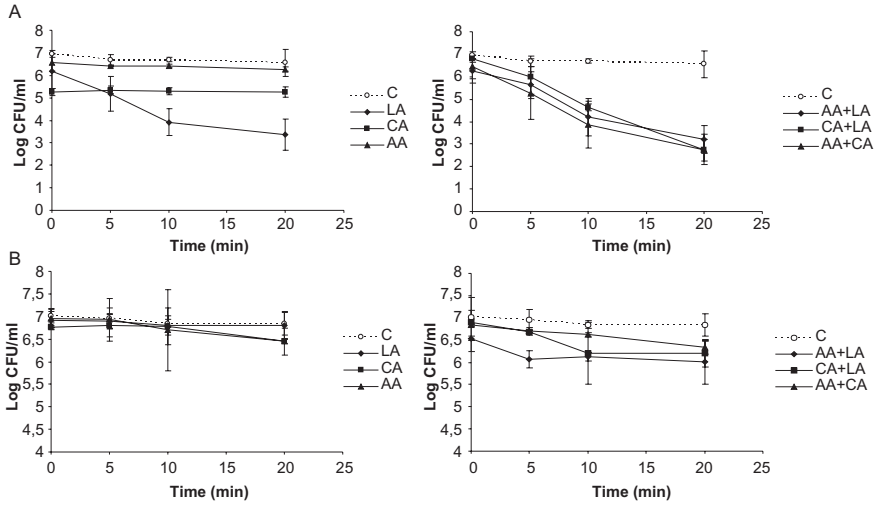


FIG. 2. EFFECT OF ORGANIC ACIDS TREATMENTS IN THE SURVIVAL OF *YERSINIA ENTEROCOLITICA* IN LABORATORY-PREPARED ORANGE JUICE AT DIFFERENT TEMPERATURES: (A) 25C AND (B) 4C
AA, ascorbic acid; LA, lactic acid; CA, citric acid; C, untreated control.

reduction obtained with LA was significantly lower when the temperature was 25C. At this temperature, no colonies were observed for 10 min, with any of the three acid mixtures on MC agar. At 4C, no significant reductions of colony counts were obtained when the organic acids were tested alone. The reductions obtained with AA plus CA and CA plus LA were 3.4 and 3.33 log units, which were significantly different from 2.63 log units obtained with AA plus CA (Fig. 3).

As sublethal injury makes bacteria more sensitive to inhibitory factors, this sublethal injury was studied at 10 and 20 min of treatment with the acid mixtures. The injured cells observed with the CA plus LA mixture were $3.35 \pm 1.13$ at 10 min and $3.92 \pm 0.18$ log units at 20 min. Besides, the counts obtained with AA plus CA mixture were $3.68 \pm 1.62$ and $3.52 \pm 0.43$ log units, respectively, and with AA plus LA mixture, the injured cells were $3.67 \pm 0.29$ and $2.97 \pm 0.92$, respectively (data not shown).

In order to know the real effect of the organic acid mixtures at 25C, the incubation time was reduced to 5 min and the samples were taken at 0, 2, 4 and 5 min. In this case, the AA plus LA mixture was, at the final time, the most effective treatment with a reduction of 5.18 log units. CA plus LA and AA plus CA gave count reductions of 4.13 and 3.56 log units, respectively (Fig. 4).

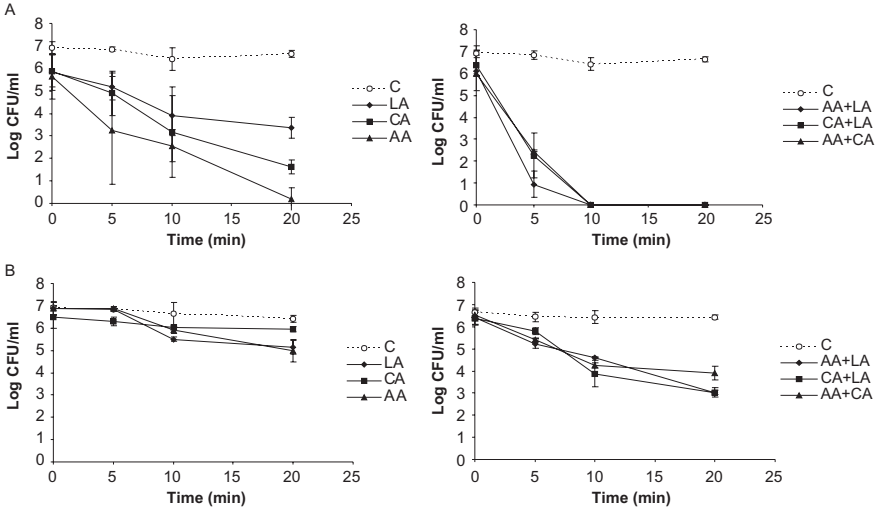


FIG. 3. EFFECT OF ORGANIC ACIDS TREATMENTS IN THE SURVIVAL OF *YERSINIA ENTEROCOLITICA* IN COMMERCIAL ORANGE BEVERAGE AT DIFFERENT TEMPERATURES: (A) 25C AND (B) 4C
AA, ascorbic acid; LA, lactic acid; CA, citric acid; C, untreated control.

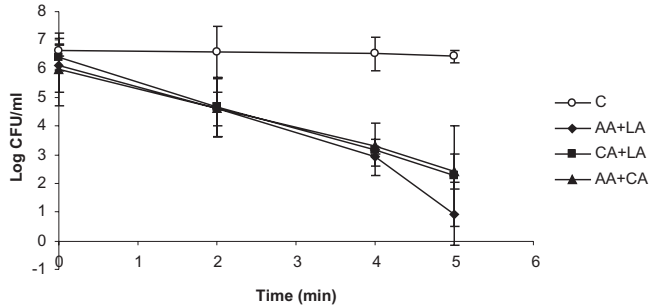


FIG. 4. EFFECT OF ORGANIC ACIDS TREATMENTS IN THE SURVIVAL OF *YERSINIA ENTEROCOLITICA* IN COMMERCIAL ORANGE BEVERAGE AT 25C
AA, ascorbic acid; LA, lactic acid; CA, citric acid; C, untreated control.

## Effect of Antimicrobial Compounds

The results of the inhibitory activity of antimicrobial compounds on *Y. enterocolitica* in the laboratory-prepared orange juice are shown in Table 2. At the final time, 24 h, EDTA proved to be bactericidal as no colonies were observed on MC, TSAYE + NaCl or TSAYE, at 25C; this effect was

TABLE 2.
PLATE COUNTS OF *YERSINIA ENTEROCOLITICA* IN INOCULATED LABORATORY-PREPARED ORANGE JUICE BEFORE AND AFTER TREATMENTS WITH DIFFERENT ANTIMICROBIAL COMPOUNDS[a]

| *Y. enterocolitica* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| T (C) | Time | Untreated | Nisin | Lysozyme | EDTA | Nisin + Lysozyme | Nisin + EDTA | Lysozyme + EDTA |
| 25 | 0 min | 6.95 ± 0.10 Aa | 6.71 ± 0.25 Aa | 6.73 ± 0.20 Aa | 6.65 ± 0.24 Aab | 6.59 ± 0.21 Aab | 6.29 ± 0.32 Ab | 6.65 ± 0.08 Aab |
| | 5 min | 6.73 ± 0.06 ABa | 6.39 ± 0.26 ABab | 6.43 ± 0.04 Bab | 6.73 ± 0.26 Aa | 6.61 ± 0.17 Aa | 5.92 ± 0.67 ABb | 6.71 ± 0.02 Aa |
| | 10 min | 6.75 ± 0.06 ABa | 6.44 ± 0.22 Aa | 6.35 ± 0.05 Bab | 6.77 ± 0.25 Aa | 6.35 ± 0.27 ABab | 5.77 ± 0.69 ABb | 6.26 ± 0.30 Aab |
| | 20 min | 6.66 ± 0.12 Bab | 6.35 ± 0.27 ABab | 6.49 ± 0.07 Bab | 6.70 ± 0.43 Aa | 6.15 ± 0.22 Bab | 5.18 ± 0.43 ABc | 6.05 ± 0.43 Ab |
| | 6 h | 6.76 ± 0.12 ABa | 6.08 ± 0.03 Bc | 6.04 ± 0.03 Cc | 3.18 ± 0.13 Bd | 6.20 ± 0.03 Bc | 3.95 ± 0.21 Bb | 3.87 ± 0.51 Bb |
| | 24 h | 6.84 ± 0.07 Ba | 4.86 ± 0.08 Cb | 4.20 ± 0.03 Db | NG Cc | 4.00 ± 0.15 Cb | 0.81 ± 1.26 Cc | 0.86 ± 1.34 Cc |
| 4 | 0 min | 7.32 ± 0.04 Ab | 7,02 ± 0.04 Aa | 7.04 ± 0.01 Aa | 7.03 ± 0.03 Aa | 7.05 ± 0.05 Aa | 7.05 ± 0.03 Aa | 6.93 ± 0.05 Aa |
| | 5 min | 7.18 ± 0.03 ABb | 6.99 ± 0.04 Aab | 6.92 ± 0.11 Aab | 6.99 ± 0.04 Aab | 7.01 ± 0.06 Aab | 6,96 ± 0.05 Aab | 6.91 ± 0.05 Aa |
| | 10 min | 7.16 ± 0.07 BCc | 6.91 ± 0.02 Aabc | 6.93 ± 0.03 Abc | 6.90 ± 0.12 Aab | 6.91 ± 0.05 Aabc | 6,80 ± 0.01 Aab | 6.66 ± 0.07 ABa |
| | 20 min | 7.23 ± 0.03 BCb | 6.76 ± 0.12 Aa | 6.76 ± 0.01 ABa | 6.83 ± 0.09 Aa | 6.74 ± 0.04 Ba | 6,74 ± 0.04 Aa | 6.57 ± 0.10 Ba |
| | 6 h | 7.03 ± 0.03 Cc | 6.30 ± 0.03 Bb | 6.43 ± 0.02 Ba | 4.86 ± 0.19 Ba | 6.41 ± 0.01 Cb | 6.41 ± 0.01 Ba | 4.63 ± 0.05 Ca |
| | 24 h | 7.06 ± 0.05 Cd | 5.16 ± 0.07 Cc | 4.91 ± 0.20 Cc | 0.97 ± 0.06 Ca | 5.29 ± 0.02 Dc | 5.29 ± 0.02 Cb | 2.29 ± 0.11 Dc |

[a] Data represent means ± standard deviations of three replications. Means with the same capital letter in the same column are not significantly different ($P < 0.05$). Means with the same lowercase letter in the row are not significantly different ($P < 0.05$).
NG, no growth.

significantly higher than those obtained with nisin and lysozyme. EDTA mixed with nisin or lysozyme resulted in count reductions of 5.48 and 5.79 log units on MC, respectively, not showing significant difference with the results obtained with EDTA alone. In contrast, the combination of nisin plus lysozyme showed a significantly lower inhibitory effect than those obtained with the other mixtures. At 4C, the results were similar to those obtained at 25C, but the count reductions were always lower than those obtained at the higher temperature.

Table 3 shows the plate counts obtained with antimicrobial compounds in the commercial orange beverage. At both temperatures, the effectiveness of EDTA was more pronounced than in laboratory-prepared orange juice, where no *Y. enterocolitica* strains were recovered, since 6 h of incubation. No sub-lethally injured cells were observed after this time either. The activity of EDTA was significantly better than that of nisin and lysozyme alone. The EDTA plus nisin and EDTA plus lysozyme mixtures led to total *Y. enterocolitica* inhibition since 6 h of incubation, whereas the count reductions obtained with nisin plus lysozyme mixture were significantly lower.

## DISCUSSION

In the present study, the temperature did not affect the number of viable *Y. enterocolitica* W1024 pYV(+) cells that could survive for more than 2 weeks in the laboratory-prepared orange juice, which has pH near 3.5. It has been reported that *Y. enterocolitica* may survive in fruit yogurt, which has a pH close to 4, after 10 days at 8C (Canganella *et al.* 1998). The limited survival in the commercial beverage observed in the present study (about 3 days) was probably due to the presence of additional ingredients in the sample which may have acted as inhibitory compounds enhancing the antimicrobial effect of the low pH. According to the information in the commercial beverage package, the ingredients include mineral water, concentrated orange juice, sweeteners (high-fructose corn syrup and sugar), CA (which provides tartness), flavor (orange essence) and AA (as antioxidant). The fact that *Y. enterocolitica* survived in both kinds of orange drinks for an extended period of time suggests the risk of accidental ingestion. Hence, orange drinks could be vehicles for yersiniosis when the hygiene conditions of its processing, manipulation and storage are not adequate.

The AA concentration was 29.6 $\pm$ 0.75 mg/100 mL and the titratable acidity was 0.15 g/100 mL CA anhidre; these results were lower than the ones obtained with the laboratory-prepared orange juice. It is not therefore likely that these organic acids were responsible for the more inhibitory effect of the commercial beverage. As the concentrations of the other ingredients were not specified, it was not possible to discriminate the action of each one.

TABLE 3.
PLATE COUNTS OF *YERSINIA ENTEROCOLITICA* IN INOCULATED COMMERCIAL ORANGE BEVERAGE BEFORE AND AFTER TREATMENTS WITH DIFFERENT ANTIMICROBIAL COMPOUNDS[a]

| *Y. enterocolitica* | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| T (C) | Time | Untreated | Nisin | Lysozyme | EDTA | Nisin + Lysozyme | Nisin + EDTA | Lysozyme + EDTA |
| 25 | 0 min | 6.66 ± 0.19 Aa | 6.67 ± 0.28 Aa | 6.68 ± 0.10 Aa | 6.59 ± 0.17 Aa | 6.51 ± 0.22 Ab | 6.65 ± 0.19 Aa | 6.07 ± 0.19 Aa |
| | 5 min | 6.44 ± .022 ABab | 6.47 ± 0.11 Aa | 6.64 ± 0.02 Ba | 6.51 ± 0.21 Aa | 6.59 ± 0.18 Aa | 6.46 ± 0.22 Aab | 6.13 ± 0.24 Ab |
| | 10 min | 6.43 ±. 0.22 ABa | 6.39 ± 0.19 Aa | 6.57 ± 0.03 Ba | 6.50 ± 0.21 Aa | 6.54 ± 0.21 Aa | 5.88 ± 0.55 Bb | 5.84 ± 0.15 Ab |
| | 20 min | 6.43 ± 0.15 ABab | 6.41 ± 0.21 Aab | 6.59 ± 0.02 Ba | 6.35 ± 0.23 Aab | 6.19 ± 0.14 Bb | 5.10 ± 0.26 Cc | 5.23 ± 0.28 Bc |
| | 6 h | 6.40 ± 0.12 ABa | 5.52 ± 0.02 Bc | 5.71 ± 0.01 Cb | NG Bd | 5.66 ± 0.03 Cb | NG Dd | NG Cd |
| | 24 h | 6.24 ± 0.32 Ba | 3.97 ± 0.03 Cc | 3.82 ± 0.20 Dc | NG Bd | 5.01 ± 0.02 Db | NG Dd | NG Cd |
| 4 | 0 min | 6.98 ± 0.08 Ab | 6.89 ± 0.04 Ab | 6.86 ± 0.12 Aab | 6.84 ± 0.05 Aab | 6.85 ± 0.02 Aab | 6.61 ± 0.02 Aa | 6.82 ± 0.04 Aab |
| | 5 min | 6.97 ± 0.18 Aa | 6.72 ± 0.02 Aa | 6.75 ± 0.12 Aa | 6.80 ± 0.07 Aa | 6.78 ± 0.04 Aa | 6.09 ± 0.55 Aa | 6.48 ± 0.04 Ba |
| | 10 min | 6.97 ± 0.01 Ac | 6.77 ± 0.02 Ac | 6.79 ± 0.02 Ac | 6.61 ± 0.21 Abc | 6.86 ± 0.02 Ac | 6.21 ± 0.08 Aab | 5.90 ± 0.15 Ca |
| | 20 min | 6.87 ± 0.13 Ac | 6.71 ± 0.03 Ac | 6.79 ± 0.01 Ac | 5.96 ± 0.92 Abc | 6.72 ± 0.18 Ac | 4.70 ± 0.10 Bab | 4.37 ± 0.01 Dab |
| | 6 h | 6.69 ± 0.08 Ac | 5.51 ± 0.26 Bb | 5.50 ± 0.07 Bb | NG Ba | 5.88 ± 0.18 Bb | NG Ca | NG Ea |
| | 24 h | 6.59 ± 0.06 Ad | 4.40 ± 0.16 Cb | 4.51 ± 0.05 Cbc | NG Ba | 4.75 ± 0.12 Cc | NG Ca | NG Ea |

[a] Data represent means ± standard deviations of three replications. Means with the same capital letter in the same column are not significantly different ($P < 0.05$). Means with the same lowercase letter in the row are not significantly different ($P < 0.05$).
NG, no growth.

In the present study, organic acids were effective against *Y. enterocolitica* at 25C, but did not have any effect at 4C. Virto *et al.* (2005) obtained similar results with CA and LA against *Y. enterocolitica*. At 25C, the order of inhibitory activity of the different organic acids in the laboratory-prepared juice was LA > AA > CA. This result is in agreement with Little *et al.* (1992) who demonstrated that LA was better than CA for the reduction of *Y. enterocolitica* in TSB, pH 3.5, at 23C and in every combination of pH and temperature they tested. Nakai and Siebert (2004) observed that LA was more effective than CA for inhibition of *Listeria innocua*, *Listeria ivanovii*, *Pseudomonas aeruginosa* and *Oenococcus oeni* in broth medium. On the other hand, in the commercial beverage, the effectiveness of organic acids was different, with the following efficacy order: AA > CA > LA.

The treatment with AA produced the best reduction in the commercial beverage, but it was not so effective in the laboratory-prepared juice. There seem to be no reports on the effect of AA in the reduction of *Y. enterocolitica*.

The pH reduction by addition of organic acids in the commercial orange beverage was more significant than that obtained in the laboratory-prepared juice. The high inhibitory effect of these acids in this kind of drink might be partly due to its low pH value. In order to compare the effect of growth inhibitory pH levels due to different organic acids, Little *et al.* (1992) used a pH range that varied between 4.5 and 3.0, and obtained better reductions of *Y. enterocolitica* with the lowest pH.

The three organic acid combinations assayed in this study proved to have an inhibitory effect in the survival of *Y. enterocolitica*, and they were more effective than when they were studied separately.

Selective media such as MC agar or TSAYE + NaCl contain agents that can inhibit injured target microorganisms. No selective media such as TSAYE can recover sublethally injured cells. In our work, *Y. enterocolitica* sublethally injured cells were recovered after treatment with all the acid mixtures tested. Lee *et al.* (2002) recovered *Salmonella typhimurium* after the treatment with sodium hypochlorite and LA. Similar results were obtained by Ryu *et al.* (1999) with *Escherichia coli* O157:H7.

Ryu *et al.* (1999) observed that *E. coli* O157:H7 acid-adapted cells were more acid tolerant than unadapted cells when inoculated in TSA acidified with acetic acid or LA. Because acid adaptation was not performed in the present study, future research should be required to determine *Y. enterocolitica* response to acid adaptation.

In the present work, when EDTA was tested alone, it produced a bactericidal effect in both drinks. These results agree with those of other authors who observed a complete inhibitory effect of EDTA, in broth medium, against *E. coli*, *Enterococcus faecalis*, *Shewanella putrefaciens* and *Salmonella enteritidis*, among other bacteria (Boziaris and Adams 1999; Gill and Holley 2003).

EDTA is a chelating agent that removes stabilizing divalent cations that interlink adjacent lipopolysaccharide (LPS) molecules of the bacterium outer membrane. It results in the release of a significant proportion of LPS from the cell and the consequent increase of the permeability (Vaara 1992). Furthermore, its high chelating activity can also strongly chelate trace metals from the medium and cause suppression of bacteria metabolism (Boziaris and Adams 1999). Taking into account that orange juice and juice-based drinks are media that contain few sources of nutrients which bacteria need to repair themselves, their survival in these beverages is more difficult once they have been damaged by EDTA. This could explain why EDTA was bactericidal in our work.

The bactericidal activity of nisin is increased at pH levels below 5 (Bari *et al.* 2005). This makes nisin suitable for use on fruit or fruit juices and beverages, which have pH levels in the 3 to 6 range. Furthermore, lysozyme activity is highest from pH 3.5 to 7.0, although it is active over a pH range from 2.0 to 10.0. Lysozyme has been reported (Pellegrini *et al.* 1997) to be active against gram-negative bacteria. Considering these reports, we decided to investigate the effect of nisin and lysozyme against *Y. enterocolitica* in orange beverages. In our work, neither nisin nor lysozyme was bactericidal to the *Y. enterocolitica* strain tested.

In the laboratory-prepared juice, antimicrobial activity was not increased by the nisin and lysozyme mixture compared with the activity obtained when these compounds were tested separately; however, this mixture was antagonistic in the commercial orange beverage. Because the effects obtained with EDTA alone were bactericidal to *Y. enterocolitica*, we were not able to determine whether the activity of nisin or lysozyme was enhanced by this chelator. The results obtained by other authors are controversial; many of them have reported an increment in the activity of lysozyme (Boland *et al.* 2003) or nisin against gram-negative bacteria in the presence of chelating compounds (Boziaris and Adams 1999; Branen and Davidson 2004) but (Gill and Holley 2003) did not observe this interaction.

EDTA and the organic acids tested in this study proved to have different *Y. enterocolitica*-reducing effects in laboratory-prepared and commercial beverage. The observed differences could also be the result of the presence of the above-mentioned additives in the commercial beverages as opposed to the laboratory-prepared juice.

The results obtained show that the activity of organic acids on the inactivation of *Y. enterocolitica* depends on temperature. Every organic acid mixture tested was bactericidal in the commercial beverage, while in the prepared juice, the most inhibiting combination was LA plus CA. Besides, EDTA demonstrated to be the most effective antimicrobial agent against *Y. enterocolitica* in both kinds of juices.

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