Journal of Biology, Agriculture and Healthcare
ISSN 2224-3208 (Paper) ISSN 2225-093X (Online)
Vol.5, No.3, 2015

Antimicrobial Effect of Citric, Acetic, Lactic Acids and Sodium Nitrite against Escherichia Coli in Tryptic Soy Broth

S. OULKHEIR* KHADIJA OUNINE N. E. EL HALOUI B. ATTARASSI
Laboratory of Biology and Health, Team of Microbiology Applied, Department of Biology, Faculty of Science, Ibn Tofaïl University, Box 133, 14000 Kénitra, Morocco
E.mail: oulkheir_said@yahoo.fr

Abstract
Antimicrobial effect of organic acids against *E. coli* (EC_1 and EC_2) was studied in tryptic soy broth (pH 5) adjusted to various concentrations 1, 2, 3, 4 or 6% of the citric, acetic and lactic acid then incubated at 5, 20 and 37ºC during 96 hours. In the second study, the effect of sodium nitrite on the survival of *E. coli* cells was investigated at pHs (4.5 and 6). In the majority of the cases, the order of effectiveness of the organic acids was: citric >acetic > lactic. At low temperature, 1 to 6% of lactic and acetic acid does not seem sufficient concentrations to influence cellular viability significantly. In contrast, addition of citric acid (3%) to TSB medium reduced *E. coli* from approximately 6.3 log CFU/ml to an undetectable number. Furthermore, antimicrobial effect of all organic acids increased with increasing the temperature of incubation. This study has confirmed that the lethal effect of sodium nitrite was much enhanced by low pH. Thus, the most bactericidal effect was obtained at pH = 4.5 and the concentrations $NaNO_2$ (0.4 or 0.6).
Key words: *Escherichia coli*, Antimicrobial effect, citric acid, acetic acid, lactic acid, sodium nitrite.

1. Introduction

Organic acids occur throughout nature and are used extensively in food systems. Although these acids are usually added to foods, some are also intrinsic to foods in that they are produced during microbial growth [15]. The major antimicrobial objective in using weak organic acids is to inhibit both the growth of microorganisms and the germination of microbial spores [5]. Thus, numerous organic acids were successfully tested against Enterobacteriaceae in experimental studies. For *E. coli*, inactivation in acidified tryptic soy broth (TSB) and agar was demonstrated for citric, malic, and tartaric acids [11, 19]. Chung and Goepfert [9] showed that various organic acids are bacteriostatic to *Salmonella* spp. at different pH levels.

In addition to their use as microbial inhibitors, organic acids can serve as defoaming agents and emulsifiers, aid in setting of pectin gels, and have a strong effect on the taste of a food [13]. With a characteristically sour taste, organic acids have an important role in the flavor of fruits and their juices by balancing the sugar/acid ratio [2].

In addition, sodium nitrite is one of the most common preservative agents used in the food industry. This additive is regulated usable in the products of meat salting believed or cooked, for their fundamental role on organoleptic qualities of the products: color and taste. The lethal effect of sodium nitrite is strongly dependent on the pH of theenvironment. Buchanan and Bagi [6] reported that the effect of NaO_2 on *E coli* O157:H7 was observed for pH< 5.5 and this effect is more high as the temperature of conservation is low [5]. The purpose of this study was to examine the effectiveness of organic acids on the survival of two strains of *E. coli* into tryptic soy broth stored at different temperatures. Furthermore, the antimicrobial effect of sodium nitrite was investigated.

2. Materials and Methods

2.1. Bacterial strains and growth conditions

Two isolates of *E. coli* were used: EC_1, isolated from rejections of slaughterhouses and EC_2, isolated from domestic wastewater of Kenitra city (Morocco). The *E. coli* strains were isolated by direct plating onto Tergitol-7 TTC medium. Identification was confirmed by Gram strain morphology, a negative oxidase reaction, the ability to ferment lactose 7- with gas production and indole at 44 ºC and by using the API 20E diagnostic system. All 8- strains were stored frozen (–20 ºC) in tryptic soy broth-glycerol 50% (vol/vol). Each stock 9- culture of *E. coli* was revived by two successive transfers in tryptic soy broth at 37°C for 10- 18 h before used in the present experiments.

2.2. Inoculum preparation

The inoculum was prepared by pipetting 5 ml of an overnight static culture (37 ºC) of *E. coli* EC_1 or EC_2 into 45 ml of tryptic soy broth. This preparation was incubated at 37ºC with shaking for 18 h. They were then harvested by centrifugation (3 min at 13,000 x g) and washed in an equal volume of sterile saline water (0.9% [wt/vol] NaCl in distilled water). After a second washing, one millilitre of washed cell suspension (approximately 2.10^6 cells/ml) was used to inoculate 99 ml of medium.

2.3. Preparation of media

Media used in this study are tryptic soy broth (TSB) and tryptic soy agar (TSA). They are sterilized at 120 ºC for 20 min. Broth preparations were kept for 2 days at room temperature to reveal possible contamination. Contaminated preparations were discarded.

2.4. Effect of organic acids

Sterile Erlenmeyer flasks (250 ml) containing the 99ml of TSB were prepared and stored at the room temperature until inoculation the following day. The acids (lactic, acetic or citric) were added to sterile media after being filter sterilized (0.45 µm pore size filter) to give final concentration of 1, 2, 3, 4 or 6%. After addition the organic acids, the TSB medium was adjusted at pH = 5. Thus, one ml of 18 hours cultures of strain EC_1 or EC_2 3. was added to the test media to give an initial level of approximately 2.10^6 CFU/ml. Following inoculation, all treatments were stored at 5, 20 or 37°C during 96 hours. Viable cell populations were enumerated by plating (0.1 ml) appropriate dilutions, in duplicate, on tryptic soy agar. Colonies were counted after the plates had been incubated at 37°C for 48 h.

2.5. Effect of sodium nitrite

The antibacterial effect of sodium nitrite on the survival of *E.coli*, was examined in TSB adjusted to different $NaNO_2$ concentrations 0.1, 0.2, 0.4 or 0.6%, and then acidified to pHs 4.5 or 6. Erlenmeyer flasks (250 ml) containing 99 ml of TSB were inoculated with 1 ml of 18 hours cultures of strain EC1 or EC2 (initial cell density was 2.10^6 CFU/ml), then they stored at (20 ± 2°C)

2.6. Viable counts

Cell suspensions were serially diluted in saline water (0.9% [wt/vol] NaCl in distilled water) and plated onto tryptic soy agar. Colonies were counted after the plates were incubated at 37 ºC for 48 h. The survival curves were based on mean values obtained from two experiments.

3. Results

3.1. Antimicrobial Effect of organic acids

The effect of citric, acetic and lactic acid on the survival of *E. coli* EC_1 and EC_2 in TSB acidified to pH 5, and then stored at different temperatures are shown in Figures 1, 2 and 3. Rate of *E. coli* reduction was dependent on both nature of acid and the temperature of incubation.

At 5°C, concentrations of lactic and acetic acids (1 to 6%) did not affect bacterial populations of strain EC_1. The highest reductions were seen in the presence of 6%. However, treatment with 4% of citric acid ($p < 0.05$) reduced the viability of the cells from approximately 6.3 $\log_{10}$ CFU/ml to undetectable levels. Thus, the antibacterial effect of the organic acids on the survival of strain EC_2, show that this strain is less resistant. At 3 and 6% respectively of citric and lactic acid, bacterial survival is completely inhibited. In the presence of the acetic acid the maximum of loss of viability is obtained at concentration of 6%.

At 20°C, for strain EC_1, at concentration equal to 6% the number of the viable cells decrease by 5 units $\log_{10}$ in the presence of the acetic acid against 3 units $\log_{10}$ in the presence of the lactic acid. On the contrary, viability of strain EC_2 was completely inhibited at 6% of acetic or lactic acid. However, treatments with citric acid show that the rates of inactivation of both strains were approximately similar. The concentration needed to reduce viable cells to an undetectable number was 2 and 3% respectively for strain EC_2 and EC_1.

At 37°C, the antibacterial effect of organic acids was much greater. At 1% of citric acid, the viability of the cells decreased by 2.7 units $\log_{10}$, but at 2 or 3% cellular viability is completely inhibited. The presence of 4% lactic acid was necessary to decline the numbers of survivors to undetectable number. At 4% of acetic acid, the viability of the cells decreased by 4 units $\log_{10}$ for strain EC_1. For this same concentration of acetic acid the viability of strain EC_2 was completely inhibited.

3.2. Antimicrobial Effect of sodium nitrite

The effect of the sodium nitrite (0.1, 0.2, 0.4 or 0.6%) on the survival of *E. coli* EC_1 and EC_2 in TBS acidified to pH 4.5 or 6 was assessed by the comparison of survival level after 10 days at 20°C (Table I). Generally the antibacterial effect of the sodium nitrite was higher at pH 4.5 than at pH 6.

At pH = 4.5, the addition of sodium nitrite to concentrations of 0.1 and 0.2% did not produce a significant inhibiting effect. The number of viable *E. coli* cells decreased by 2 to 3 units log respectively for strain EC_1 and strain EC_2 after 10 days of survival. When the cells are cultivated in the presence of 0.4% the decrease of viability is rapid. In addition, a high effect of sodium nitrite was obtained with 0.6%, no viable cells were recovered after 10 days for strain EC_1 and 8 days for strain EC_2.

At pH 6 the numbers of survivors declined slowly, a high degree of sodium nitrite was obtained only with 0.6%.

4. Discussion

The results of this study indicated that the antibacterial effect of organic acids against *E. coli* at pH 5 depends of the nature of the acid and the temperature of incubation: the highest reductions were seen at 20 and 37°C. The treatments with 2 or 3% of citric acid reduced *E. coli* to undetectable levels, but strain EC_1 appeared to be resistant than strainEC_2. However, treatments with acetic and lactic acids were less effective in reducing *E. coli*. Indeed, the inhibitory effect of organic acids depends on the undissociated form, as well as its ability to donate hydrogen ions in an aqueous system [22]. The degree of dissociation for a particular acid is related to its dissociation constant and the acidity of the product. Citric acid having (pKa 4.8) has higher antibacterial effect than of the acetic (pKa 4,6) or lactic acid (pKa 3.86). In this form, the cell membrane is more permeable to the acid, allowing it to enter the cell. Upon entering the cytoplasm, the acid dissociates, thus lowering the internal pH of the cell and disrupting cellular functions (i.e.enzyme stability) [16]. In addition to affecting enzymes, excess protons in the cytoplasm upset the membrane potential necessary for energy production and transport across the cell membrane.

In both culture media and food system, the varying bacteriostatic and bactericidal effects of organic acids have been demonstrated. Fischer et *al.* [14] reported a 0.75% solution of citric acid to sufficiently reduce *S. typhimurium*, *Yersinia enterocolitica*, *E. coli*, and *Staphylococcus aureus* on hard-boiled eggs. A previous study Sorrells [21] had shown that the order of effectiveness of the acidulants against *L monocytogenes* at 10, 25 or 35°C was: acetic > lactic > citric. Similar observations had reported by Conner and *al.* [10] and Vasseur and *al.* [23]. Other authors Ogawa and *al.* [17], reported that the cytotoxic effect of the lactic acid not dissociated on 3 strains of *E. coli* sérotype O157:H7, is divided into 2 phases: a bacteriostatic phase (from 3,2 to 62 mM) then bactericidal, for concentrations in lactic acid higher than 62 mM. Castillo and *al.* [8] revealed that the application of sprays before (15 seconds with 55°C, 250 ml to 2 % of lactic acid) and after ressuyage (30 seconds with 55°C, 500 ml of lactic acid to 4 %) contributes to the decrease the populations of *E. coli* in the chopped meat obtained from the treated carcasses. In contrast, work by Brackett and *al*. [4] indicated that hot (55°C) sprays of acetic, citric, and lactic acids did not affect survival of *E. coli* on raw beef. Cutter and Siragusa [12] reported that organic acid carcass washes did not completely inactivate the pathogen on beef tissues. Growth of *E. coli* can also occur in fermented dairy products [1], further indicating that the pathogen is resistant to organic acids. In the other hand, Samelis *et al.* [20] mentioned that the application of such treatments can cause the adaptation of cells of *E. coli*, resulting from sublétaux acid shocks. In addition, the effectiveness of organic acids also depends on the history of the strains and in particular of their adaptation or not to an acid environment preliminary to the contamination [3].

Our results confirmed that the effectiveness of sodium nitrite was much enhanced by low pH. The highest reductions were obtained at pH 4.5 and 0.4 or 0.6% of $NaNO_2$. These results are in agreement with those of Casey and Condon [7], who showed the importance of nitrite in the inactivation of E. *coli* in fermented sausages. These authors noted an effect bacteriostatic at pH>5 and a bactericidal effect at pH < 5 for nitrite rates of 100 mg/kg and more. At 200 mg/kg of nitrite, the cellular density of E. *coli* decrease 100 times more quickly than in the absence of nitrite. Riordan and *al.* [18] studied the behaviour of *E. coli* during the manufacture of pepperoni, following various formulations: salt (2.5 to 4 %), sodium nitrite (100 to 400 mg/kg) and adjustment of the pH (4.4 to 5.6) by addition of dextrose. The manufacturing process implemented, no formulation made it possible to reach the 5 decimal reductions of *E. coli* recommended by Food Safety Inspection Service (FSIS, the United States).

In this study the relationship between the effectiveness of organic acids and temperature of incubation was demonstrated. So, citric acid may dramatically affect the
survival of *E. coli* compared to other acids. The positive relationship obtained between the antibacterial effect of sodium nitrite and acid pH, results that the nitrite is converted into nitric oxyde (NO), compound able to inhibit several micro-organisms.

5. References

1- Arocha, MM., McVey, M., Loder, S D., Rupnow, JH., Bullerman, LB. (1992) Behavior of hemorrhagic *Escherichia coli* O157:H7 during the manufacture of cottage cheese. *J. Food Prot*. 55:379–381.
2- Arthey, D., Ashurst, P.R. (1996). *Fruit Processing*. Chapman and Hall, Great Britain
3- Berry, E.D., Cutter, C.N. (2000). Effects of acid adaptation of *E. coli* O157:H7 on efficacy of acetic acid spray washes to decontaminate beef carcass tissue. *Appl Environ Microbiol* 66: 1493-1498.
4- Brackett, RE., Hao, YY. (1994). Ineffectiveness of hot acid sprays to decontaminate *Escherichia coli* O157:H7 on beef. *J. Food Prot*. 57:198–203.
5- Brul, S., Coote, P. (1999). Preservative agents in foods: mode of action and microbial resistance mechanisms. *Int. J. Food Microbiol*. 50: 1-17.
6- Buchanan, R.L., Bagi, L.K. (1994).Expansion of response surface models for the growth of *Escherichia coli* O157:H7 to include sodium nitrite as a variable. *Int J Food Microbiol* 23: 317-332.

7- Casey, P., Condon, S. (2000).Synergistic lethal combination of nitrite and acid pH on a verotoxinnegative strain of Escherichia coli O157. *Int J Food Microbiol* 55: 255-258.
8- Castillo, A., Lucia, L.M., Roberson, D.B., Stevenson, T.H., Mercado, I., Acuff, G.R. (2001). Lactic acid sprays reduce bacterial pathogens on cold beef carcass surfaces and in subsequently produced ground beef. *J Food Prot* 64: 58-62.
9- Chung, K.C., Goepfert J.M. (1970). Growth of *Salmonella* at low pH. *J. Food Sci*. 35:326-328.
10- Conner, D.E., Scott, V.N., Bernard, D.T. (1990). Growth, inhibition, and survival of *Listeria monocytogenes* as affected by acidic conditions. *J.Food Protect*. : 53 : 59-652- 655.
11- Conner, D.E. Kotrola, J.S. (1995). Growth and survival of *Escherichia coli* O157:H7 under acidic conditions. *Appl. Environ. Microbiol*. 61(1):382-385.
12- Cutter, C N., Siragusa, GR. (1994). Efficacy of organic acids against *Escherichia coli* O157:H7 attached to beef carcass tissue using a pilot scale model carcass washer. *J. Food Prot.* 57:97–103.
13- Fennema, O.R. (1996). (ed). *Food Chemistry*. 3rd ed. Marcel Dekker, Inc., New York..
14- Fischer, J.R., Fletcher, D.L., Cox, N.A., Bailey, J.S. (1985). Microbiological properties of hard-cooked eggs in a citric acid-based preservation solution. *J. Food Prot*. 4:252.
15- Hill, C., O'Driscoll, B., Booth, I. (1995). Acid adaptation and food poisoning microorganisms. *Int. J. Food Microbiol.* 28:245-254.
16- Lück, E., Jager, M. (1997). *Antimicrobial food additives: characteristics, uses, effects*. 2nd ed. Springer-Verlag, Germany.
17- Ogawa, M., Shimizu, K., Nomoto, K., Tanaka, R., Hamabata, T., Yamasaki, S., Takeda, T., Takeda, Y. (2001). Inhibition of in vitro growth of Shiga toxin-producing Escherichia coli O157:H7 by probiotic Lactobacillus strains due to production of lactic acid. *Int J Food Microbiol* 68: 135-140.
18- Riordan, D.C., Duffy, G., Sheridan, J.J., Whiting, R.C., Blair, I.S., McDowell, D.A. (2000). Effects of acid adaptation, product pH, and heating on survival of *Escherichia coli* O157:H7 in pepperoni. *Appl Environ Microbiol* 66: 1726-1729.
19- Ryu, J.H., Deng, Y., Beuchat, L.R. (1999). Behavior of acid-adapted and unadapted *Escherichia coli* O157:H7 when exposed to reduced pH achieved with various organic acids. *J. Food Prot*. 62(5):451-455.
20- Samelis, J., Sofos, J.N., Kendall, P.A., Smith, G.C. (2002). Effect of acid adaptation on survival of *Escherichia coli* O157:H7 in meat decontamination washing fluids and potential effects of organic acid interventions on the microbial ecology of the meat plant environment. *J Food Prot* 65: 33-40.
21- Sorrells, K.M., Enigl, D.C., Hatfield, J.R. (1989). Effect of pH, acidulant, time, and temperature on the growth and survival of *Listeria monocytogenes. J.Food Protect*. : 52 : 571-573.
22- Uljas, H.E., Ingham, S.C. (1998). Survival of *Escherichia coli* O157:H7 in synthetic gastric fluid after cold and acid habituation in apple juice or trypticase soy broth acidified with hydrochloric acid or organic acids. *J Food Prot* 61: 939-947.
23- Vasseur, C., Baverel, L. Hébraud, M., Labadie, J. (1999). Effect of osmotic, alkaline, acid or thermal stresses on the growth and inhibition of *Listeria monocytogenes. J. Appl.Microbiol*. 86 : 469-476.

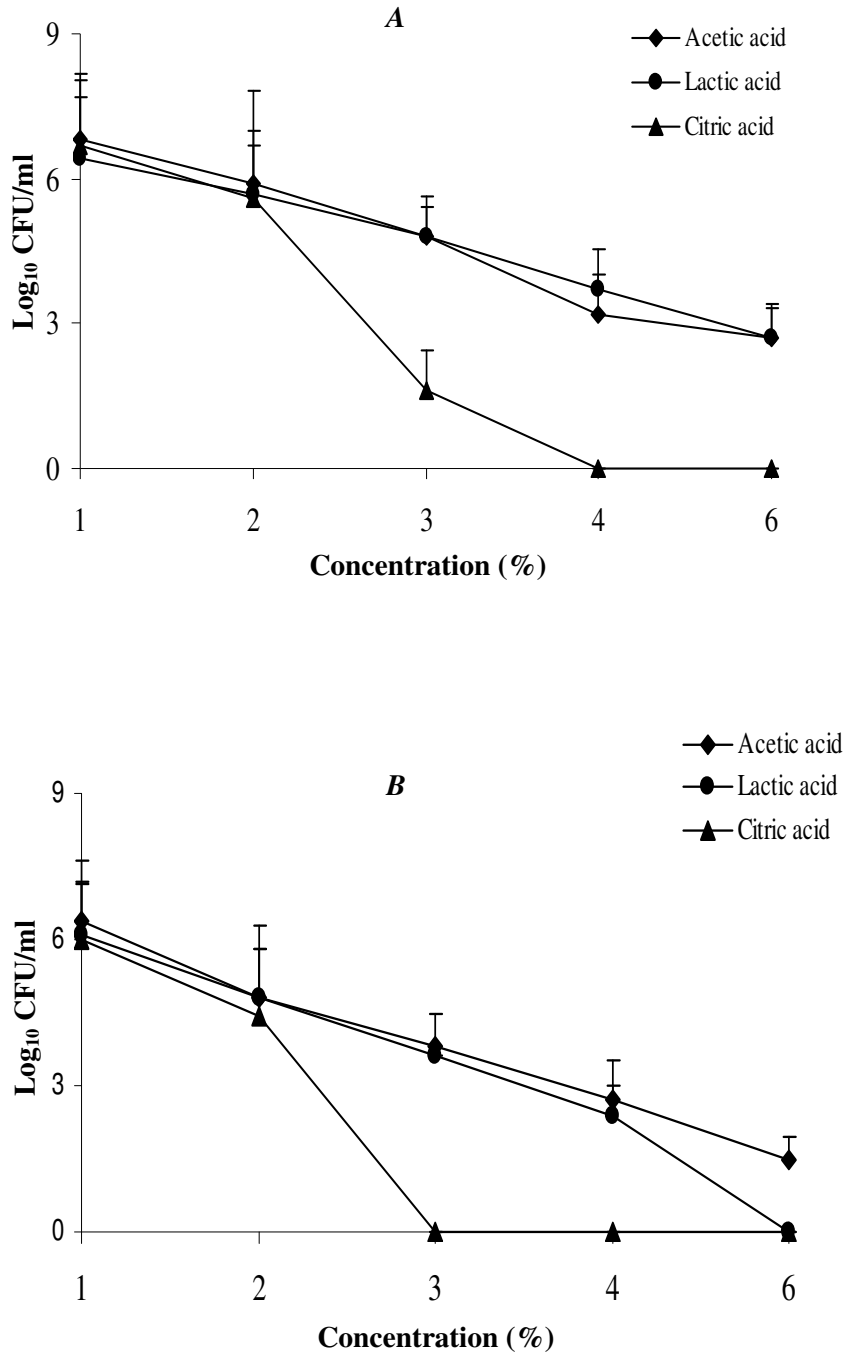

Figure 1. Antibacterial effect of organic acids on the survival of *E. coli* strains. Cells were grown to the stationary phase in TSB, harvested by centrifugation, suspended (2.10^6 CFU ml^{-1}) in TSB (pH 5) adjusted to different citric, acetic or lactic acids concentrations (1, 2, 3, 4 and 6%), and held at 5ºC for 96 hours. Each curve shows the average values obtained from two independent experiments. The strains used were *E. coli* EC_1 (A) and *E. coli* EC_2 (B).

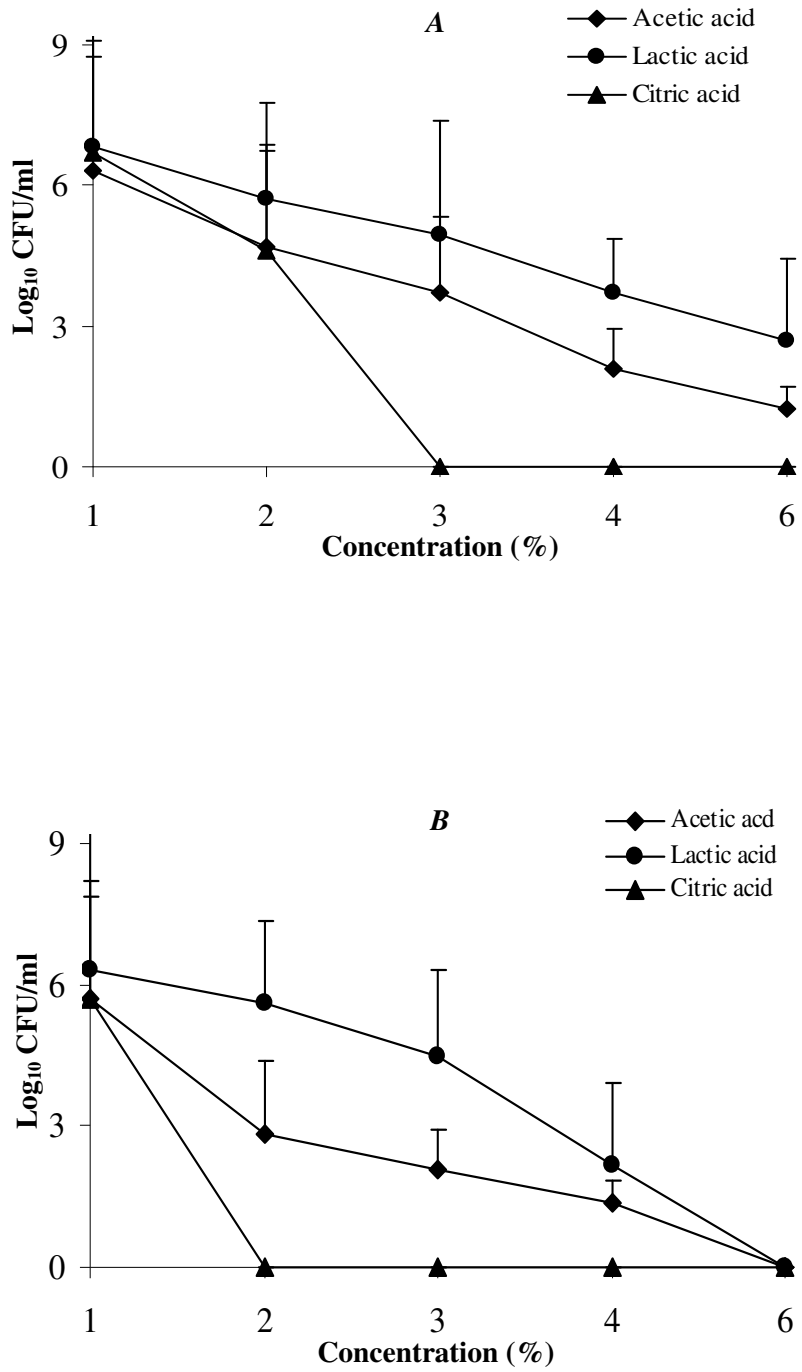

Figure 2. Antibacterial effect of organic acids on the survival of *E. coli* strains. Cells were grown to the stationary phase in TSB, harvested by centrifugation, suspended (2.10^6 CFU ml^{-1}) in TSB (pH 5) adjusted to different citric, acetic or lactic acids concentrations (1, 2, 3, 4 and 6%), and held at 20ºC for 96 hours. Each curve shows the average values obtained from two independent experiments. The strains used were *E. coli* EC_1 (A) and *E. coli* EC_2 (B).

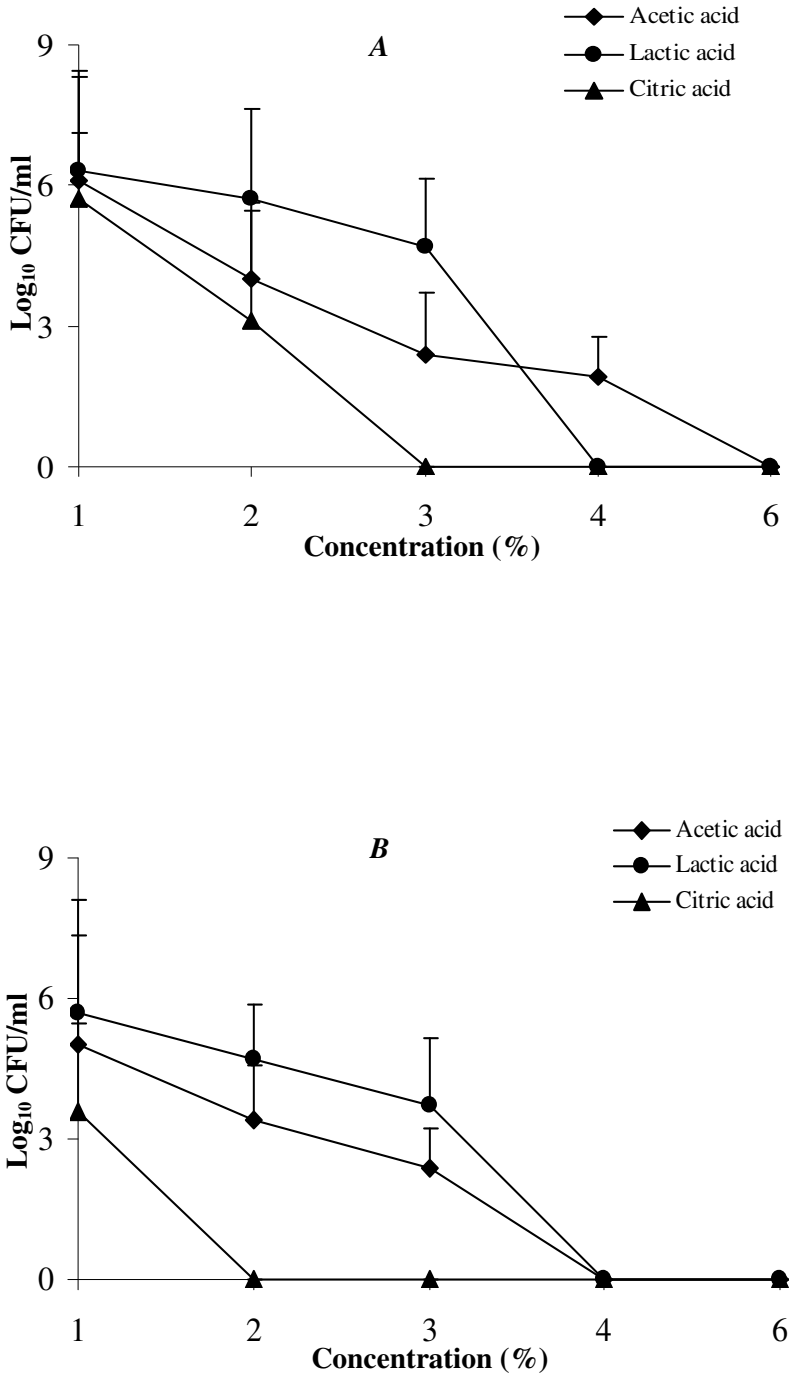

Figure 3. Antibacterial effect of organic acids on the survival of *E. coli* strains. Cells were grown to the stationary phase in TSB, harvested by centrifugation, suspended (2.10^6 CFU ml^{-1}) in TSB (pH 5) adjusted to different citric, acetic or lactic acids concentrations (1, 2, 3, 4 and 6%), and held at 37ºC for 96 hours. Each curve shows the average values obtained from two independent experiments. The strains used were *E. coli* EC_1 (A) and *E. coli* EC_2 (B).

Table I

Effect of the sodium nitrite on the survival of *E. coli.* Cells were grown to stationary phase in TSB, suspended (2.10^6 CFU ml^{-1}) in TSB (pH 4.5 or 6) adjusted to different $NaNO_2$ concentrations (0.1, 0.2, 0.4 and 0.6%), and then stored at a temperature of 20 ºC.

pH	% of $NaNO_2$	Time of treatments (days)	Population density (log_{10} CFU ml^{-1})	
			Strain EC_1	Strain EC_2
	0.1	2	6.3	6.3
	0.1	4	5.7	4.7
	0.1	6	4.7	3.7
	0.1	8	4.08	3.7
	0.1	10	4	3
	0.2	2	6.3	5.7
	0.2	4	4.7	4.65
	0.2	6	4.65	3.7
	0.2	8	3.7	2.7
	0.2	10	2.7	2.7
4.5	0.4	2	5.7	5.7
	0.4	4	3.10	3.1
	0.4	6	2.7	2.18
	0.4	8	2.08	2.08
	0.4	10	1.26	-
	0.6	2	4.48	3.7
	0.6	4	2.7	2.18
	0.6	6	2.18	2.08
	0.6	8	1.26	-
	0.6	10	-	-
	0.1	2	6.3	6.3
	0.1	4	5.7	4.7
	0.1	6	4.7	4.08
	0.1	8	4.65	3.7
	0.1	10	4	3.7
	0.2	2	6.3	5.7
	0.2	4	5.7	4.08
	0.2	6	4.7	3.7
6	0.2	8	4.08	3
	0.2	10	3.7	2.7
	0.4	2	5.7	5.7
	0.4	4	4.65.	3.10
	0.4	6	2.7	2.7
	0.4	8	2.7	2.18
	0.4	10	2.08	2.08
	0.6	2	4.65	4.48
	0.6	4	3.7	2.7
	0.6	6	2.18	2.08
	0.6	8	2.08	1.26
	0.6	10	1.26	-

The IISTE is a pioneer in the Open-Access hosting service and academic event management. The aim of the firm is Accelerating Global Knowledge Sharing.

More information about the firm can be found on the homepage:
http://www.iiste.org

CALL FOR JOURNAL PAPERS

There are more than 30 peer-reviewed academic journals hosted under the hosting platform.

Prospective authors of journals can find the submission instruction on the following page: http://www.iiste.org/journals/ All the journals articles are available online to the readers all over the world without financial, legal, or technical barriers other than those inseparable from gaining access to the internet itself. Paper version of the journals is also available upon request of readers and authors.

MORE RESOURCES

Book publication information: http://www.iiste.org/book/

Academic conference: http://www.iiste.org/conference/upcoming-conferences-call-for-paper/

IISTE Knowledge Sharing Partners

EBSCO, Index Copernicus, Ulrich's Periodicals Directory, JournalTOCS, PKP Open Archives Harvester, Bielefeld Academic Search Engine, Elektronische Zeitschriftenbibliothek EZB, Open J-Gate, OCLC WorldCat, Universe Digtial Library , NewJour, Google Scholar