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ScienceDirect
Food Chemistry 109 (2008) 137–143
Food Chemistry
www.elsevier.com/locate/foodchem

Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157:H7 in laboratory medium and carrot juice

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Received 5 July 2007; received in revised form 13 November 2007; accepted 17 December 2007

Abstract

Outbreaks of food-borne pathogens, such as *Escherichia coli* O157:H7 and *Salmonella,* continue to draw public attention to food safety. Several reports have demonstrated the efficacy of using natural ingredients to control the growth of food-borne pathogens. The objective of this study was to investigate antimicrobial effects of lactic acid and copper, alone and in combination, on the survival and growth of *Salmonella* spp. and *E. coli* O157:H7 in laboratory medium and carrot juice. Survival and growth of 38 *Salmonella* spp. and six *E. coli* O157:H7 strains were compared when grown in brain heart infusion (BHI) broth and carrot juice under conditions including either lactic acid (0.2%) alone, copper sulfate (50 ppm) alone or the combination of the two. The growth inhibition was negligible when copper sulfate was added to BHI broth and carrot juice. Lactic acid (0.2%) retarded the growth of bacterial strains. However, the growth of bacterial strains was significantly inhibited when both lactic acid and copper were in BHI broth and carrot juice within the time frame of this study. These findings indicated that lactic acid, in combination with copper sulfate, could be used to inhibit the growth of pathogens. Natural ingredients, such as lactic acid and low dose of copper ions, can be used to improve the safety of food products.

Keywords: Lactic acid; Copper; *Salmonella*; *Escherichia coli* O157:H7; Antimicrobial activity

1. Introduction

With greater availability of information about the health impacts and better reporting standards, consumers are now, not only paying closer attention to the risk of food-borne pathogens, but also to the presence of artificial chemical preservatives included to control food-borne pathogens. As a result, consumers are calling for greater use of natural ingredients to ensure the safety of consumable products. In response to this demand, the food industry is looking to use more natural food preservatives that have strong antimicrobial activity to ensure safe wholesome food products. Two of these natural additives are lactic acid and copper. Traditionally, lactic acid, a weak-organic acid, has been widely used to control growth of pathogenic bacteria in foods for several decades. The antimicrobial activity occurs through the diffusion of lactic molecules into microbial cells until equilibrium is reached, in accordance with the pH gradient, causing membrane disruption, inhibition of essential metabolic reactions, stress on intracellular pH homeostasis and accumulation of toxic anions and ultimate death of microbial cells (Brul & Coote, 1999).

It is common knowledge that microorganisms require low concentrations of copper ions as essential micronutrients and as vital cofactors for processing of metalloproteins

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doi:10.1016/j.foodchem.2007.12.035

and certain enzymes (Gadd, 1993; Nies, 1999). Higher concentrations of copper (250 ppm) can cause inhibition of growth or even death of microorganisms. The toxic effect of copper on microorganisms occurs by the displacement of essential ions, thereby obstructing functional groups of proteins, inactivating enzymes, producing hydroperoxide free radicals, and altering membrane integrity (Nies, 1999). Copper compounds have been widely used as algicide, fungicide, molluscide and acaricide agents in agriculture (Borkow & Gabbay, 2005).

Copper plated surfaces have been shown to have a significant antibacterial activity against a wide range of microorganisms, including *Salmonella enteric* and *Campylobacter jejuni* (Faúndez, Troncoso, Navarrete, & Figueroa, 2004). Copper (3.72%) added to stainless steel was found to impede the adhesion of microorganisms during an initial 48 h period, but this antimicrobial effect disappeared after longer incubation periods (Kielemoes & Verstraete, 2001). Copper was added to phosphate-based glasses to combat oral microorganisms and to inhibit the adherence of several microorganisms (Mulligan, Wilson, & Knowles, 2003). Therefore, there is a potential for copper to be used as a preservative to inhibit pathogens in food products. Currently, limited information is available on the effect of copper on the growth inhibition of food-borne pathogens in food products. Previous work, using pigs, suggests that the combination of lactic acid and copper may be an effective combination against undesired microbes. In a study by Beal, Niven, Campbell, and Brooks (2003), lactic acid (150 mM) and copper sulfate (50 ppm) were added to liquid pig feed and found to cause a 10-fold decrease in the D_{value} of *Salmonella typhimurium* DT104:30. Given the growing attention being paid to natural antimicrobial compounds, which may be more readily accepted by consumers, it is sensible to explore the possibility of combining compounds to obtain a better and more cost efficient reduction of pathogenic microbes. Therefore, the objective of this study was to investigate the effect of lactic acid alone, or in combination with copper sulfate, on the growth inhibition of *Salmonella* spp. and *Escherichia coli* O157:H7 in laboratory medium and carrot juice.

2. Materials and methods

2.1. Bacterial strains

Thirty eight strains of *Salmonella* spp. strains (Table 1) and six strains of *E. coli* O157:H7 (Table 2) were used in this study. *Salmonella* spp. strains were obtained from the Food Microbiology Laboratory in North Carolina A&T State University. *E. coli* strains were supplied by Dr. S.S. Summer, Department of Food Science and Technology at Virginia Tech. These strains were maintained on tryptic soy agar (TSA, Difco Laboratories, Becton Dickinson, Sparks, MD) slants at 4 °C. Strains were then transferred to fresh tryptic soy broth (TSB) before use. Cultures of each strain were grown separately in TSB (Difco, Becton Dickinson, Sparks, MD) at 37 °C and transferred at 24 h intervals.

2.2. Inoculum preparation

Overnight cultures of the strains were centrifuged (8000*g*, 15 min) and supernatant of each strain was decanted and the cell pellet was resuspended (approximately 8 log CFU/ml) in 0.1% (v/v) 10 ml peptone water. Several serial decimal dilutions were made to achieve an initial inoculum level of ~3.0 log CFU/ml before inoculation into broth. To determine the effect of lactic acid and copper on bacterial population in BHI broth and carrot juice, a separate cocktail mixture of *E. coli* O157:H7 or *Salmonella* spp. was used. Strains were prepared by mixing 100 μl of each overnight strain in 10 ml peptone water. Several serial decimal dilutions were then made to achieve an initial inoculum level of ~3.0 log CFU/ml before inoculation into BHI broth and the carrot juice.

2.3. Media preparation

One litre of BHI broth was prepared and split into two 500 ml portions. To one portion, lactic acid (Fisher Scientific, Pittsburgh, PA) was added to obtain a 0.2% (v/v) concentration. The second portion was left untreated. These two portions were then split into two equal portions and copper sulfate ($CuSO_4 \cdot 5H_2O$, 50 ppm) was added to one of the two lactic acid portions (forming the combination lactic acid/copper sample) and one of the untreated portions (forming the copper only sample). The remaining two samples represented the lactic acid sample only and control. Seven millilitre batches of each portion sample were dispensed into test tubes and sterilized at 121 °C for 15 min. Each set of experimental tests was conducted three times.

2.4. Measuring bacterial growth

Bacterial growth was monitored by measuring the turbidity every two hours time intervals using a spectronic 21 Milton Roy spectrophotometer (Thermo Electron Scientific Co., Madison, WI) at the wavelength of 610 nm.

2.5. Preparation of carrot juice

Fresh pasteurized carrot juice was purchased from a local store (Greensboro, NC) the day before each use and stored at 4 °C. As preparation for its use as a growth media, 1 l of the carrot juice was steam-sterilized at 95 °C for 10 min to inactivate natural flora and then cooled to 37 °C. The sterilized juice was then split into two 500 ml portions and then divided into Acidified and Control portions, with and without receiving the same treatment as the BHI broth.

Table 1
Antimicrobial activity of lactic acid (LA) and copper (Cu) on growth of *Salmonella* in laboratory medium (optical density: 610 nm)

Strain	Control			Cu			LA			Cu/LA		
	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
S. vellore	0.07	0.44	0.57	0.09	0.45	0.57	0.07	0.38	0.45	0.05	0.14	0.22
S. muenster	0.06	0.52	0.59	0.08	0.48	0.54	0.08	0.40	0.46	0.05	0.14	0.23
S. Newport	0.06	0.50	0.56	0.09	0.47	0.52	0.05	0.36	0.41	0.05	0.14	0.20
S. Typhimurium	0.04	0.45	0.49	0.06	0.45	0.48	0.04	0.42	0.31	0.03	0.13	0.20
S. Newport	0.06	0.49	0.63	0.09	0.49	0.53	0.05	0.39	0.46	0.05	0.16	0.22
S. muenster	0.05	0.48	0.54	0.06	0.47	0.49	0.05	0.36	0.24	0.04	0.15	0.19
S. Typhimurium	0.04	0.45	0.54	0.05	0.45	0.54	0.05	0.44	0.35	0.03	0.15	0.15
S. mbandaka	0.05	0.56	0.62	0.06	0.55	0.52	0.05	0.42	0.50	0.04	0.21	0.28
S. kentucky	0.04	0.46	0.54	0.04	0.47	0.45	0.03	0.43	0.35	0.03	0.12	0.24
S. juzendorf	0.01	0.49	0.51	0.03	0.47	0.53	0.04	0.45	0.34	0.04	0.15	0.18
S. heidelberry	0.05	0.53	0.58	0.03	0.56	0.58	0.04	0.40	0.42	0.04	0.23	0.24
S. Typhimurium	0.05	0.49	0.53	0.04	0.55	0.55	0.05	0.46	0.40	0.04	0.18	0.21
S. heidelberrg	0.05	0.53	0.54	0.06	0.49	0.52	0.04	0.42	0.42	0.04	0.18	0.22
S. Typhimurium	0.05	0.51	0..55	0.06	0.54	0.57	0.03	0.42	0.40	0.04	0.20	0.23
S. heidelberrg	0.05	0.53	0.54	0.06	0.49	0.52	0.05	0.42	0.44	0.04	0.20	0.19
S. hadar	0.06	0.49	0.58	0.07	0.55	0.61	0.04	0.47	0.55	0.05	0.25	0.18
S. newport	0.05	0.54	0.58	0.06	0.54	0.57	0.02	0.43	0.46	0.05	0.23	0.26
S. enteritidis	0.04	0.55	0.61	0.06	0.55	0.63	0.04	0.47	0.51	0.05	0.23	0.23
S. gaminara	0.05	0.53	0.63	0.06	0.56	0.64	0.05	0.44	0.54	0.05	0.17	0.24
S. urbana	0.06	0.56	0.64	0.08	0.54	0.65	0.05	0.44	0.44	0.05	0.21	0.21
S. anatum	0.06	0.51	0.66	0.08	0.53	0.57	0.05	0.38	0.47	0.04	0.16	0.22
S. enteritidis	0.06	0.51	0.57	0.08	0.49	0.58	0.07	0.39	0.48	0.04	0.14	0.22
S. Typhimurium	0.06	0.48	0.57	0.08	0.51	0.53	0.04	0.40	0.43	0.05	0.18	0.23
S. arizonae	0.06	0.50	0.53	0.09	0.52	0.58	0.05	0.38	0.44	0.05	0.19	0.25
S. enteritidis	0.05	0.54	0.56	0.07	0.53	0.59	0.04	0.42	0.45	0.04	0.22	0.29
S. Typhimurium	0.06	0.47	0.59	0.09	0.50	0.55	0.05	0.41	0.39	0.03	0.16	0.13
S. Typhimurium	0.05	0.46	0.55	0.08	0.47	0.54	0.04	0.43	0.42	0.04	0.16	0.17
S. abony	0.06	0.47	0.55	0.08	0.47	0.54	0.04	0.37	0.38	0.04	0.16	0.19
S. Typhimurium	0.05	0.44	0.55	0.06	0.44	0.48	0.04	0.42	0.31	0.04	0.15	0.20
S. Typhimurium	0.06	0.49	0.51	0.08	0.51	0.52	0.05	0.50	0.38	0.04	0.17	0.22
S. Worthington	0.05	0.48	0.51	0.07	0.47	0.53	0.03	0.42	0.46	0.03	0.14	0.21
S. ohio	0.05	0.48	0.56	0.06	0.49	0.53	0.04	0.40	0.45	0.03	0.12	0.20
S. Thompson	0.05	0.49	0.54	0.07	0.51	0.50	0.04	0.41	0.39	0.04	0.14	0.21
S. Tennessee	0.04	0.43	0.55	0.05	0.47	0.57	0.04	0.48	0.34	0.04	0.16	0.18
S. Typhimurium	0.05	0.49	0.56	0.07	0.53	0.54	0.04	0.42	0.42	0.04	0.15	0.21
S. Schwarzengrund	0.05	0.48	0.54	0.07	0.50	0.53	0.04	0.43	0.41	0.04	0.13	0.18
S. montervideo	0.04	0.49	0.55	0.05	0.49	0.55	0.05	0.46	0.35	0.03	0.12	0.17
S. Typhimurium	0.06	0.59	0.66	0.09	0.59	0.56	0.05	0.50	0.48	0.03	0.21	0.22

Table 2
Antimicrobial activity of lactic acid (LA) and copper (Cu) on growth of *E. coli* O157:H7 in laboratory medium as measured by optical density (O.D. 610 nm)

E. coli O157:H7 strains	Control			Cu			LA			LA/Cu		
	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h	0 h	4 h	8 h
944	0.07	0.49	0.57	0.09	0.47	0.52	0.07	0.38	0.39	0.05	0.12	0.21
Cider	0.06	0.51	0.63	0.08	0.45	0.58	0.05	0.41	0.46	0.05	0.15	0.20
E0019	0.06	0.51	0.56	0.09	0.47	0.51	0.05	0.43	0.43	0.05	0.12	0.21
F4546	0.04	0.51	0.59	0.06	0.45	0.55	0.04	0.37	0.45	0.03	0.12	0.22
H1730	0.06	0.54	0.56	0.09	0.46	0.52	0.05	0.36	0.41	0.05	0.14	0.23
380	0.05	0.51	0.58	0.06	0.45	0.55	0.05	0.44	0.44	0.04	0.09	0.19

2.6. Bacterial enumeration

Bacterial populations were determined by plating onto tryptic soy agar (TSA). In this procedure, samples (1 ml) were withdrawn from inoculated samples at 6 h intervals for up to 12 h, serially diluted in 0.1% peptone water; then appropriate dilutions were surface-plated (100 μl) onto duplicate TSA. Colonies were counted after plates were incubated at 35 °C for 24 h to determine the bacterial populations.

2.7. Statistical analysis

Each set of experimental tests were conducted three times for each bacterial strain to determine the effect of lactic acid alone and in combination with copper sulphate on

the survival and growth of the tested bacterial strains. Data were analyzed by a factorial analysis of variance of duplicate samples at a significance level of $P < 0.05$. Statistical results were subjected to a Bonferroni adjustment for conservative analysis.

3. Results and discussion

3.1. Preliminary study

A preliminary study was conducted to determine the lowest concentration of copper and lactic acid alone that caused slight growth inhibition of *Salmonella* spp. and *E. coli* O157:H7. Table 3 shows the effect of four different concentrations (0.0 ppm, 50 ppm, 100 ppm, and 200 ppm) of copper sulfate on the growth of *Salmonella* spp. and *E. coli* O157:H7. Results showed that slight growth inhibition was obtained with 50 ppm. A significant growth inhibition was observed with 100 ppm and 200 ppm.

A similar process was followed for testing the effect of lactic acid on bacterial growth. Table 4 shows the effects of four different concentrations; 0.0%, 0.2%, 0.3% and 0.4% of lactic acid on the growth of *Salmonella* spp. and *E. coli* O157:H7. Results showed that slight growth inhibition was obtained with 0.2%. Therefore, combinations of 0.2% lactic acid and 50 ppm copper sulfate was selected to determine whether there would be a synergistic effect on the growth of *Salmonella* and *E. coli* O157:H7 in laboratory medium and carrot juice.

Tables 1 and 2 show the growth of individual strains of *Salmonella* spp. and *E. coli* O157:H7 in BHI broth in the presence of copper and lactic acid, respectively. In the control samples, the initial growth, as observed by the turbidity (O.D. 61 nm), was low (O.D. ~ 0.05). The bacterial strains continued to grow during the incubation period and reached stationary phase within 6–8 h. The turbidity readings reached an absorbance of 0.66. When copper was added to BHI broth at a concentration of 50 ppm, a slight delay in the growth was observed ($P < 0.1$) in most of the tested samples. For some of the samples (e.g., *Salmonella heidelberrg*) copper sulfate did not have an effect on the bacterial growth (see Table 1). The addition of 0.20% lactic acid retarded the growth of the tested strain ($P < 0.07$). Our results indicated that copper or lactic acid alone had a slight effect on the growth of *Salmonella* spp. and *E. coli* O157:H7 in laboratory medium. The combination of lactic acid and copper sulfate showed significant growth inhibition for all tested strains ($P < 0.05$) indicating that combination of lactic acid and copper sulfate could be used to control the growth of *Salmonella* and *E. coli* O157:H7.

Table 3
Effect of copper sulphate on the growth inhibition of *Salmonella* and *E. coli* O157:H7 (optical density: 610 nm)

Copper sulphate (ppm)	*Salmonella*	*E. coli* O157:H7
0	0.58	0.60
50	0.53	0.58
100	0.22	0.24
200	0.12	0.20

Table 4
Effect of lactic acid on the growth inhibition of *Salmonella* and *E. coli* O157:H7 (optical density 610 nm)

Lactic acid (%)	*Salmonella*	*E. coli* O157:H7
0	0.61	0.62
0.2	0.55	0.54
0.3	0.15	0.27
0.4	0.03	0.05

The effects of lactic acid and copper, alone or in combination, on the growth of *Salmonella* sample, which is a cocktail of 38 *Salmonella* spp. in BHI broth, are shown in Fig. 1. In the control samples, the number of *Salmonella* increased from 10^3 to 1.5×10^7 CFU/ml after 6 h at 37 °C and then 2.5×10^7 CFU/ml after 12 h. With the addition of copper (50 ppm), the growth of *Salmonella* spp. was not significantly inhibited ($P > 0.05$). The bacterial population reached 1.4×10^7 CFU/ml after 6 h. The number of cells further increased to 2.0×10^7 CFU/ml after 12 h. The concentration of copper is one of the most important factors for bactericidal effect (Deveer, Wilde, & Ruden, 1993). Obviously, copper at 50 ppm, is not significantly bactericidal for *Salmonella*. The addition of lactic acid at a concentration of 0.2% produced a retarding effect on the growth of *Salmonella* (Fig. 1). After 6 h, the number of cells reached only 3.0×10^4 CFU/ml and, after 12 h, the number was 4.5×10^5 CFU/ml. Therefore, with 0.2% lactic acid, the growth of *Salmonella* was delayed. When a combination of both 50 ppm copper and 0.2% lactic acid was used, a significant growth inhibition of *Salmonella* ($P < 0.01$) (Fig. 1) was observed. After 6 h, the number of cells was only 1.1×10^2 CFU/ml while, after 12 h, the number was 2.8×10^2 CFU/ml.

In this study, effects of lactic acid and/or copper on the growth of *Salmonella* in carrot juice were also investigated. In the control sample, the number of blended 38 *Salmonella* spp. was increased from 10^3 to 4.0×10^7 CFU/ml after 6 h at 37 °C and then reached 2.1×10^8 CFU/ml after 12 h (Fig. 2). When there was 50 ppm of copper in carrot juice, the number was 1.2×10^7 CFU/ml after 6 h and then 1.7×10^8 CFU/ml after 12 h. The growth of *Salmonella* with the presence of 50 ppm was not significantly affected ($P > 0.05$). When there was only 0.2% lactic acid in carrot juice, the number was 4.9×10^4 CFU/ml after 6 h and then 3.3×10^5 CFU/ml after 12 h. The growth of *Salmonella* with the presence of 0.2% lactic acid was retarded ($P < 0.05$). When both lactic acid (2%) and copper (50 ppm) were added to carrot juice, the number of *Salmonella* spp. was 3.6×10^2 CFU/ml after 6 h and then 6.3×10^2 CFU/ml after 12 h. The growth of *Salmonella* with the presence of both lactic acid and copper was significantly inhibited ($P < 0.05$). Similar results were also obtained with *E. coli* O157:H7 in both BHI (Fig. 3)

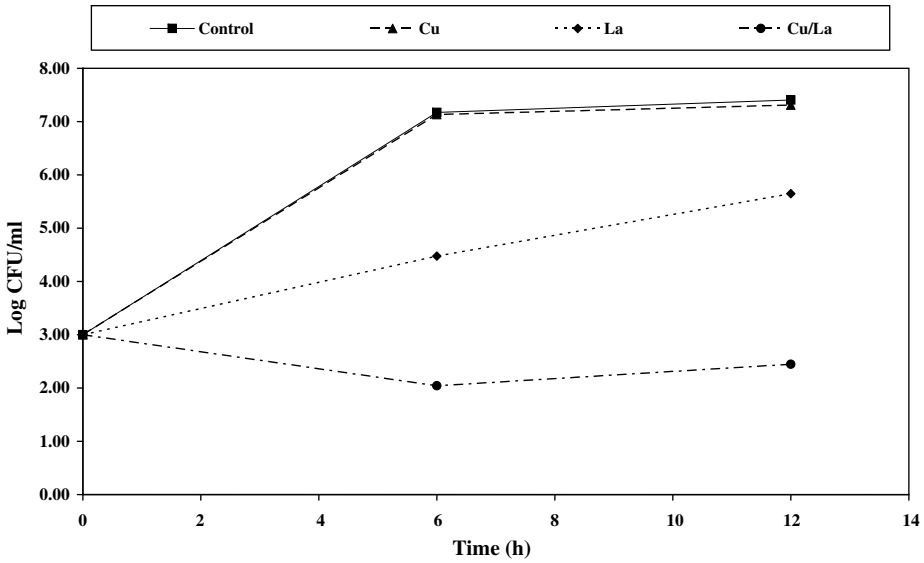

Fig. 1. Survival and growth of mix 38 *Salmonella* strains in BHI broth with lactic acid (LA) (0.2%) and/or copper (Cu) (50 ppm).

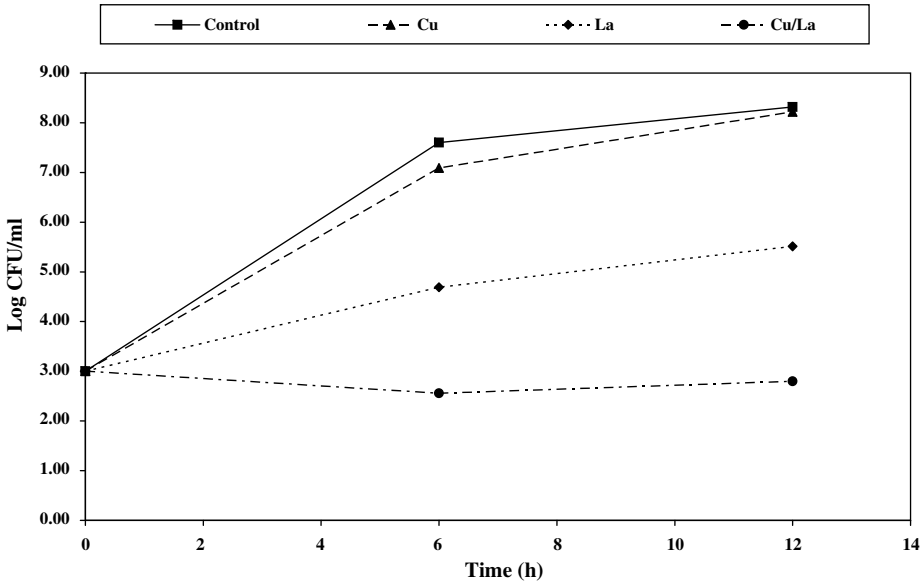

Fig. 2. Survival and growth of mix 38 *Salmonella* strains in carrot juice with LA (0.2%) and/or copper Cu (50 ppm).

and carrot juice (Fig. 4) in the presence of copper and lactic acid, indicating a significant inhibitory effect when combination of 0.2% lactic acid and 50 ppm copper were used.

Salmonella and *E. coli* O157:H7 have become recognized as important food-borne pathogens because of the high mortality rates associated with infections in susceptible populations (Mead et al., 1999; Williams, Sumner, & Golden, 2004). Both pathogens have been implicated in several outbreaks involving several food products, including juices. Therefore, it is very important to control the growth of these pathogens in food products. Commercially, natural preservatives, such as organic acids, essential oils and herbs (Ibrahim, Salameh, Phetsomphou, Yang, & Seo, 2006) have been widely applied to add to food products to prevent the growth of microorganisms. Copper has been used to control the growth of microorganisms; however, the mechanism of the antimicrobial activity of copper and lactic acid on the survival and growth of bacterial cells is not well understood. High copper diet has been shown to support the resistance of pigs to *E. coli* infection during the post-weaning period (HØjberg, Canibe, Poulsen, Hedemann, & Jensen, 2005). Beal et al. (2003) reported that acid stress decreased the tolerance of bacterial cells to free copper (Cu^{2+}) since these bacterial cells were not sensitive to copper alone. Rodgers and Ryser (2004) reported that a 5-log reduction was achieved for *Listeria monocytogenes* and *E. coli* O157:H7 in apple cider with the combination of copper and sodium hypochlorite, followed by sonication treatment.

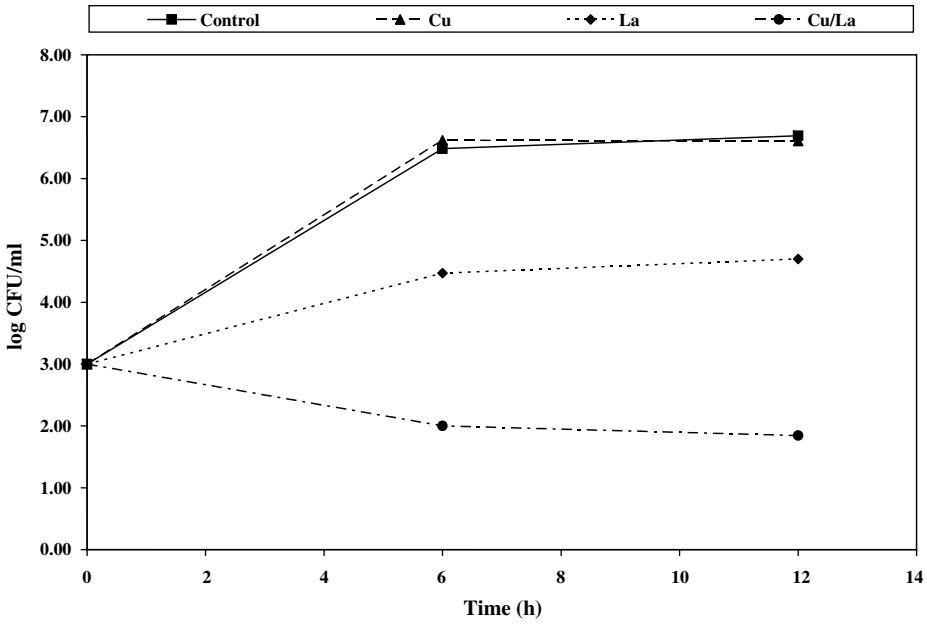

Fig. 3. Survival and growth of mix 6 *E. coli* O157:H7 strains in BHI broth with LA (0.2%) and/or copper Cu (50 ppm).

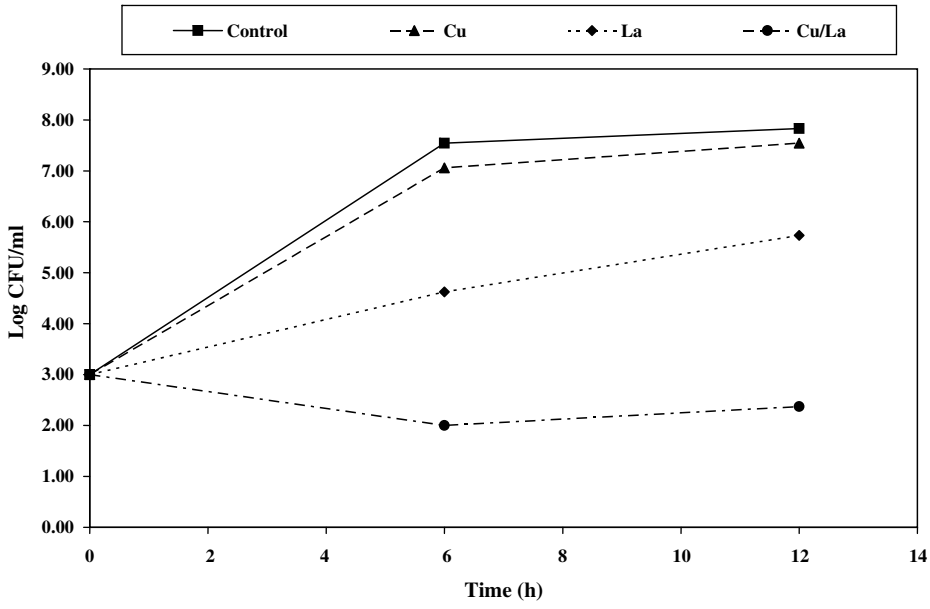

Fig. 4. Survival and growth of mix 6 *E. coli* O157:H7 strains in carrot juice with LA (0.2%) and/or copper Cu (50 ppm).

In the present study, lactic acid (0.2%) was found to significantly retard the growth of *Salmonella* and *E. coli* O157:H7 in laboratory media and carrot juice. The presence of 50 ppm copper does not influence the growth of *Salmonella* and *E. coli* O157:H7. Normally, copper at high concentration is toxic to microorganisms because it can mediate cell membrane damage, interact with nucleic acids and mediate protein damage (Borkow & Gabbay, 2005; Brul & Coote, 1999). The concentration of 50 ppm, which is a safe level for human beings, may not be lethal enough to inhibit the growth of *Salmonella* and *E. coli* O157:H7. However, when both lactic acid and copper were added to laboratory medium and carrot juice, the growths of *Salmonella* spp. and *E. coli* O157:H7 were significantly inhibited. These results clearly demonstrate that combination of lactic acid and copper produces a synergistic inhibition effect on the microbial growth.

Acknowledgments

The research work was funded through the Cooperative State Research, Education and Extension Service of the United States Department of Agriculture, Project No. NC.X-173-5-02-170-1, in the Agricultural Research

Program, North Carolina Agricultural and Technical State University. We would like to express our gratitude to Dr. C.S. Turner, Associate Dean for Research for her support and Dr. K. Gruber for technical editing.

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