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Synergistic effect of copper and organic acids on growth of *E. coli* and *Salmonella* sp. from fresh fruits and vegetables.

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ABSTRACT

Fresh fruits and vegetables are an indispensable aspect of the diet today. But foodborne outbreaks associated with them have also increased over decades. The present study was designed to reduce the microbial load as a step towards strengthening food safety. Copper and organic acids including lactic acid, acetic acid and citric acid were used to determine their inhibitory effect on *E. coli* and *Salmonella* sp. isolated from fruits and vegetables. The effect was determined individually as well as in combinations. It was observed that lactic acid at 0.2%, acetic acid at 0.2% and citric acid at 0.3% inhibited the bacterial growth. Combination of copper with the three organic acids was investigated separately. The combinations were applied onto carrot and apple to determine the microbial reduction. Copper (50 mg/ml) in combination with 0.2% lactic acid was found to inhibit the microbial growth the most. Results indicated that the combination yielded 2 log reductions in the case of *E. coli* on carrot and 4 log reductions on apple respectively. Thus, the combined treatment of copper and lactic acid can be applied in food industries to prevent the occurrence of outbreaks associated with fresh fruits and vegetables.

Keywords: Fresh fruits and vegetables, *E. coli*, *Salmonella* sp., copper, organic acids

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INTRODUCTION

Increased consumption of fruits and vegetables in raw form in the recent years has been found to be accompanied by an increase in the number of human infections and outbreaks [1]. Fruits and vegetables are generally considered to be low-risk foods with respect to safety. However, sporadic outbreaks of foodborne diseases associated with fresh fruits and vegetables have raised concern that these products may be an increasing source of foodborne infection [2]. Fruits and vegetables can become contaminated during growing, harvesting, processing or distribution stages. An increasing number of foodborne outbreaks of human illness have been linked to the consumption of raw fruits, vegetables contaminated with gastrointestinal pathogens [3]. *Salmonella* and *Escherichia coli* O157:H7 are the major pathogens contributing to outbreaks of foodborne illness associated with fresh fruits and vegetables [4; 5; 6]. Several pathogen-substrate combinations more frequently occur in outbreaks: *Salmonella* and cantaloupes, tomatoes or sprouts; *E. coli* O157:H7 and leafy green vegetables; *Cyclospora* and raspberries; and hepatitis A with green onions [7; 8].

The incidence of outbreaks has led to the development ofvarious methods to either prevent or reduce the microbial load associated with fresh fruits and vegetables. Chlorine, hydrogen peroxide and other chemicals have been conventionally used in industries to wash the produce, but the efficacy of treatments has been low. Also, the threat of chemicals being retained on product is high. Foods preserved with natural additives have become popular in recent years due to consumer awareness and concern about synthetic chemical additives. Copper is one of the oldest metals with various beneficial uses to the mankind. Copper has been shown to be a broad-spectrum antimicrobial agent effective against a range of pathogens. The mechanism of antimicrobial action of copper begins by rupture of the bacterial outer membrane on contact followed by entry of copper ions into the cell and obstructing cell metabolism. Copper has shown its antimicrobial action against a wide range of microorganisms including *Salmonella* and *E. coli* O157:H7. Faundez et al., 2004 [9] showed that copper ions induce and inhibit growth in bacteria and have a toxic effect on most microorganisms. Recent reports also showed that copper nanoparticles can kill *E. coli* and has a great potential as antimicrobial agents [10]. Organic acids approved by the Federal Drug Administration (FDA) are generally recognized as safe (GRAS). They have been documented to possess antimicrobial activities against different pathogens such as *E. coli*, *Salmonella* sp and *Listeria monocytogenes*. They have both bacteriostatic and bactericidal effects which hamper the growth of bacteria and leading to loss of viability [11]. The hydrophobicity and un-dissociated acid level of an organic acid decide the efficacy of antimicrobial effect they exert. Most effective organic acids are acetic, lactic, propionic, sorbic, and benzoic acid. Alvarado-Casillas [12] showed that there was a significant reduction in the number of *S. typhimurium* and *E. coli* O157:H7 on fresh cantaloupes with 2% lactic acid spray. Application of organic acids in food preservation has long been known and should be taken to the commercial level as most of the available data is only at research level. Combination treatments have been reported to inactivate *Salmonella sp* and *E. coli* O157:H7 on different food products. Beal et al., 2004 [13] reported that the addition of 150 mM lactic and 50 ppm of Cu to liquid pig feed resulted in a 10-fold increase in the death rate of *Salmonella typhimurium*. Objective of the study is to investigate the effect of copper alone and in combination with organic acids in controlling the growth of pathogenic microflora on fresh fruits and vegetables.

MATERIALS AND METHODS

Bacterial strains

E. coli and *Salmonella* sp. isolated from fresh fruits and vegetables which are consumed raw in the form of salads were selected for the study. Five pathogenic strains including three *E. coli* and two *Salmonella* sp. were selected from the culture collection at Food Science and Technology, Pondicherry University. These cultures were maintained on nutrient agar slants at 4°C and transferred to LB broth before use.

Effect of Cu and organic acids on bacteria

All the five strains were tested individually for determining the effect of copper and the three organic acids on them. Copper at concentrations starting from 25 to 200 mg/ml and organic acids at concentrations from 0.1% to 0.4% were added to the bacterial culture in LB broth and incubated at 37°C for 24h. The bacterial growth in terms of turbidity was measured by using spectrophotometer at 600 nm.

Bacterial growth pattern with Cu and organic acids

The strains under study were grown in BHI broth supplemented with copper (50 mg/ml), lactic acid[LA] (0.2%), acetic acid[AA] (0.2%) and citric acid[CA] (0.3%) individually in triplicates with the control containing the bacterial culture in BHI broth. Also, each strain was subjected to three different combinations of copper with lactic acid, acetic acid and citric acid separately as follows: Cu (50 mg/ml) plus 0.2% LA, Cu (50 mg/ml) plus 0.2% AA and Cu (50 mg/ml) plus 0.3% CA. Bacterial growth was monitored by measuring the turbidity every two hours time intervals using ELICO CL63 photometer at a wavelength of 600 nm.

Treatment with Cu and LA

Fresh samples of apple and carrot were purchased from local market of Puducherry and brought into the laboratory for the experiment. Samples were rinsed thoroughly in sterile distilled water followed by drying under sterile conditions in the laminar flow chamber. Overnight culture of *E. coli* O157:H7 (MTCC 727) and *Salmonella typhi* (ATCC 14028) were prepared to yield cell density of 10^3 CFU/ml, followed by dipping the samples into the culture in separate beakers and then allowed to dry. Samples were cut in aseptic conditions (25 g), immersed in 225 ml of BHI broth supplemented with 50 mg/ml copper and 0.2% lactic acid and stored at room temperature for a period of 12 h.

Bacterial enumeration after treatment

Bacterial growth was then determined by plating onto tryptic soy agar (TSA). In this procedure, samples (1 ml) were withdrawn from inoculated samples at 0h, 6 h and 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 37°C for 24 h to determine the bacterial growth.

Statistical analysis

All the experiments were performed in triplicate sets and the mean values were analyzed by using one way-ANOVA.

RESULTS

The effect of copper and the three organic acids on microbial growth was examined individually. Table 1 shows the effect of different concentrations of copper, viz., 50, 100 and 200 mg/ml on the growth of five strains under study. Results indicated that copper at 50 mg/ml was the lowest concentration which inhibited growth while higher concentrations of 100 and 200 mg/ml inhibited growth significantly.

Table 1. Effect of Copper on growth of *E. coli* and *Salmonella* sp.

Isolates	Copper (O.D at 600 nm) mg/ml			
	0	50	100	200
E. coli				
E1	0.49	0.42	0.39	0.29
E2	0.58	0.50	0.47	0.42
E3	0.55	0.47	0.45	0.36
***Salmonella* sp.**				
S1	0.48	0.45	0.40	0.31
S2	0.52	0.45	0.46	0.40

Similarly, table 2 shows the effect of different concentrations of all the three organic acids, lactic acid, acetic acid and citric acid at 0.1%, 0.2%, 0.3% and 0.4% on the bacterial strains. It has been observed that lactic acid at 0.2%, acetic acid at 0.2% and citric acid at 0.3% inhibited the bacterial growth and higher concentrations inhibited the growth further. Thus, the lowest concentrations which inhibited microbial growth, each of copper (50 mg/ml), lactic acid (0.2%), acetic acid (0.2%) and citric acid (0.3%) were taken into consideration for further experiments in the study to determine their synergistic effect in the inhibition of growth of *E. coli* and *Salmonella* sp. isolated from fresh fruits and vegetables.

Table 2. Effect of the three organic acids on growth of *E. coli* and *Salmonella* sp.

			Isolates				
			E. coli			*Salmonella* sp.	
			E1	E2	E3	S1	S2
Organic acids (O.D at 600 nm)	Lactic acid (mg/ml)	0.1	0.49	0.58	0.55	0.48	0.52
		0.2	0.40	0.52	0.49	0.43	0.49
		0.3	0.40	0.49	0.45	0.38	0.46
		0.4	0.39	0.38	0.30	0.38	0.40
	Acetic acid (mg/ml)	0.1	0.49	0.58	0.55	0.48	0.52
		0.2	0.46	0.56	0.53	0.47	0.51
		0.3	0.43	0.43	0.49	0.43	0.49
		0.4	0.39	0.39	0.45	0.40	0.38
	Citric acid (mg/ml)	0.1	0.49	0.58	0.55	0.48	0.52
		0.2	0.49	0.58	0.54	0.48	0.51
		0.3	0.45	0.52	0.49	0.42	0.45
		0.4	0.40	0.47	0.46	0.38	0.42

For determining the effect of copper with different organic acids, we examined the growth of individual strains in BHI broth in the presence of copper and each of the three organic acids separately, followed by in combination. The bacterial growth was monitored for a period of 12 h with readings taken at 0, 6 and 12 h. Both *E. coli* and *Salmonella* sp. showed only marginal reduction in growth in the presence of copper (50 mg/ml). Similar pattern was observed in the case of samples treated with lactic acid, citric acid and acetic acid individually. Lactic acid showed more inhibition than acetic and citric acids. In the case of combinations, copper with lactic acid showed significant inhibition in growth when compared to copper with acetic acid or citric acid. Combination of copper with lactic acid showed almost 40% reductions in growth of *E. coli* while that of copper with acetic acid and citric acid showed only 26% and 4% respectively (Fig 1). In the case of *Salmonella*, it was found that copper with lactic acid showed 36% reduction while acetic acid showed only 2% and citric acid did not affect the growth at all (Fig 2).

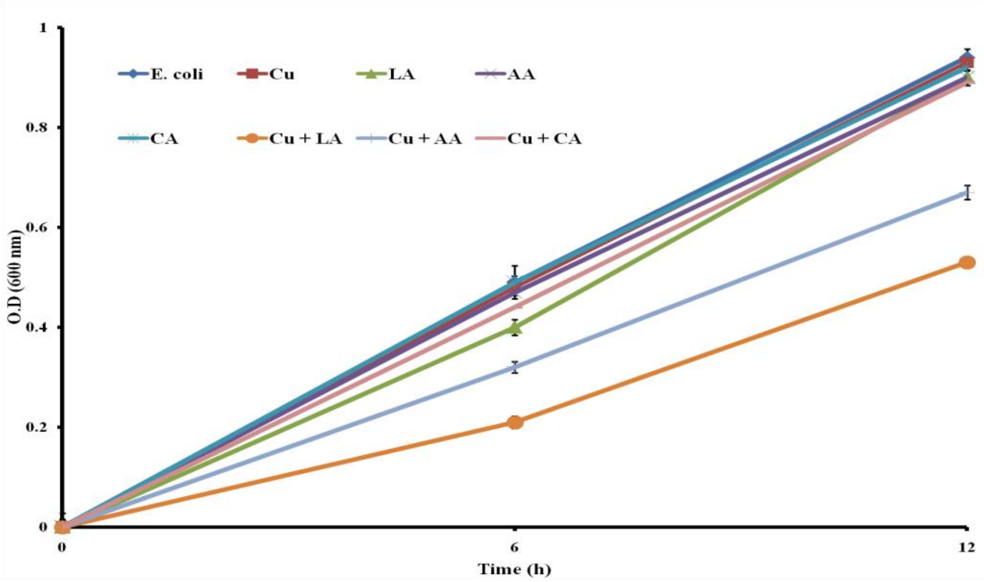

Fig 1. Effect of various treatments on *E. coli*. Cu- Copper, LA- Lactic acid, AA- Acetic acid, CA- Citric acid, Cu + LA- Copper(50 mg/ml) in combination with 0.2% lactic acid, Cu + AA- Copper(50 mg/ml) in combination with 0.2% acetic acid, Cu + CA- Copper(50 mg/ml) in combination with 0.3%citric acid.

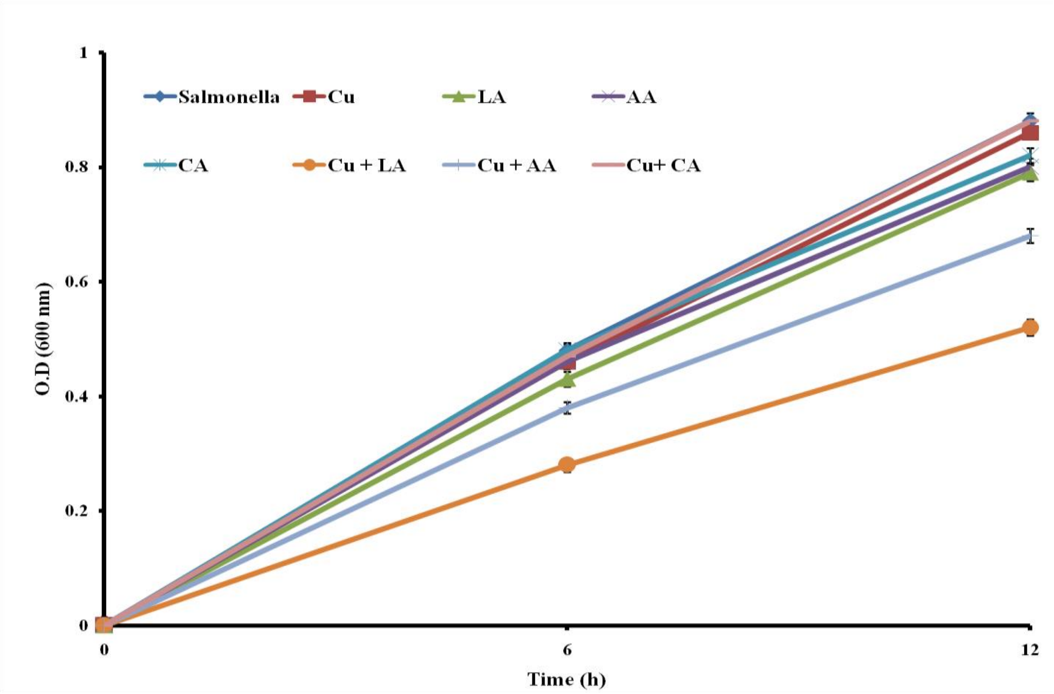

Fig 2. Effect of various treatments on *Salmonella sp*. Cu- Copper, LA- Lactic acid, AA- Acetic acid, CA-Citric acid, Cu + LA- Copper (50 mg/ml) in combination with 0.2% lactic acid, Cu + AA- Copper (mg/ml) in combination with 0.2% acetic acid, Cu + CA- Copper (50 mg/ml) in combination with 0.3%citric acid

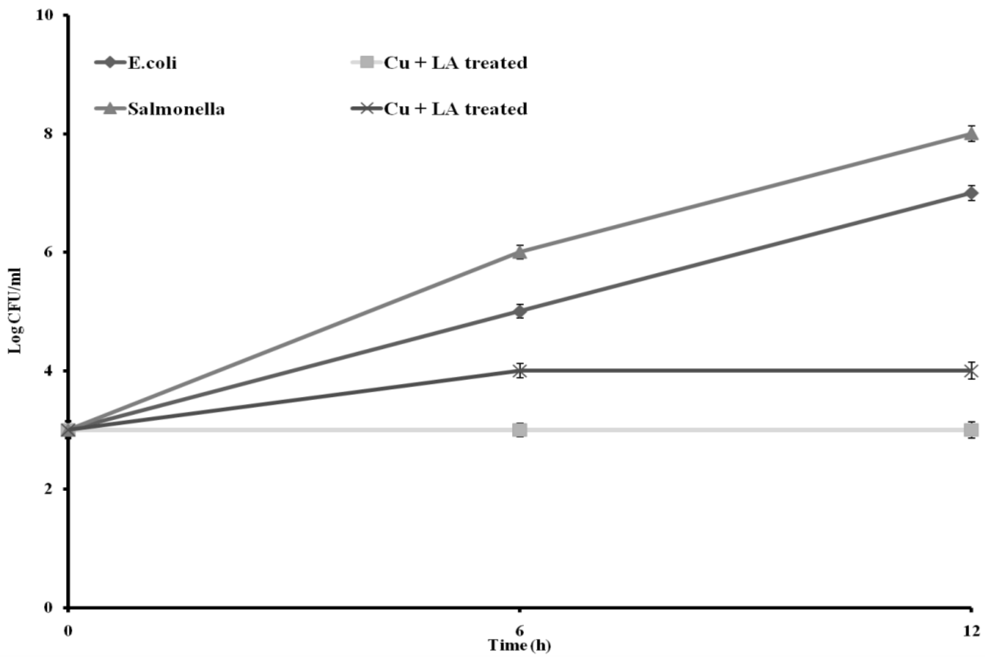

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Fig 3. Effect of Cu + LA treatment on *E. coli* and *Salmonella* sp. in Carrot. Cu+ LA- Copper (50 mg/ml) + 0.2% Lactic acid

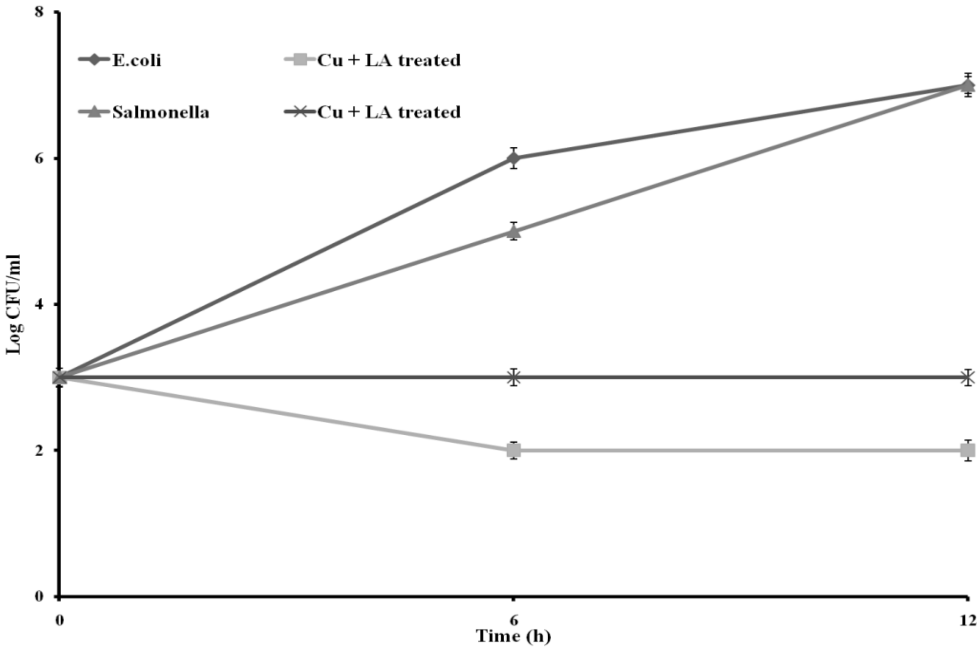

Fig 4. Effect of Cu + LA treatment on *E. coli* and *Salmonella* sp. in Apple. Cu+ LA- Copper (50 mg/ml) + 0.2% Lactic acid

Based on the above results, it was observed that 50 mg/ml Cu in combination with 0.2% LA was the most effective treatment in the reduction of microbial growth. In the case of *E. coli* on carrot, it was found that the initial microbial count of 10^3 increased to 1.2 x 10^5 in 6 h and 2.6 x 10^7 in 12 h in the control sample. After the combination treatment of 50 mg/ml copper and 0.2% lactic acid, the count reduced to 2.1 x 10^3 in 6 h and 3.2 x 10^3 in 12 h. This infers that the treatment significantly inhibited the microbial growth. Similar results were also observed in the case of *Salmonella* sp. (Fig 3). The effect of combination treatment on apple also yielded similar results. *E. coli* growth was found to be srongly inhibited when compared to *Salmonella* sp. as the microbial count decreased significantly from 2.3 x 10^6 in control sample to 1.4 x 10^2 in the treated sample (Fig 4).

DISCUSSION

Copper has been a well known natural antimicrobial agent. Copper at higher concentration is toxic to microorganisms as it can mediate cell membrane damage, interact with nucleic acids and mediate protein damage [14; 15]. Present study investigated the role of copper along with three different organic acids, namely, lactic acid, acetic acid and citric acid in reducing the microbial load. Copper and the three organic acids individually exhibited antimicrobial effect against *E. coli* and *Salmonella* sp. isolated from fresh fruits and vegetables. The essential mechanism of antimicrobial activity in copper is because of its changing oxidation states, generating highly reactive hydroxyl radicals damaging the biomolecules and affecting the cell membrane integrity [16]. The minimum inhibitory concentration (MIC) of copper was found to be 50 mg/ml while higher concentrations led to higher inhibition. Reyes-Jara [17] reported the antimicrobial activity of copper on microorganisms isolated from raw milk, where 250 mg/ml was the MIC for *E. coli.* But higher antimicrobial activity was observed when copper was used in combination with the organic acids. Organic acids are generally effective at low concentrations. Marriot and Gravani [18] reported that broad spectrums of bacteria are rapidly killed by organic acids within in a wide range of temperature. In the present study, lactic acid was found to be the most effective organic acid against *E. coli* and *Salmonella* sp. when compared to acetic acid and citric acid. Similar reports have been documented by Doyle et al. [19] who showed that lactic acid treatment provided the greatest biocidal effect against *E. coli* O157:H7. Earlier studies have suggested that a combination of two organic acids or organic acid with other natural antimicrobial compounds could have a stronger inhibitory effect compared to the effect of single acid [20]. Copper combined with each organic acid produced significant inhibition in microbial growth. The most effective combination in retarding the microbial growth was found to be 50 mg/ml copper with 0.2% lactic acid. The combined treatment yielded 4 log and 5 log reductions in the case of *Salmonella* sp. and *E. coli* respectively. Similar results have been reported by Ibrahim et al. [21] where the growth of *Salmonella* sp. and *E. coli* O157:H7 were significantly inhibited when both lactic acid and copper were added to laboratory medium and carrot juice.

CONCLUSIONS

The results of the study clearly demonstrated that copper and organic acids at low concentrations showed inhibitory activity against *E. coli* and *Salmonella* sp. from fresh fruits and vegetables. Lactic acid, acetic acid and citric acid at concentrations of 0.2, 0.2 and 0.3% respectively inhibited microbial growth while copper showed inhibition at a concentration of 50 mg/ml. It has been proved that a combination of 0.2% lactic acid and 50 mg/ml copper exhibited pronounced inhibition of microbial growth. One of the possible reasons for the synergistic effect of copper and lactic acid may be that the permeabilizing nature of lactic acid enables the entry of Cu ions into the bacterial cell there by affecting microbial metabolism and inhibiting their growth. Harmful chemical sanitizers used in cleaning of fresh fruits and vegetables pose a threat to their safety. Thus, application of copper in combination with organic acids on fresh fruits and vegetables can be commercialized in food sectors, which can help reduce the microbial load and thereby helping in the prevention of foodborne disease outbreaks.

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REFERENCES

[1] Olaimat AN, Holley RA. Factors influencing the microbial safety of fresh produce: a review. Food Microbiology 2012; 32 (1): 1–19.
[2] Tauxe R, Kruse H, Hedberg C, Potter M, Madden J, Wachsmuth K. Microbial hazards and emerging issues associated with produce: a preliminary report to the National Advisory Committee on Microbiological Criteria for Foods. Journal of Food Protection 1997; 60(11):1400-8.
[3] Liao, CH, Fett WF. Analysis of native microflora and selection of strains antagonistic to human pathogens on fresh produce. Journal of Food Protection 2001; 64: 1110–1115.
[4] Buck JW, Walcott RR, Beuchat LR. Recent trends in microbiological safety of fruits and vegetables. Plant Health Progress 2003; http://dx.doi.org/10.1094/PHP-2003-0121-01-RV (online) Availableat: http://www.plantmanagementnetwork.org/pub/php/review/2003/safety/.
[5] Food and Drug Administration (FDA). Guidance for industry, guide to minimize microbial food safety hazards for fresh fruit and vegetables. 1998. Available at: http://www.fda.gov/downloads/Food/GuidanceComplianceRegulatoryInformation/GuidanceDocuments/ProduceandPlanProducts/UCM169112.pdf (accessed on 09/08/2016).
[6] Warriner K, Huber A, Namvar A, Fan W, Dunfield K. Recent advances in the microbial safety of fresh fruits and vegetables. Advances in Food and Nutrition Research 2009; 27: 155–208.
[7] Dewaal CS, Bhuiya F. Outbreaks by the numbers: fruits and vegetables 1990–2005. Washington DC, Center for Science in the Public Interest. 2009. Available from: http: //www.cspinet.org/foodsafety/IAFPPoster.pdf (accessed on 29/09/2014).
[8] Lynch MF, Tauxe RV, Hedberg CW. The growing burden of foodborne outbreaks due to contaminated fresh produce: risks and opportunities. Epidemiology and Infection 2009; 137 (3): 307–315.
[9] Faundez G, Troncoso M, Navarrete P, Figueroa G. Antimicrobial activity of copper surfaces against suspensions of *Salmonella enterica* and *Campylobacter jejuni.* BMC Microbiology 2004; 4: 19–25.
[10] Delgado K, Quijada R, Palma R, Palza H. Polypropylene with embedded copper metal or copper oxide nanoparticles as a novel plastic antimicrobial agent. Letters in Applied Microbiology 2011; 53: 50–54.
[11] Entani E, Asai M, Tsujihata S, Tsukamoto Y, Ohta M. Antibacterial action of vinegar against food-borne pathogenic bacteria including *Escherichia coli* O157:H7. Journal of Food Protection 1998; 61: 953-959.
[12] Alvarado-Casillas S, Ibarra-Sanchez S, Rodriguez-Garcia O, Martinez-Gonzales N, Castillo A. Comparison of rinsing and sanitizing procedures for reducing bacterial pathogens on fresh cantaloupes and bell peppers. Journal of Food Protection 2007; 70: 655-660.

[13] Beal J, Niven S, Campbell A, Brooks P. The effect of copper on the death rate of *Salmonella typhimurium* DT104: 30 in food substrates acidified with organic acids. Letters in Applied Microbiology. 2004; 38: 8-12.
[14] Brul S, Coote P. Preservative agents in foods modes of action and microbial resistance mechanisms. International Journal of Food Microbiology 1999; 50: 1–17.
[15] Borkow G, Gabbay J. Cooper as biocidal tool. Current Medicinal Chemistry 2005; 12: 2163–2175.
[16] Santo C, Taudte N, Nies D, Grass G. Contribution of copper ion resistance to survival of *Escherichia coli* on metallic copper surfaces. Applied and Environmental Microbiology 2008; 74: 977.
[17] Reyes-Jara A, Cordero N, Aguirre J, Troncoso M, Figueroa G. Antibacterial Effect of Copper on Microorganisms Isolated from Bovine Mastitis. Frontiers in Microbiology 2016; 7: 626. doi: 10.3389/fmicb.2016.00626.
[18] Marriott NG, Gravani RB. 2006. Cleaning compounds. In; 5th Ed. Principles of food sanitation, Gaithersburg, 2006; 149–151.
[19] Doyle MP, VenkitanarayananKS, Zhao T. Inactivation of *Escherichia coli* O157:H7 by combinations of GRAS chemicals and temperature. Food Microbiology 1999; 16: 75-82.
[20] Hayek SA, Gyawali R, Ibrahim SA. Antimicrobial Natural Products. In; A. Méndez-Vilas, Ed. Microbial pathogens and strategies for combating them: science, technology and education, Badajoz, Spain; Formatex Research Center, 2013; 910-921.
[21] Ibrahim SA, Yang H, Chung WS. Antimicrobial activity of lactic acid and copper on growth of *Salmonella* and *Escherichia coli* O157: H7 in laboratory medium and carrot juice. Food Chemistry, 2008; 109: 137-143.