2010

Antimicrobial Activity Of Copper Alone Or In Combination With Lactic Acid On The Growth Of Escherichia Coli O157:H7 In Laboratory Medium And On The Surface Of Lettuce And Tomatoes

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ANTIMICROBIAL ACTIVITY OF COPPER ALONE OR IN COMBINATION WITH LACTIC ACID ON THE GROWTH OF *ESCHERICHIA COLI* O157:H7 IN LABORATORY MEDIUM AND ON THE SURFACE OF LETTUCE AND TOMATOES

by

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A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Department: Family and Consumer Sciences
Major: Food and Nutritional Sciences
Major Professor: Dr. Salam A. Ibrahim

North Carolina A&T State University
Greensboro, North Carolina
2010

School of Graduate Studies
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North Carolina Agricultural and Technical State University

Greensboro, North Carolina
2010

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BIOGRAPHICAL SKETCH

Rabin Gyawali was born in Himalayan country Nepal in south Asia. He received the Bachelor of Science in Zoology from Tribhuvan University, Nepal and came to the U.S. to pursue a higher education. He enrolled at North Carolina A&T State University in the Food and Nutritional Sciences program. During his study, his research was focused on food safety under the supervision of Dr. Salam A. Ibrahim. Mr. Gyawali has also served as a Research Assistant in the Family and Consumer Sciences Department. He received the Wadaran L. Kennedy Scholar award which honors academic excellence (2009). He was inducted into Gamma Sigma Delta honor society of Agriculture in 2009. His research findings were presented at various national meetings including American Chemical Society, Institute of Food Technologists, National Institute for Farm Safety Conference, and Ronald E. McNair Research Symposium on the campus of NCA&T State University. He was in a team that participated in the Collegiate Dairy Products Evaluation Contest held in Chicago, 2009. After graduating, he plans to pursue a doctoral degree in food science. Though, he finds most aspects of food science fascinating, he is particularly interested in the study of foodborne pathogens and controlling survival and growth of microorganisms in foods.

ACKNOWLEDGMENTS

First and foremost, I would like to thank Dr. Salam A. Ibrahim, my advisor and major professor, for providing guidance, support, and motivation during my study. This thesis would not have been possible without his encouragement. His faith and encouragement helped me tremendously to advance my degree despite being from a different major.

Sincere appreciation goes to my committee members, Dr. Ipek Goktepe and Dr. Patricia A. Lynch for the time and energy they committed to my project. I would also like to thank Dr. Giddings for her support. I would specially like to thank Dr. Tammy Song for assisting me in statistical analysis and reviewing my report and Dr. Tajkarimi for his valuable input.

I would like to thank all the faculty members in the Food and Nutritional Sciences program for their guidance during my degree. Last but not least, great thanks also go to my friends, Biyi, Isam, Khalda, Sulaiman, Bashier and Madhavi for their help and support. You are all truly wonderful and I am very happy to call you my friends.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURES **PAGE**

LIST OF TABLES

TABLES **PAGE**

NOMENCLATURE

E. coli	*Escherichia coli*
ºC	Celsius
O.D	Optical density
CFU	Colony Forming Units
CFU/mL	Colony Forming Units per Milliliters
GI	Gastrointestinal track
HUS	Hemolytic-uremic Syndrome
hr	Hours
nm	Nanometers
ppm	Parts per million
µL	Microliters
BHI	Brain Heart Infusion
LA	Lactic Acid
Cu	Copper
GRAS	Generally Recognized As Safe
USDA	United States Department of Agriculture
CDC	Center for Disease Control
FDA	Food and Drug Administration
FD&C	Federal Food, Drug and Cosmetic
TSB	Tryptone Soy Broth
Spp	Species

ABSTRACT

Gyawali, Rabin. ANTIMICROBIAL ACTIVITY OF COPPER ALONE OR IN COMBINATION WITH LACTIC ACID ON THE GROWTH OF *ESCHERICHIA COLI* O157:H7 IN LABORATORY MEDIUM AND ON THE SURFACE OF LETTUCE AND TOMATOES. (**Major Advisor: Dr. Salam A. Ibrahim**), North Carolina Agricultural and Technical State University.

In recent years, *Escherichia coli* O157:H7 has been a major foodborne pathogen associated with fresh produce. Many outbreaks of foodborne illnesses have raised concerns in the microbial safety of fresh produce. Several different treatment solutions are being used to decontaminate fresh produce, but still it is very difficult to guarantee the microbiological safety. Therefore, the development of effective treatment for reducing *E. coli* O157:H7 on fresh produce is needed. The objectives of this study were to investigate the effect of copper alone or in combination with lactic acid on the survival and growth of *E. coli* O157:H7 in laboratory medium and to evaluate the potential of copper and lactic acid solution to reduce the bacterial population of *E. coli* O157:H7 on the surface of lettuce and tomatoes. Four strains of *E. coli* O157:H7 were individually inoculated into BHI broth containing different concentrations of copper (5, 10, 20 and 40 ppm), lactic acid (0.1, 0.2, and 0.25%), and their combinations. Samples were then incubated at 37 ºC for 8 hours. Bacterial growth was monitored at 0, 4 and 8 hours by measuring turbidity levels. At the end of the incubation, samples were plated on BHI agar to determine the *E. coli* population. Results showed that lactic acid at 0.2% or higher retarded the growth of bacterial strains. The growth inhibition was negligible when copper was used alone.

However, significant growth inhibition ($p < 0.05$) was observed when 0.2% lactic acid was used in combination treatments of copper at 20 ppm and 40 ppm.

The pH value of individual or combined samples was determined. Results showed that individual or combined treatments had little effect on pH (5.48-5.74) values ($p < 0.05$) indicating that acid is not the main inhibiting factor in this study. A four-strain mixture of *E. coli* O157:H7 was inoculated separately on lettuce and tomatoes, which were treated with copper and lactic acid solution. Log reductions of 3.39 and 3.93 were obtained for tomato and lettuce treated with combination of copper 40 ppm and 0.2% lactic acid respectively. The results showed higher log reduction when surfaces were rinsed with treatment solution containing 0.1% Tween 80. Hence, the treatment with copper and lactic acid was effective in reducing substantial populations of foodborne pathogens. This indicates its potential application to inhibit *E. coli* O157:H7 and could be used as natural ingredients for the decontamination of fresh produce surfaces.

CHAPTER 1

INTRODUCTION

Escherichia coli O157:H7 (*E. coli* O157:H7) has been considered to be an important pathogen as a major cause of hemorrhagic colitis and the hemolytic-uremic syndrome (HUS) in the United States. In recent years there have been widespread outbreaks of *E. coli* O157:H7 illnesses linking it to fresh produce. As produce consumption has increased in the United States, so have the number of produce-related outbreaks of *E. coli* O157:H7. Fresh produce surfaces are not free from natural contaminants during growing and harvesting, and the safety of fresh produce has always been a concern as it represents the second leading cause of foodborne illnesses in the U.S. (López-Gálvez, Allende, Selma, & Gil, 2009).

Different chemical agents (chlorine, hydrogen peroxide, organic acids, ozone etc.) have been used to decontaminate fresh produce surfaces (Koseki & Itoh, 2001; Park, Hung, Doyle, Ezeike, & Kim, 2001). The sanitization of fresh produce plays an important role in the improvement of food quality and microbial safety. However, the efficacy of some of those treatments in inactivating microorganisms on the surfaces of fresh produce is minimal. Most of the recent studies have been focused on the search of alternative sanitizers and has resulted in increased numbers of studies that analyze the efficiency of different methods for reducing the pathogens on fresh produce (Parish et al., 2003). There is also a growing consumer demand for natural methods of controlling microorganisms associated with food. The food industry is, therefore, looking for natural food

preservatives such as, copper and lactic acid with strong antimicrobial activity that can be used as a washing treatment to assure microbial safety of food products.

The most common preservative agents are organic acids especially lactic acids, which have "generally recognized as safe" status (Brul & Coote, 1999). The use of lactic acid has been successful in decontaminating beef, lamb, pork, and poultry carcasses. Application of organic acid washes or sanitizers to the surface of vegetables for the purpose of reducing populations of viable microorganisms also has potential (Alzamora, Tapia, & Lopez-Malo, 2000). Copper is an active ingredient in many different types of antimicrobial products, in agriculture, in marine environments, in healthcare systems, and in the home. Copper is an essential nutrient for humans as well as bacteria, but in high doses, copper ions can cause a series of negative effects in bacterial cells (Nies, 1999). Even though there is limited information on its ability to inactivate foodborne pathogens, copper has great potential as an antimicrobial agent in food products.

Since both copper ion and lactic acid possess some antibacterial properties and are natural compounds, their synergistic effect could be significant in the fight against foodborne pathogens, such as *E. coli* O157:H7. Therefore, the objectives of this study were: (a) to investigate the effect of copper alone or in combination with lactic acid on the growth of *E. coli* O157:H7 in laboratory medium, and (b) to evaluate the potential of copper and lactic acid as natural sanitizers to reduce the bacterial population of *E. coli* O157:H7 on the surface of lettuce and tomatoes.

CHAPTER 2

LITERATURE REVIEW

2.1 Foodborne Pathogen *Escherrichia coli* O157:H7

Escherichia coli (*E. coli*) are facultatively anaerobic, gram-negative rods within the family Enterobacteriacea and a common microorganism that live in the intestine of healthy animals and humans. Most of them are harmless. However, *E. coli* O157:H7 was one type which was first recognized as a pathogen in 1982 during an outbreak investigation of hemorrhagic colitis (Riley et al., 1983). *E. coli* O157:H7 produces a powerful toxin that can cause diarrhea (Kaper, Nataro, & Mobley, 2004), abdominal cramps, fever or even death. Therefore *E. coli* O157:H7 serotype is among the most dangerous when people are exposed. There is no harm to cattle because it does not bind to the walls of their gastrointestinal (GI) track but in human, *E. coli* O157:H7 binds to the cells that line our GI track, leading to vascular damage resulting in hemorrhagic colitis and the hemolytic uremic syndrome (Pruimboom-Brees et al., 2000). People can become infected with *E. coli* O157:H7 through contaminated food and water. Preventive measures during food processing are very important to control the spread of this pathogen. Mainly, vegetables and fruits may become contaminated with *E. coli* O157:H7 from soil, water, or from individuals who have handled the produce.

The Center for Disease Control (CDC) estimates that there are 76 million cases of foodborne illness in the United States annually, with 14 million cases attributed to known pathogens (Doyle, 2000). *E. coli* O157:H7 alone is estimated to account for 76,000 cases

of foodborne illness and 76 deaths annually. Fresh salad and vegetables are generally considered safe to eat by consumers. However, the CDC found that vegetables contributed to 5% of the foodborne diseases caused by *E. coli* bacteria.

Outbreaks of foodborne illness caused by *E. coli* O157:H7 and other bacterial foodborne pathogens in produce have raised concerns about the safety of fresh fruits and vegetables that are consumed raw or have been only minimally processed to reduce or eliminate pathogens. It was only after 1993 that *E. coli* O157:H7 became broadly recognizable as an important and threatening pathogen after a large multistate outbreak linked to undercooked ground beef patties sold from a fast-food restaurant chain (Bell et al., 1994). Food remained the predominant transmission path from 1982-2002 for several foodborne outbreaks. Based on the report submitted to the CDC (Rangel, Sparling, Crowe, Griffin, & Swerdlow, 2005), 49 states reported 350 outbreaks, representing 8,598 cases, 17% hospitalizations, 4% hemolytic uremic syndrome cases, and 0.5% deaths. The transmission route for 52% was foodborne, 21% unknown, 14% person to person, 9% waterborne, 3% animal contact, and 0.3% was laboratory related. The food vehicle for 41% of the foodborne outbreaks was ground beef and 21% was produce. Produce-associated outbreaks were first reported in 1991 and have remained a prominent food vehicle. Thirty four percent of the total produce outbreaks were associated with lettuce, 18% with apple cider, 16% with salad, 11% with coleslaw, 11% with melons, 8% with sprouts, and 3% of the outbreaks were associated with grapes. Produce associated outbreaks most commonly occurred in restaurants and were reported to be due to cross-contamination during food preparation (Rangel et al., 2005).

Contamination of fruits and vegetables can occur when ruminant animals enter fields or when improperly composted cow manure has been applied as fertilizer in the fields. In recent years, *E. coli* O157:H7 has been isolated with increasing frequency from fresh produce, including bean sprouts, cantaloupes, apples, and leaf lettuce (Ackers et al., 1998; Hillborn et al., 1999). The plants became contaminated when grown in fields fertilized with improperly treated manure (Beuchat, 1999). A second route by which *E. coli* O157:H7 may be introduced is flood irrigation with water contaminated with cattle feces or contact with contaminated surface runoff (Ackers et al., 1998; Hillborn et al., 1999).

In the last few years there have been many documented cases of foodborne illnesses associated with fresh fruits and vegetables (Olsen, MacKinnon, Goulding, Bean & Slutsker, 2000). There have been a large number of microbiological contaminations involving whole fresh produce (Mukherjee, Speh, Dyck, & Diez-Gonzalez, 2004; FDA, 2001, 2003). Olsen et al. (2000) reported that *Salmonella* and *E. coli* O157:H7 are the leading causes of produce related outbreaks in the USA. Outbreaks have been linked to lettuce (Ackers et al., 1996) and apple cider (Besser et al., 1993; CDC, 1996; Steele, Murphy, Arbus, & Rance, 1982). Nathan (1997) reported an outbreak in radish sprouts, and alfalfa sprouts. Enterotoxigenic *E. coli* has also been linked to carrots (Burnett & Beuchat, 2001). Enterohemmorrhagic *E. coli* can grow on several types of produce and fruits, for example, shredded lettuce (Diaz & Hotchkiss, 1996), salad vegetables (Abdul-Raouf, Beuchat, & Ammar, 1993) and apple cider (Hilborn et al., 2000) and can cause several illnesses. In 2006, *E. coli* O157:H7 outbreak in spinach affected 26 U.S. states

with 3 deaths and 200 cases of reported illnesses including hemolytic uremic syndrome (FDA, 2006). Among the different types of produce, lettuce is the single most consumed commodity, and there are several examples of outbreaks and recalls of *E. coli* O157:H7 linked to different varieties of lettuce believed to be contaminated at the production or processing level from 1995-2008. Varieties that have been contaminated include Romaine lettuce (Ackers et al., 1998), Mesclun lettuce (Hilborn et al., 1999), and Lettuce unspecified (Welinder-Olsson et al., 2004). Similarly, fresh tomatoes have been associated with major outbreaks of salmonellosis; multistate outbreaks occurred in 1990, 1993, 1998, 2002, and 2004 were responsible for more than 1300 cases (CDC, 2002; 2005; Dewaal, Barlow, & Hicks, 2002; FDA, 2003, 2004; Srikantiah et al., 2005). Outbreaks of *E. coli, Listeria monocytogenes, Salmonella*, and *Bacillus cereus* have been linked to the consumption of contaminated raw salad vegetables (Zhuang, Beuchat, & Angulo, 1995; Beuchat, 1996), raw tomatoes, and raw vegetable seed sprouts (Beuchat, 1996) respectively.

Fresh fruits and vegetables are an essential part of the human diet, and in recent years, the consumption of fresh produce and vegetables has increased. The number of foodborne illness outbreaks linked to the presence of pathogenic microorganisms such as *E. coli* O157:H7 on fresh produce has increased over the past years. The concern about these pathogens has also increased the consumer's desire for minimally processed fruits and vegetables.

2.2 Applications of Organic Acids

Organic acids (lactic, acetic, malic, citric, caprylic, propionic, succinic) in many foods are being utilized as food additives and preservatives. Different organic acids have been used as antimicrobials to control foodborne pathogens. Major applications which are being used to inhibit microorganisms include: (a) organic acids as antimicrobials in acidified foods, (b) organic acids produced during food fermentations, (c) food preservatives in high pH foods, and (d) organic acids in meat decontamination (Roller, 2000).

Organic acids are used in different food systems. Acetic acid is the main component of vinegars and is used for flavoring in mustard, salad dressings, and mayonnaise and in sausages. Acetic acid is generally recognized as safe (GRAS) and has been used as a disinfectant. Caprylic acid is used as a flavoring agent for baked goods, cheese, fats and oils, frozen dairy desserts, gelatins and puddings, meat products, candies and snack foods. Citric acid is used in ice cream, beverages, salad dressings, fruit preservatives, jams and jellies, and also used as acidulant in dairy products and canned vegetables. Lactic acid, a weak organic acid, is used in the food industry to make jams, jellies, candies and beverages. It is also applied in olives and pickles to adjust acidity. Malic acid is also used in flavoring and acidification characteristics. Propionic acid is used to inhibit mold in breads. It is found in Swiss cheese up to 1% due to the growth and metabolism of propionibacteria which gives the characteristic flavor. Similarly, succinic acid is used to alter the plasticity of bread doughs and in the production of edible fats. It also serves as a flavor enhancer due to its slight bitter test (Roller, 2000).

Organic acids have been used for decades in feed preservation for broiler production because of their ability to reduce pH in the intestinal tract interrupting the growth of harmful bacteria and altering the intestinal flora (Al-Kassi & Mohssen, 2009). Acids such as sorbic and fumaric acids have some antifungal activity. Use of organic acids to control *Listeria monocytogenes* in meat have been well documented in previous studies (Doyle, 1999). Since most pathogens generally do not grow at a pH lower than 4.5, acidification might help to prevent proliferation of microbes. Therefore, organic acids are commonly used as antimicrobial acidulants to preserve food either by direct addition or through microbiological fermentation (Foegeding & Busta, 1991). Organic acids have a long history of use in the food industry as food preservatives and have been shown to be effective under a wide variety of food processing conditions. Organic acids have several advantages and uses in different stages of food production. However, some microorganisms are acid resistant and are responsible for foodborne pathogens. Therefore, studying pathogen behavior in food production environments and the use of organic acids will provide a greater potential to develop effective control measures of such microorganisms.

2.3 Lactic Acid as an Antimicrobial Agent

Lactic acid (2-hydroxypropanoic acid) is a carboxylic acid and has a chemical formula of $C_3H_6O_3$. It is frequently found in various life forms including animals, plants, and microorganisms as a weak organic acid with pKa of 3.86 at 25 °C (Bogaert & Naidu, 2000). Lactic acid is an effective antimicrobial agent and is classified by the U.S. Food

and Drug Administration (FDA) as a Generally Recognized as Safe (GRAS) food additive (Sapers, 2005). The treatment solution pH and the degree of organic acid dissociation determine the antimicrobial effectiveness (Smulders, 1995; Tamblyn & Conner, 1997). In solution, weak acid preservatives exist in a pH dependent equilibrium between the undissociated and dissociated state. Weak acid like lactic acid has an optimal inhibitory activity at low pH because this favors the uncharged, undissociated state of the molecule, which is freely permeable across the plasma membrane and able to enter the cell. Hence, inhibitory action is mainly due to the compound crossing the plasma membrane in the undissociated state. After encountering the higher pH inside the cell, the molecule will dissociate releasing charged anions and protons, which cannot cross the plasma membrane. In a high acid environment (low pH), lactic acid remains undissociated and is in its most active antimicrobial form. So the preservative molecule like lactic acid diffuses into the cell until equilibrium is reached according to the pH gradient across the membrane and results in the accumulation of anions and protons inside the cell. In conclusion, inhibition of growth by weak acid preservative such as lactic acid is due to a number of actions including membrane disruption, inhibition of metabolic reactions, stress on intracellular pH homeostasis and, the accumulation of toxic anions (Brul & Coote, 1999).

Lactic acid solutions are one of the most widely applied organic acid treatments to decontaminate foods (Anang, Russel, Bakar, & Ling, 2007). Lactic acid is a multipurpose GRAS chemical that has an antimicrobial effect on a variety of bacteria pathogens (Anderson & Marshall, 1990, Greer & Dilts, 1992). Several researchers have determined

that *E. coli* O157:H7 has unusual tolerance to acidic conditions and found that *E. coli* has resistant mechanisms against inorganic or organic acids by the modifications of sigma factors or membrane fatty acid composition (Jo & Rim, 2007). Therefore, organic acids have been tested with other antimicrobial agents to overcome bacterial acid resistance (Olasupo, Fitzgerald, Narbad, & Gasson, 2004). Doyle, Venkitanarayanan, and Zhao (1999) reported that the antimicrobial activity of organic acids can be increased or potentiated when combined with other food preservatives.

The use of washes and sprays containing organic acids, particularly lactic acid has been successful in decontaminating beef, lamb, pork, and poultry carcasses to reduce populations of viable microorganisms. They also have a great potential to serve as a practical means of decontamination as a sanitizing agent. Organic acids are the most commonly used chemical decontaminant for foods (Belk, 2001). Lactic acid is a biocidal to a wide range of microorganisms. However, *E. coli* O157:H7 is unusually tolerant to its antimicrobial properties and has survived up to 56 days in Tryptone Soy Broth (TSB) acidified to pH 4.7 (Conner & Kotrola, 1995). Similarly, Abdul-Raouf et al. (1993) determined that *E. coli* O157:H7 survived well in beef slurries with lactic acid. In a study by Doyle et al. (1999), lactic acid (1.5%), hydrogen peroxide (0.1%), sodium benzoate (0.1%), and glycerol monolaurate (0.005%) as alone treatment on *E. coli* O157:H7 in 0.1% peptone water indicated that none of these chemicals could reduce bacterial population by 5.0 log CFU/ml. However, among four of the treatments, lactic acid had the greatest biocidal effect in reducing *E. coli* O157:H7. Studies have reported the decontamination effects of weak organic acid treatments on microorganisms in meats,

especially in poultry and beef processing. According to Castillo et al. (2001), the treatment of 2% lactic acid reduced levels of *Salmonella typhimurium* and *E. coli* O157:H7 on inoculated carcass surfaces. Castillo et al. (2001) also reported spraying 4% lactic acid on chilled carcasses in a commercial environment reduced about 3 log in aerobic plate counts. Lactic acid dips and spray washes of prechilled birds have also been used in the poultry industry and the bacterial reduction was from 5.2 to 3.7 log CFU/g when immersed for 15 seconds in 1 or 2% LA at pH 2.2 (Van der Marel, Van Logtestijn, & Mossel, 1988). Lactic acid can also be used as a surface treatment of ready to eat (RTE) meats for the control of *L. monocytogenes* (Samelis et al., 2002; Barmpalia et al., 2004; Geornaras et al., 2006).

Lactic acid as a weak organic acid is successfully used as a sanitizer on food animal carcasses and may have the potential to reduce microorganisms on produce surfaces. A solution containing 1.5% lactic acid and 1.5% hydrogen peroxide as a 15 minute soak at 40 °C was reported to give greater than 5 log reductions in the population of *E. coli* O157:H7, *Salmonella enteritidis*, and *L. monocytogenes* on spot inoculated apples, oranges, and tomatoes (Sapers, 2005). The populations of *S. typhimurium* and *E. coli* O157:H7 were not detected inside tomatoes, which had been sprayed with purac lactic acid (250 ml of 2% lactic acid) on four tomatoes at 5 °C. It was believed that the lack of detection of pathogen populations were mainly due to the fast bacterial reduction on the surface, hindering active pathogens from diffusing into the tomatoes (Ibarra-Sanchez, Alvarado-Casillas, Rodriguez-Garcia, Martinez-Gonzales, & Castillo, 2004). Similarly, there was a significantly lower number of *S. typhimurium* and *E. coli* O157:H7

on fresh cantaloupes with 2% lactic acid spray (Alvarado-Casillas, Ibarra-Sanchez, Rodriguez-Garcia, Martinez-Gonzales, & Castillo, 2007). It was also documented that 0.5% of lactic acid eliminates *Yersinia enterocolitica* on shredded lettuce. The 5-minute dip wash treatment of 0.5% lactic acid showed a 2.5 log reduction of *Y. enterocolitica* (Escudero, Velazquez, Di Genaro, & de Guzmán, 1999).

Therefore, the use of lactic acid demonstrates a broad spectrum of antimicrobial activity against different kinds of foodborne pathogens including gram-negative pathogens such as *E. coli* O157:H7 and *Salmonella* spp. In the food industry, the antimicrobial activity of lactic acid is generally applied in decontamination of meat carcasses at slaughterhouses and for the shelf life enhancement of fresh or semi-processed foods, which includes fresh fruits and vegetables. Hence, the worldwide use of lactic acid and its products accounts to 100,000 metric tons annually with a continuous growth rate of 12 to 15% per annum (Bogaert & Naidu, 2000).

2.4 Applications of Copper

Copper is an essential trace mineral necessary for healthy plants, animals, and humans and has a strong biocidal property, which has been used to control harmful organisms such as bacteria and fungi. Today copper compounds are being used in different sectors mainly the antimicrobial properties of copper as fungicides, pesticides, antifouling paints, antimicrobial medicines, oral hygiene products, antiseptics and several other useful applications (Borkow & Gabbay, 2005).

2.4.1 Copper as a water purifier. It was found that water distributions systems made of copper surfaces have a greater potential for suppressing growth of harmful microorganisms. Cupric chloride inactivated 9 of the 13 bacteria strains by more than 5 logs within 30 minutes (Albright & Wilson, 1974). It was also reported that bacteria levels were reduced on copper surfaces compared with glass and other plumbing materials. According to the report of the efficacy of copper-silver ionization in eradiacting *Legionella* species published in 1998, more than 30 hospitals in the USA are using copper-silver ionization to control *Legionella* in their water distribution systems (Liu et al., 1994). In developing countries the public water distribution systems are poorly maintained and even treated water gets recontaminated due to unsafe storage. Therefore the use of copper pots to store drinking water is highly desirable in those populations (Borkow & Gabbay, 2005).

2.4.2 Copper in agricultural applications. Copper as an algaecide, fungicide, molluscicide and acaricide are extensively applied in agriculture (Borkow & Gabbay, 2005). Copper sulfate was used on farms as a disinfectant against storage rot and for the control and prevention of foot rot in sheep and cattle. Copper sulfate is also used to prevent the growth of alga, control green slime and other algae found in ponds, rice fields, irrigation and drainage canals, rivers, lakes and swimming pools. Copper sulfate has been found to be an important molluscicide and used to control snails. Copper is also used to treat seed grains to avoid different kinds of seed borne diseases. Today formulations of copper such as copper-8-quinolinate, copper octate, nanocopper oxide and alkaline copper quat and copper azole are used to control fungus in crops, textiles,

paints and woods (Lewis, 2005). Because of its antifungal properties, many plants diseases could be prevented with small amounts of copper.

2.4.3 Antifouling surfaces and paints. Microbiologists and cell culture scientists have depended on copper walled incubators to resist microbial growth, fungal growth and to resist contamination of sensitive of human and animal cell lines during cultured in humidified laboratory incubators. On the other hand, copper antifouling properties help to prevent undesirable organisms in fish farming. Antifouling copper based paints can reduce bacterial populations by 99.9975% within 24 hours (Lewis, 2005). The antifouling benefits of copper sheathing on the bottom of boats have been known for many years.

2.4.4 Consumer products. Products made of copper have been used in our daily households for a long time. In Japan, copper sink strainers are very common, and tabletops and water storage vessels made from copper are still in use in Middle Eastern countries (Lewis, 2005). Copper is also used for its bactericidal properties in medicine such as antiplaque agents in mouthwashes and toothpastes. Copper tongue scrapers are used in India and other south eastern countries to prevent bad breath resulting from oral bacteria and traditional copper pots are still in use for purification of drinking water in some parts of rural areas in those countries (Lewis, 2005).

2.4.5 Health related facilities. The efficacy of copper and copper alloys to inactivate hospital borne microbes is well documented (Casey et al., 2010). For example, copper alloys can replace touch surfaces at health related facilities to reduce the microbial infection due to cross contamination. It also has the potential to reduce Methicillin-resistant *Staphylococcus aureus* (MRSA), which is a serious public health threat and has

defied nearly all antibiotics. MRSA is usually spread through direct contact via the hands of healthcare employees and is extremely difficult to eradicate. A research team from the University of Southampton, UK has found that using copper alloys for touch surfaces in hospitals can reduce MRSA infections (Lewis, 2005). Therefore, antimicrobial surfaces of copper can play an important role in reducing contamination and limiting cross infection.

2.4.6 Food processing facilities. Foods are always in contact with various touch surfaces during processing. Copper and its alloys can eliminate foodborne pathogens due to its antimicrobial properties. The most common touch surface materials that are being used in the food processing industry including slaughter houses are made up of stainless steel which have no efficacy in destroying pathogenic microbes (Faundez, Troncoso, Navarrete, & Figueroa, 2004). Despite the fact that stainless steel looks clean, it does not have any properties to combat *E. coli* O157:H7 and other harmful microbes and therefore can be a potential source of cross contamination (Lewis, 2005). It would be of greater benefit if these surfaces can be replaced with copper plates.

2.5 Copper as an Antimicrobial Agent

Copper is a very important trace metal and has played a significant and surprising role in improving public health. It is an essential element required for the activity of several biological enzymes. Microorganisms require low concentrations of copper ions as cofactors for processing of metalloproteins and certain enzymes (Nies, 1999). Copper is an essential nutrient for humans as well as bacteria, but in high doses, copper ions

induces and inhibits growth in bacteria and have a toxic effect on most microorganisms (Faundez et al., 2004).

Earlier studies have shown that metallic copper and copper alloys have antibacterial activity over the harmful *E. coli* O157:H7 and also inhibits the adhesion of bacteria on biofilm development (Faundez et al., 2004). The addition of copper to drinking glasses has been shown to reduce biofilm formation of *Streptococcus sanguis*, reducing the risk of oral infections. Currently copper is used as a water purifier, algaecide, fungicide, nematocide, molluscicide, and antifouling and antibacterial agent (Borkow & Gabbay, 2005). Sudha, Singh, Prasad, and Venkatasubramanian (2009) found that water inoculated with *E. coli*, *S. typhi* and *V. cholera* after overnight storage in copper pots was difficult to recover while there was significantly higher number of microbial growth in water stored in glass bottles. *E. coli* O157:H7 strain survives for much shorter periods on copper and a brass known as Muntz metal than on stainless steel. Copper surfaces have an antimicrobial activity against *Salmonella enterica* and *Campylobacter jejuni* and have potential application as an inhibitory agent in different stages of food processing operations (Faundez et al., 2004). Beal, Niven, Campbell, and Brooks (2004) found a 10-fold decrease in the D_{value} of *S. typhimurium* DT104:30 in a liquid pig feed with 150 mM Lactic acid and 50 ppm copper. According to Ibrahim, Yang, and Seo (2008), slight growth inhibition of *Salmonella* spp. and *E. coli* O157:H7 was obtained with 50 ppm of copper and there was significant inhibition observed with 100 ppm and 200 ppm copper in carrot juice.

The bactericidal action of Cu is dependent on concentration of free ionic copper (Menkissoglu & Lindow, 1991; Zevenhuizen, Dolfing, Eshuis, & Scholten-Koerselman, 1979). Copper sulphate pentahydrate ($CuSO_4$. $5H_2O$) is the most common copper source and is very soluble in both water and acidic solvents (Guo et al., 2001; Pang & Applegate, 2006). A wide range of microorganisms has been shown to be susceptible to copper including *E. coli* O157:H7. The way in which copper acts on microorganisms is a complicated subject. However, a few of many proposed mechanisms include:

- The toxicity of copper is largely due to its tendency to alternate between its cuprous, Cu (I), and cupric, Cu (II), oxidation states, differentiating copper from other trace metals. Under aerobic conditions, this redox cycling leads to the generation of highly reactive hydroxyl radicals that readily and efficiently damage biomolecules, such as DNA, proteins, and lipids (Santo, Taudte, Nies, & Grass, 2008). As shown in Figure 2.1, free radicals produced from redox cycle can damage the cell integrity (Borkow & Gabbay, 2005).
- Copper reacts with proteins by combining –SH groups of enzymes and this leads to the inactivation of the proteins (Yoon, Hoon Byeon, Park, & Hwang, 2007). Copper ions will bind to the sulfhdryl, amino and carboxyl groups of amino acids, thereby denaturing the proteins they compose. This makes enzymes and other proteins ineffective, compromising the biochemical process they control. Cell surface proteins necessary for transport of materials across cell membranes also are inactivated as they are denatured. Copper will bind with the phosphate groups that are part of the structural backbone of DNA molecules. This mechanism leads

to the separation of the double helix and consequent destruction of the cell molecule (Meyer, 2001).

- It is possibly due to displacement of essential ions, hence inactivating enzymes and obstructing functional groups of proteins, producing free radicals from hydroperoxide compounds and thus affecting membrane integrity (Nies, 1999).

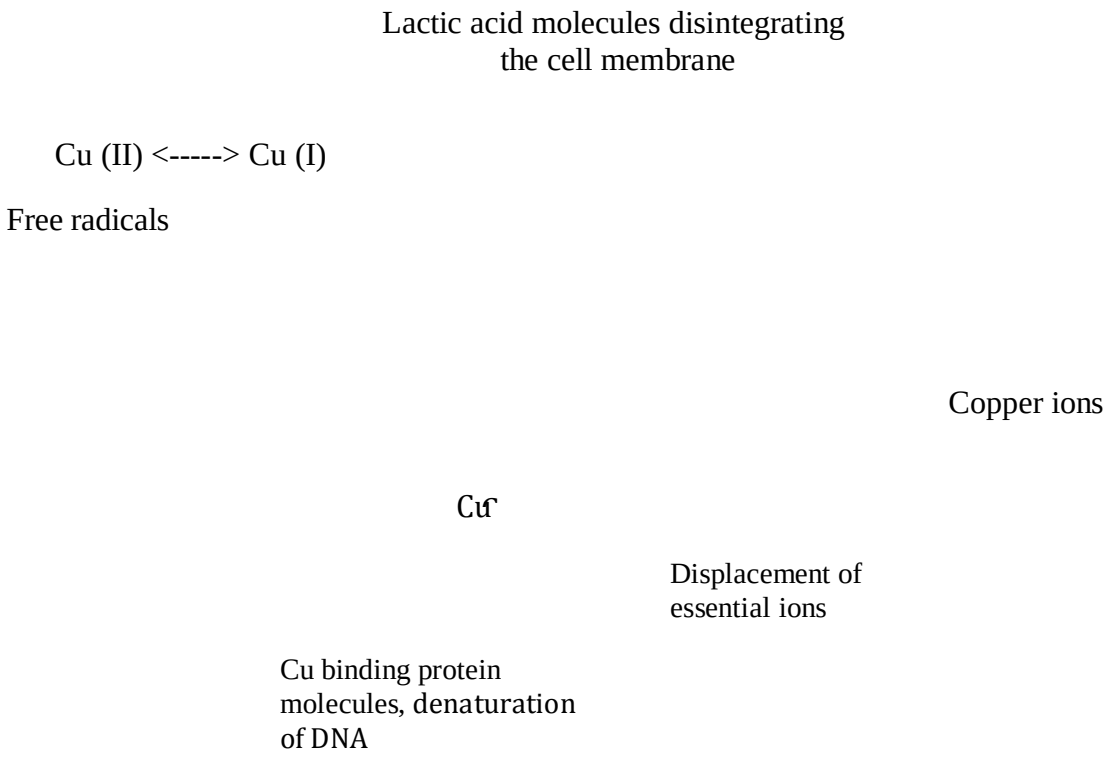

Figure 2.1. Mechanisms of Toxicity of Copper to Microorganisms

Therefore, whenever copper is used, microbes use copper containing enzymes to help drive vital chemical reactions. Based on all of these studies, it is copper's

electrochemical potential that enables its free copper ion to affect the proteins and enzymes in microbes, thereby inhibiting their activity and giving copper its antimicrobial characteristic.

2.6 Use of Sanitizers to Reduce Foodborne Pathogens in Food Systems

Sanitizers that are used to wash or treat fruits and vegetables are regulated by the U.S. Food and Drug Administration in accordance with the Federal Food, Drug and Cosmetic (FD&C) Act. Several different washing procedures for fresh produce are being used to decontaminate fruits and vegetables. Most processors and consumers have assumed that washing and sanitizing fresh fruits and vegetables will reduce the microorganisms. Since leafy green vegetables and even tomatoes are consumed raw, sanitizing washes with various treatments is the most practical means of decontamination of these products. Washing fresh produce with running tap water may remove soil and other dirt but will not kill the pathogens present in the surfaces. The use of sanitizers might reduce the pathogenic microorganisms from the vegetable tissue but the complete inactivation is not possible (Allende, Selma, Lopez-Galvez, Villaescusa, & Gil, 2008). Therefore, food industries and processing plants need different sanitizing methods as spray or dip to effectively reduce or eliminate pathogenic microorganisms on fruits and vegetables.

Chemical sanitizers have been widely used in food processing to reduce pathogens. Studies have shown that chlorine rinses can decrease the microbial load from 1 log CFU/g to 3.15 log CFU/g (Keskinen, Burke, & Annous, 2009). Chlorine dioxide

and acidified sodium chlorite have also been used as sanitizers. Rodgers, Cash, Siddiq, and Ryser (2004) found that aqueous chlorine dioxide (5 ppm) was able to achieve greater than 5 log reductions of *L. monocytogenes* and *E. coli* O157:H7 on apples, lettuce and cantaloupe. Similarly, chlorine and peroxyacetic acid based sanitizers, such as Tsunami are effective for the disinfection of processing water during rinsing (López-Gálvez et al., 2009). Lactic acid and hydrogen peroxide are generally recognized as safe chemicals. The antimicrobial activity of hydrogen peroxide alone or in combination with lactic acid on fresh produce has been reported. A study by Venkitanarayanan, Lin, Bailey, and Doyle (2002) revealed that the treatment of apples, oranges and tomatoes with 1.5% LA+ 1.5% hydrogen peroxide at 40 °C for 15 min can effectively reduce populations of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* by ≥5.0 log CFU/fruit. Organic acids (lactic acid, acetic acid, caprylic acid, and levulinic acid) and sodium dodecyl sulfate (SDS) have the ability to inactivate *Salmonella* and *E. coli* O157:H7. Results showed that the combination of 3% levulinic acid + 1% SDS for <20 s reduced *Salmonella* and *E. coli* O157:H7 population by >6.7 log CFU/g on lettuce and can be used as a washing solution for killing foodborne pathogens on fresh produce (Zhao, Zhao, & Doyle, 2009). A solution of calcium hypochlorite has reduced *E. coli* by 1.9 to 2.8 logs and by 1.7 to 2.5 logs CFU/g in dipped lettuce leaves and broccoli florets respectively (Abuladze et al., 2008).

In a study by Mahmoud and Linton (2007), the D-values of *E. coli* and *S. enterica* were 2.9±0.1 and 3.8±0.5 min, respectively at 5-mg/l ClO_2 gas. The experiment revealed that treatment with ClO_2 gas reduced pathogens on lettuce. Inactivation of pathogens

(*Salmonella* and *E. coli* O157:H7) within fresh produce has been demonstrated by a combination of ultraviolet (UV) light and hydrogen peroxide (Hadjok, Mittal, & Warriner, 2008). On the other hand, Crowe, Bushway, Bushway, Davis-Dentici, and Hazen (2007) reported that a combination effect of UV and H_2O_2 did not show a significant reduction in the microbial population present in blueberries compared with H_2O_2 alone. Therefore, the synergistic effect of UV-H_2O_2 is still not clearly understood. A combination of lactic acid with H_2O_2, sodium benzoate or glycerol monolaurate was more effective in inhibiting *E. coli* O157:H7 than when they were present alone. It is reported that these chemicals can be applied as surface treatments to inactivate *E. coli* O157:H7 on applicable raw vegetables (Venkitanarayanan et al., 2002). Electrolyzed water as a sanitizing agent for produce has been used for produce and results were varied with different studies. It is reported that microbial reductions on lettuce leaves were higher than 2.49 log units for *E. coli* O157:H7 and *L. monocytogenes*. On the other hand, there was 3.7-4.6 log reduction for *E. coli* O157:H7 on apples and only a 1-log reduction on fresh cut vegetables (Sapers, 2001). The combined effect of ozonated water (3 ppm) and 1% citric acid treatment showed 2.26 and 1.32 log reduction on *E. coli* O157:H7 and *L. monocytogenes* respectively on enoki mushroom. Ozone-organic acid treatment was more effective than individual treatments of ozone and organic acid in reducing initial population of such pathogens (Yuk, Yoo, Yoon, Marshall, & Oh, 2007).

In an experiment conducted by Deza, Araujo, and Garrido (2003), the bactericidal activity of neutral electrolyzed water (NEW) as a rinsing solution controlled the presence of *E. coli* O157:H7, *S. enteritidis* and *L. monocytogenes* on the surface of fresh tomatoes.

The results showed that bacterial populations were reduced by more than 6 log CFU/ml within 5 min exposure to NEW. This could be a potential application for the decontamination of fresh produce surfaces of lettuce and tomatoes. Similarly, acidic electrolyzed water (AEW) has strong antimicrobial activity due to its low pH and high oxidation reduction potential. AEW when applied to lettuce surfaces reduces 2 log in viable aerobes (Koseki, Yoshida, Isobe, & Itoh, 2001). It is reported that, a relatively low microbial count was present on fresh cut cilantro when washed with aqueous ozone followed by AEW as compared to aqueous ozone wash and chlorine wash (Wang, Feng, & Luo, 2004). Sanitizers like acidified sodium chlorite (100, 200, 500 ppm) have inhibited bacterial load effectively and maintained the pathogen <1 log CFU/g during storage for washing shredded carrots and also shows the great potential as a commercial sanitizing agent (Ruiz-Cruz, Acedo-Félix, Diaz-Cinco, Islas-Osuna, & Gonzalez-Aguilar, 2007). Velázquez, Barbini, Escudero, Estrada, and de Guzmán (2009) reported that 0.2% lactic acid followed by 200 ppm chlorine and 0.1 mg/ml benzalkonium chloride were the most effective sanitizing treatments with 5.08, 4.7 and 4.21 log CFU reductions on *Yersinia enterocolitica* in tomatoes. Hydrogen peroxide alone or in combination with nisin, sodium lactate, and citric acid was found to be potential sanitizers for reducing *E. coli* O157:H7 or *L. monocytogenes* on whole cantaloupe and honeydew melons (Ukuku, Bari, Kawamoto, & Isshiki, 2005). Lactic acid either alone or in combination with other sanitizers has been widely used to inactivate pathogenic microorganisms on fruits and vegetables.

There are different washing chemical agents as sanitizers to inactivate pathogenic bacteria on vegetables. However, most of the research evaluating the efficacy of sanitizers on pathogenic bacteria reported has not been promising. Food safety issues and rising foodborne illnesses have always been in the news, and as a result of increasing outbreaks, more attention has been focused on the microbial safety of fresh produce. Therefore, the food industry is interested in developing new effective sanitizing agents in order to meet consumer demands for safer raw fruits and vegetables. There is growing interest in developing safer and effective sanitizers for fruits and vegetables (Ukuku et al., 2005). Currently used and proposed methods for reducing the numbers of such pathogens on produce, which include washing with various sanitizers are often ineffective (Doyle et al., 1999). Varieties of disinfectants such as chlorine, hydrogen peroxide, organic acids, ozone etc. have been used to reduce the microbial population on fruits and vegetables. However, they cannot completely remove or inactivate microorganisms on fresh produce. Furthermore, widely used chlorinated water has minimal impact in inactivating pathogens on the surfaces of fresh produce (Lin, Moon, Doyle, & McWatters, 2002). The conventional washing technology in reducing populations of pathogens on fresh produce surfaces is limited and insufficient to ensure food safety. To overcome all these limitations, combination treatments of lactic acid and copper could be used as a rinsing solution to inactivate microbial pathogens. This could be developed as a new alternative sanitizer for washing fresh produce which could be more effective in reducing or eliminating foodborne pathogens such as *E. coli* O157:H7.

Hence, the current study could provide a basis for improved sanitizer combining lactic acid and copper in reducing gram-negative pathogens on produce as a novel idea.

2.7 Use of Surfactant (Tween 80)

Tween 80 belongs to a class of food additives and is also known as polysorbate, nonionic surface-active agent. The main problem of fresh produce washing is that attached microorganisms are difficult to remove even with strong treatments. *E. coli* O157:H7 can attach to cracks, trichome, and inside the cuticle and stomata of lettuce surfaces, which increases the adherence of such pathogens and making it harder to remove even with sanitizing treatments. The formation of biofilms by some microorganisms on the surfaces of fruits and vegetables makes the washing procedure less effective. Earlier studies have shown the effectiveness of surfactant such as Tween 80 when added with other chemical compounds. Raiden, Sumner, Eifert, and Pierson (2003) reported that a combination of 1% trisodium phosphate with 5% tween 80 was more efficient in removing *Salmonella* spp. from chicken skin. There were about 1.1-1.2 log reductions of *E. coli* O157:H7 on the surfaces of strawberries when they were sanitized with 100 and 200 ppm of tween 80 (Yu, Newman, Archbold, & Hamilton-Kemp, 2001). Tween 80 is an anionic surfactant that is approved by the U.S. Food and Drug Administration (FDA) as a wetting agent and also generally recognized as a safe (GRAS) product. It is a hydrophilic compound having an affinity to water and usually charged or have polar side group that will attract water. Many studies have shown the effectiveness of such surfactant as an aid to a sanitizing agent (Raiden et al., 2003).

Therefore the addition of surfactant like Tween 80 might enhance the lethality of copper and lactic acid solution by increasing surface contact of the sanitizer with the microbes by maximizing the release of the pathogen from inoculated vegetables.

2.8 Synergistic Effect of Copper and Lactic Acid

Several examples of combination treatments have been reported to inactivate *E. coli* O157:H7 on fresh produce. Sometimes individual treatments show different mode of action and behave differently than when combined with other chemical agents. Weak organic acids such as lactic acid also show synergistic effect when combined with copper. *E. coli* O157:H7 is tolerant to organic acids and can survive well in acidic food. It can adapt to acidic conditions and tolerate pH levels that would normally inactivate it (Eribo & Ashenafi, 2003). As a result of its resistivity to acidic media, lactic acid acts differently towards pathogens as compared to its combination effect with others and shows greater efficacy towards bacteria. Ibrahim et al. (2008) indicated that lactic acid in combination with copper sulfate could be used to inhibit the growth of pathogens. Their results showed that the combination of lactic acid and copper produces a synergistic effect against *E. coli* O157:H7 and *Salmonella* spp. The presence of copper in acidic liquid food substrates significantly decreases the death rate of *S. typhimurium* DT104:30. It was found that the addition of 150 mM lactic and 50 ppm of copper to liquid pig feed resulted in a 10-fold increase in the death rate (Beal et al., 2004). Russell (2008) reported that an acidic copper sulfate based commercial sanitizer was able to reduce *E. coli* and *Salmonell*a during scalding and able to extend the shelf life of broiler chicken carcasses.

The combination of sodium hypochlorite (NaClO) and hydrogen peroxide (H_2O_2) in the presence of copper sulfate ($CuSO_4$) has shown antifungal activity against *penicillium digitatum* (Cerioni, Rapisarda, Hilal, Prado, & Rodriguez-Montelongo, 2009).

The use of copper ion for decreasing microbial loads on fresh produce is deficient. However, several in vitro studies indicate that copper ion might be potentially useful as a produce sanitizer (Rodgers & Ryser, 2004). A study conducted by Jo and Rim (2007) showed that the addition of organic acid with silver ions enhanced the inhibitory effect on *E. coli* O157:H7 growth compared to individual treatments. Weak organic acids possess antimicrobial activity; the combined treatment with metal ions may be synergistic. This study supports the proposed recommendation that combining lactic acid with copper ion may develop a useful sanitizer for produce. Since, lactic acid not only reduces the pH of the medium but also possesses permeabilizer properties and can freely pass across the plasma membrane and enter the cell, lactic acid can facilitate the entrance of copper ions into the cell thus producing a toxic effect. It can be assumed that the permeabilizing nature of lactic acid could enable the entry of copper ions into the bacterial cells and therefore produce a synergistic effect against *E. coli* O157:H7.

Based on the previous research related to the properties and effectiveness of copper, the current study will focus on the effect of copper ion in combination with lactic acid to determine its potential to inhibit the survival and growth of *E. coli* O157:H7. This study could help to develop a potential sanitizer that combines lactic acid and copper ion to reduce the number of foodborne pathogens on fresh produce.

CHAPTER 3

MATERIALS AND METHODS

3.1 Bacterial Strains

Four different strains of *E. coli* O157:H7 (H1730, 43895+, 43895- and 86.24) used in this study were obtained from the Department of Food Science and Technology at Virginia Tech. These strains were maintained on tryptic soy agar slants at 4 ºC. Strains were then transferred to fresh Brain Heart Infusion broth (BHI, Becton Dicksinson & Co., Sparks, MD) and incubated at 37 ºC for 12 hours. Overnight strains were streaked onto a BHI agar plate and incubated for at least 24 hours at 37 ºC. Single colonies of each strain were then transferred into BHI broth and incubated at 37 ºC overnight before inoculating into the treatments.

3.2 Inoculum Preparation

Overnight cultures of the strains were centrifuged (Model 5415 R, Eppendorf North America, Inc., Westbury, NY) for 10 min (5900×g, 4 ºC). Supernatant of each strain was decanted and the cell pellet was washed twice with 1 ml of sterilized peptone water. The bacterial suspension was then serially diluted in 9 ml of peptone water to give an initial inoculum of each strain containing approximately 3 log CFU/ml before inoculating into broth. Bacterial population of each initial inoculum was determined by serially diluting in sterile 0.1% peptone water and plating appropriated dilutions (100 µl) on duplicate plates of BHI agar (Becton Dickinson and Co., Sparks, MD). Colonies were

counted after plates were incubated at 37 ºC for 24 hr to determine the initial bacterial population.

3.3 Media Preparation

Two liters of BHI broth (supplemented with 5 g Yeast extract per liter deionized water) was prepared and divided into four 500 ml portions. Lactic acid (85% Thermo Fisher Scientific, Fair Lawn, NJ) was added to obtain 0.1%, 0.2%, and 0.25% (v/v) concentrations in three portions. These three portions were again divided into five 100 ml portions and sterilized at 121 ºC for 15 min. Out of five, one portion was left alone to form a lactic acid only sample and the remaining four were treated with 5, 10, 20 and 40 ppm of copper sulphate (Fisher Scientific, Fair Lawn, NJ) forming the combination of lactic acid and copper sample. The fourth 500 ml portion was divided into five 100 ml portions. One portion left untreated represented the control sample and the remaining four were combined with 4 different concentrations of copper (5, 10, 20, and 40 ppm) forming the copper only sample. For the preparation of copper stock solution, deionized water was used and filter sterilized through 0.2-µm Nalgene filteration product (Nalge Nunc International, Rochester, NY). Seven ml of each portion sample were dispensed into sterilized test tubes. Samples were then treated with 100 µl of each active bacterial cell and incubated at 37 °C for 12 hr. Each set of experiments was conducted three times.

3.4 Measuring Bacterial Growth and pH

3.4.1 Experiment 1: Growth of *E. coli* O157:H7 measured by optical density. For each treatment, bacterial growth was monitored by measuring the turbidity at 0, 4 and 8 hour of incubation period using a spectronic 21 Milton Roy spectrophotometer at the wavelength of 610 nm.

3.4.2 Experiment 2: Colony forming units in laboratory medium. Bacterial populations were determined by plating onto brain heart infusion (BHI) agar. At the end of the incubation period (8 hr), one ml of each treated *E. coli* strain was withdrawn and 0.1 ml of the appropriate dilution was surface plated on duplicate plates of agar. The plates were incubated at 37 °C for 24 hr and bacterial colonies were counted.

3.4.3 Experiment 3: Determination of pH. The pH levels of the treatment solutions were measured after sample preparation using pH meter (Accument Excel XL15, Thermo Fisher Scientific, Pittsburgh, PA). First the pH meter was calibrated with standard buffers of pH 4.0 and 10.0 and the values of treated solution samples were recorded for selected treatments.

3.4.4 Experiment 4: Sanitizing treatment and bacterial enumeration on the surface of lettuce and tomatoes.

3.4.4.1 Inoculum preparation. A mixture of four *E. coli* O157:H7 strains was used in this study. Each active bacterial strain grown in 10 ml of BHI were combined and centrifuged (Thermo Electron Scientific, Sorvall RC 6 Plus, Asheville, NC) at 7580×g for 10 min. Pelletes were washed and resuspended in 400 ml of sterilized peptone water to give a cell number of approximately 10^9 CFU/ml.

3.4.4.2 Produce preparation. Romaine Lettuce *(Lactuca sativa L., longifolia)* and tomatoes (*Lycopersicum esculentum*) were purchased at a local grocery store (Greensboro, NC) and stored at 4 ºC until further testing. Fresh whole tomatoes of similar sizes weighing between 55-65 g each and without external defects on the skin were selected for the experiment. Tomatoes were first washed with tap water to remove external dirt. Two or three outer leaves of the lettuce plant were removed and a few inner leaves without any damage were selected before washing procedure. The internal leaves were cut into pieces of 4 cm by 4 cm and then dipped in tap water to remove any dirt present. Tomatoes and pieces of lettuce were then dried at room temperature for an hour in the biosafety cabinet (Labconco Corporation, Kansas City, MO) to remove moisture from the surface.

3.4.4.3 Produce inoculation. Whole tomatoes and pieces of 4×4 cm lettuce leaves were submerged in a four strains *E. coli* cocktail bacterial inoculum (~10^9 CFU/ml) and gently agitated by hand with a stainless steel spoon for 2 min at 22 ºC. The high inoculum rate was used to test the inhibitory efficacy of the treatments used in this study based on the recommended level of 5 log reductions by FDA. Samples were stored overnight at 4 ºC before use in experiments. Each produce type was inoculated and stored in different containers with equal volume of bacterial culture.

3.4.4.4 Rinse treatment. Using stainless forceps, each stored sample was placed on a petri dish in a laminar flow biosafety hood and was allowed to dry for one hour to facilitate bacterial adhesion prior to treatment. After an hour, 12 pieces of lettuce leaves and 12 whole tomatoes were placed in individual sterile sampling bags. Treatment

solutions of copper (20 and 40 ppm), lactic acid (0.2%) and their combinations (0.2%+20 ppm and 0.2% +40 ppm) with & without 0.1% v/v Tween 80 (Fisher Scientific, Fair Lawn, NJ) and Tween 80 only were prepared in 0.1% peptone water. All solutions without copper were sterilized at 121 ºC for 15 min. For the preparation of copper solution, deionized water was used and filter sterilized. The appropriate volume was then added to each sample of copper to give final concentrations of 20 and 40 ppm. All samples were then rinsed with 10 ml of each treatment. Two bags containing tomato and a piece of lettuce were rinsed with 10 ml of 0.1% peptone water as a control. The bags were gently shaken and each tomato was hand rubbed for 1 min to facilitate wetting by the treatment solution. Bags with lettuce pieces were shaken at 100 rpm (Orbit shaker, Lab-Line instruments, Inc. Melrose Park, IL) for 3 min to release attached cells from the surface.

3.4.4.5 Microbiological analysis. One ml of wash suspension from each bag was serially diluted with 9 ml of sterile 0.1% peptone water and 100 µl was plated on duplicate BHI agar. The plates were then incubated at 37 ºC for 24 hr for enumeration of *E. coli* O157:H7.

3.5 Experimental Design and Statistical Analysis

The experimental design for the study in laboratory media was a 4X5X4 factors factorial design, with 4 *E. coli* O157:H7 strains (H1730, 43895+, 43895- and 86.24), 5 copper concentrations (0, 5, 10, 20 and 40 ppm), and 4 lactic acid concentrations (0, 0.1, 0.2 and 0.25%). Data were analyzed using the general linear models procedure of the

Statistical Analysis Software (SAS Institute, Cary, NC) with the use of Duncan's multiple range to determine significant differences among the treatments ($p < 0.05$).

CHAPTER 4

RESULTS AND DISCUSSION

4.1 Experiment 1: Growth of *E. coli* O157:H7 Measured by Optical Density

Figures 4.1-4.4 show the effect of four different concentrations (5, 10, 20 and 40 ppm) of copper and 3 different concentrations (0.1, 0.2 and 0.25%) of lactic acid alone and their combination on the growth of four different strains of *E. coli* O157:H7 (H1730, 43895+, 43895-, and 86.24) grown in BHI broth after 8 hr of incubation at 37 ºC. When *E. coli* O157:H7 strains were grown in BHI broth without copper and lactic acid (control samples), the strains continued to proliferate during the incubation period from the initial turbidity level of ~0.03 and reached the maximum absorbance of 0.78-0.84 (O.D. 610 nm) after 8 hr of incubation. Copper, when added to BHI broth at a concentration of 5 ppm and 10 ppm, did not have a significant effect ($p > 0.05$) (Table 4.1) on the bacterial growth as measured by turbidity (0.76-0.82). The addition of 20 and 40 ppm copper showed a slight delay in bacterial growth (O.D. 0.65-0.78) for all tested strains. This indicates that copper alone is not effective in controlling the growth of *E. coli* O157:H7 in laboratory medium. The addition of 0.1% lactic acid slightly retarded the growth of the tested strains. When lactic acid was added to BHI broth at 0.2 and 0.25%, a significant growth inhibition ($p < 0.05$) was observed in all tested strains (Table 4.1).

The addition of 5 and 10 ppm of copper to lactic acid 0.1% (O.D. 0.65-0.71) did not have any more retarding effect on all bacterial strains compared to the lactic acid only sample (O.D. 0.69-0.72). Increased concentrations of copper at 20 and 40 ppm

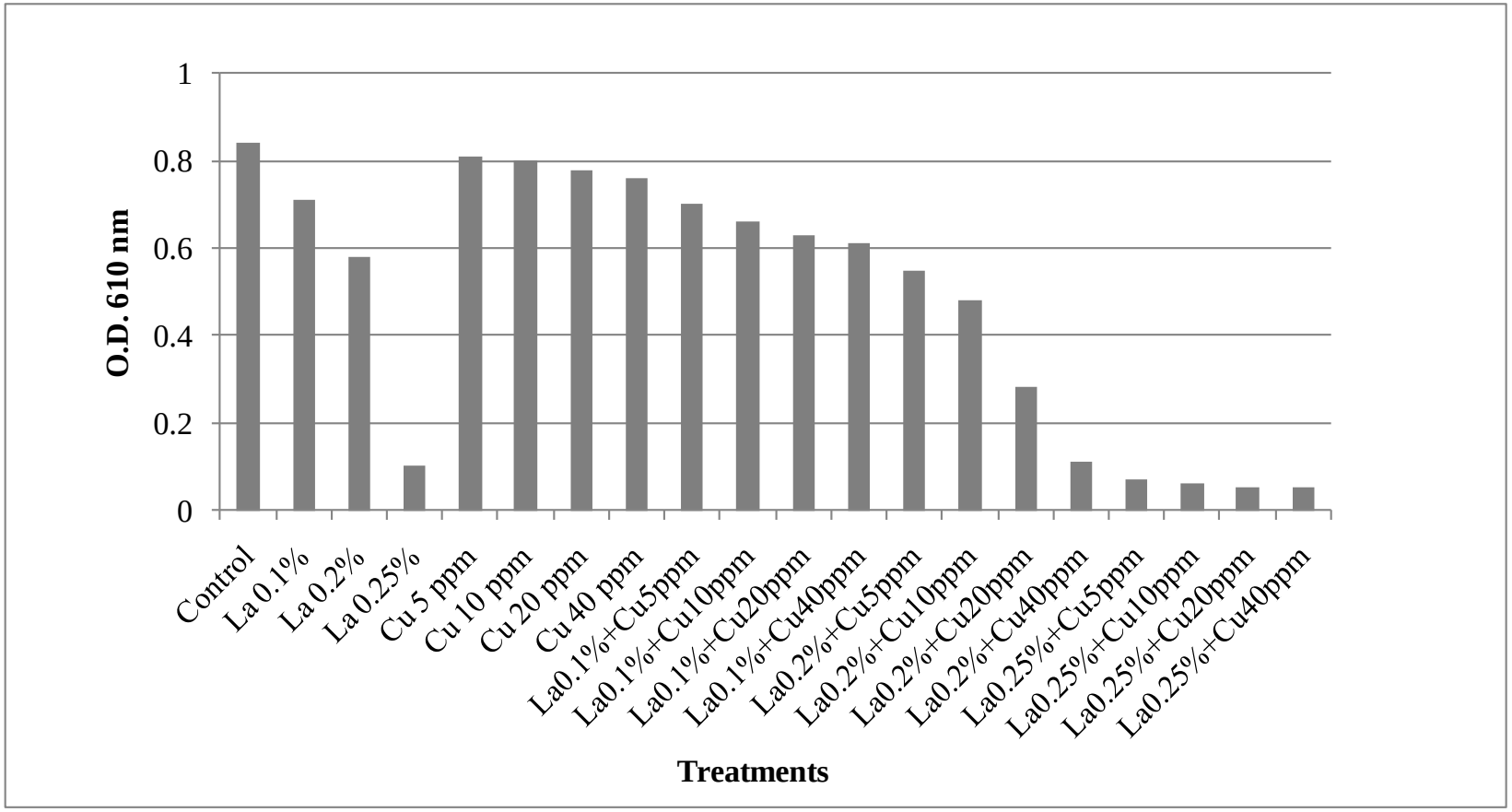

Figure 4.1. Growth of *E. coli* O157:H7 (Strain H1730) in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

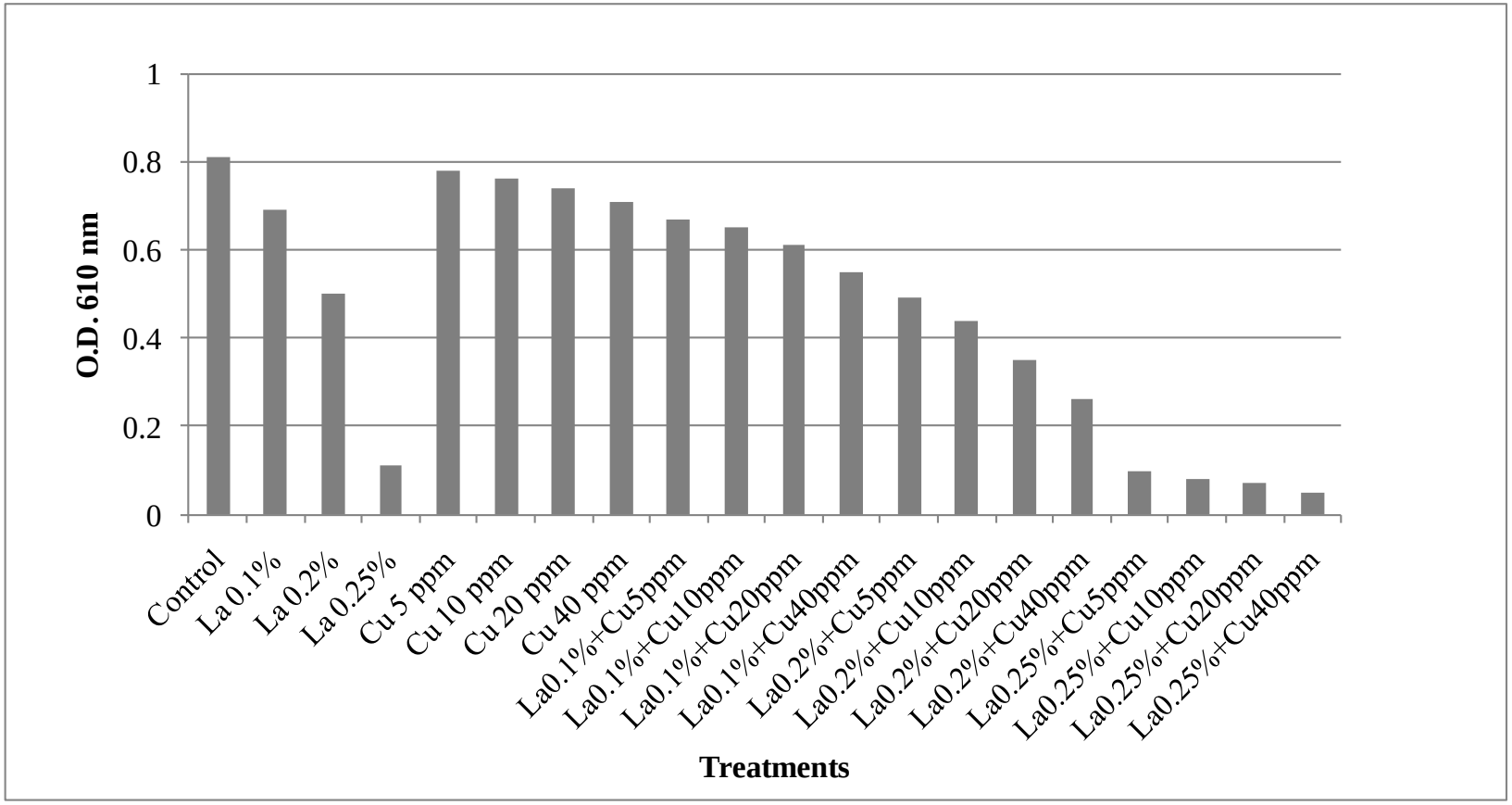

Figure 4.2. Growth of *E. coli* O157:H7 (Strain 43895+) in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

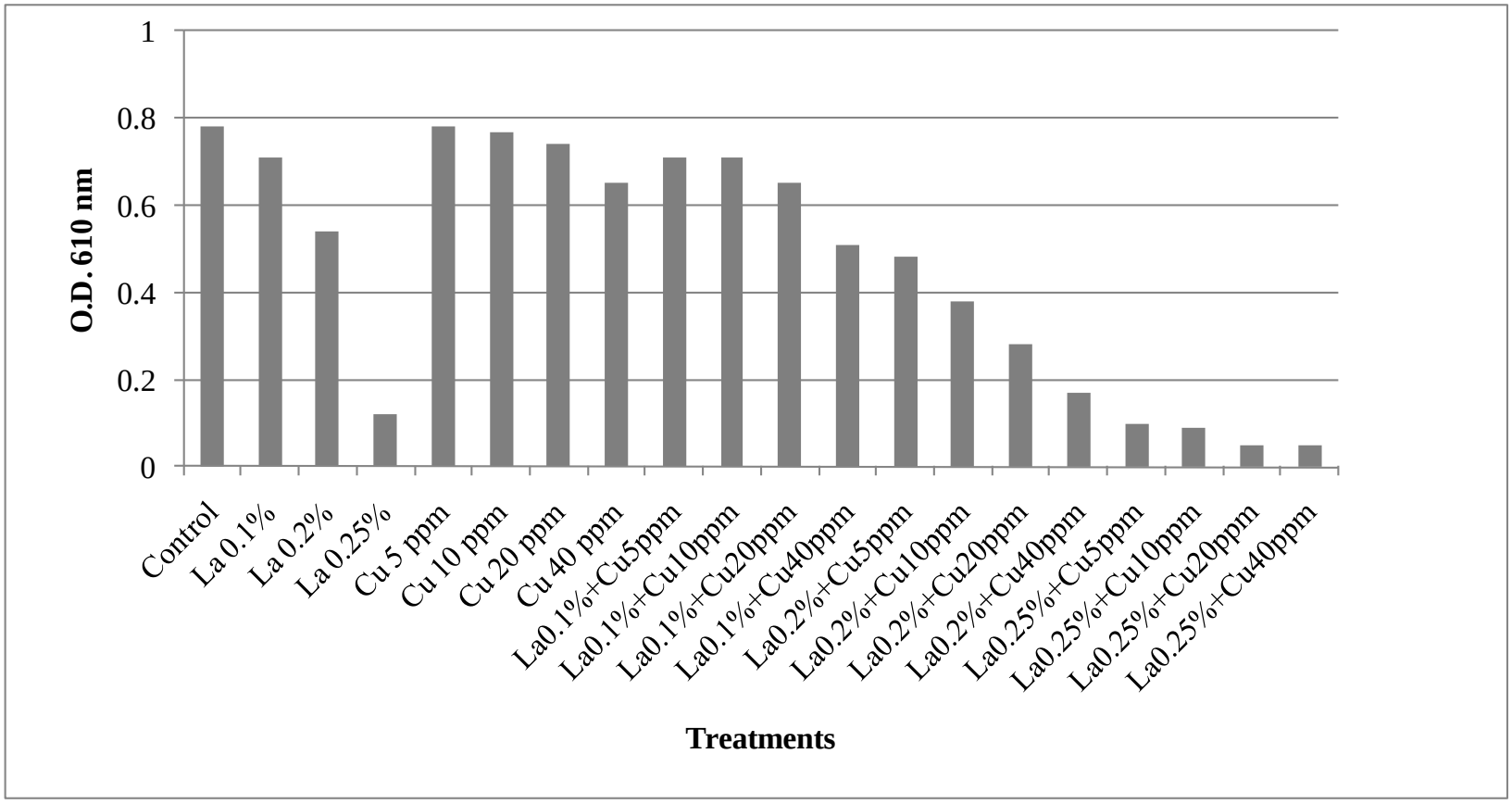

Figure 4.3. Growth of *E. coli* O157:H7 (Strain 43895-) in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

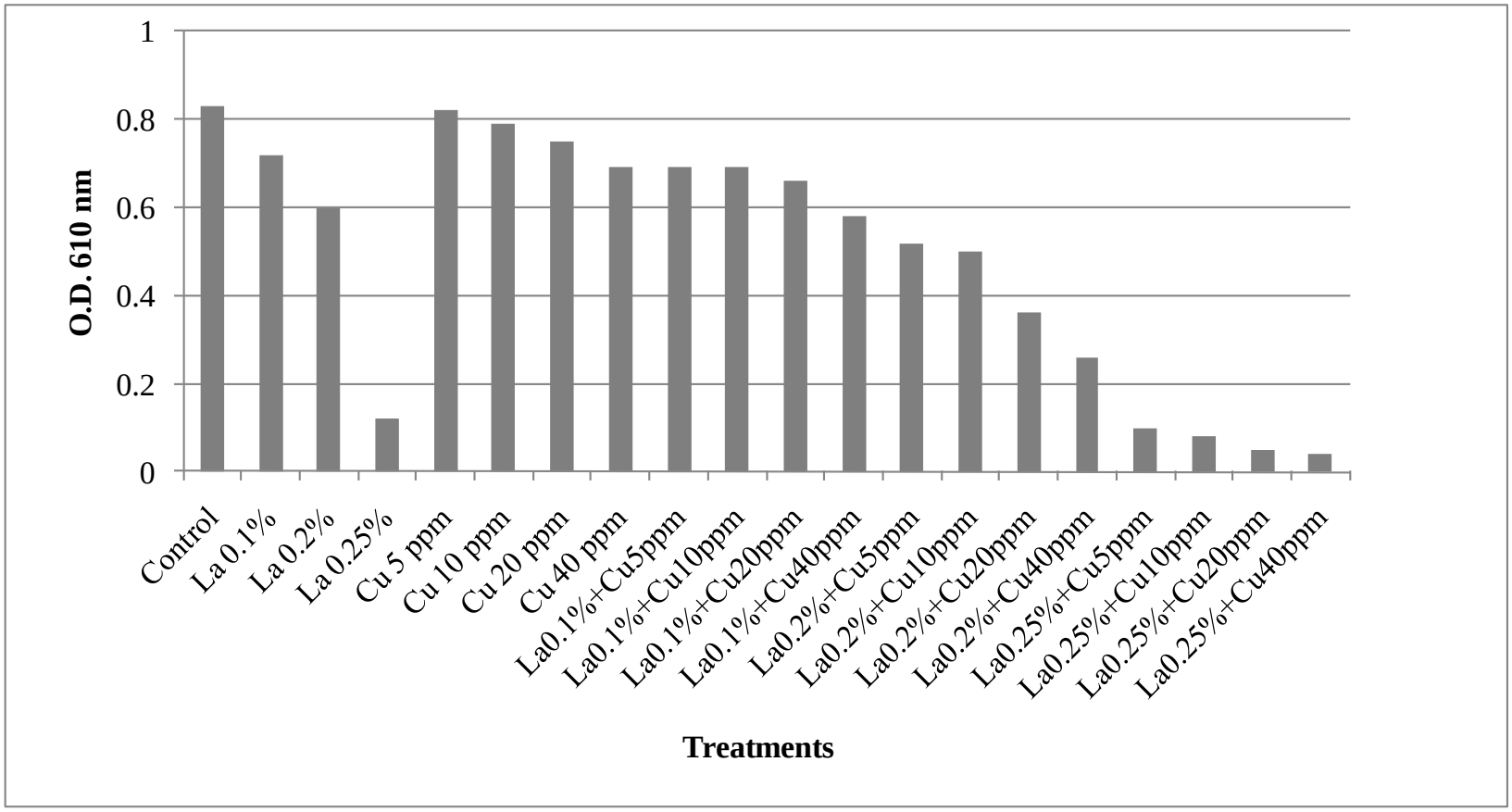

Figure 4.4. Growth of *E. coli* O157:H7 (Strain 86.24) in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

Table 4.1. Populations of *E. coli* O157:H7 Strains (O.D) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

	***E. coli* O157:H7 Strains**			
Treatments	**H1730**	**43895+**	**43895-**	**86.24**
Control	$0.84^{a} \pm 0.064$	$0.81^{a} \pm 0.014$	$0.78^{a} \pm 0.035$	$0.83^{a} \pm 0.021$
La 0.1%	$0.71^{cd} \pm 0.028$	$0.69^{e} \pm 0.021$	$0.71^{c} \pm 0.049$	$0.72^{de} \pm 0.014$
La 0.2%	$0.58^{fg} \pm 0.021$	$0.50^{i} \pm 0.007$	$0.54^{e} \pm 0.007$	$0.60^{g} \pm 0.000$
La 0.25%	$0.10^{j} \pm 0.021$	$0.11^{m} \pm 0.007$	$0.12^{j} \pm 0.014$	$0.12^{k} \pm 0.007$
Cu 5ppm	$0.81^{ab} \pm 0.035$	$0.78^{ab} \pm 0.007$	$0.77^{ab} \pm 0.021$	$0.82^{ab} \pm 0.042$
Cu 10ppm	$0.80^{ab} \pm 0.014$	$0.76^{bc} \pm 0.014$	$0.77^{ab} \pm 0.007$	$0.79^{bc} \pm 0.028$
Cu 20ppm	$0.78^{b} \pm 0.028$	$0.74^{bc} \pm 0.014$	$0.74^{ac} \pm 0.014$	$0.75^{cd} \pm 0.014$
Cu 40ppm	$0.76^{bc} \pm 0.014$	$0.71^{de} \pm 0.042$	$0.65^{d} \pm 0.007$	$0.69^{ef} \pm 0.007$
La 0.1%+Cu 5ppm	$0.70^{cd} \pm 0.014$	$0.67^{ef} \pm 0.021$	$0.71^{c} \pm 0.021$	$0.69^{ef} \pm 0.049$
La 0.1%+Cu 10ppm	$0.66^{ed} \pm 0.007$	$0.65^{f} \pm 0.014$	$0.71^{c} \pm 0.007$	$0.69^{ef} \pm 0.000$
La 0.1%+Cu 20ppm	$0.63^{ef} \pm 0.000$	$0.61^{g} \pm 0.021$	$0.65^{d} \pm 0.014$	$0.66^{f} \pm 0.028$
La 0.1%+Cu 40ppm	$0.61^{efg} \pm 0.028$	$0.55^{h} \pm 0.021$	$0.51^{ef} \pm 0.021$	$0.58^{g} \pm 0.007$
La 0.2%+Cu 5ppm	$0.55^{g} \pm 0.071$	$0.49^{i} \pm 0.014$	$0.48^{f} \pm 0.021$	$0.52^{h} \pm 0.000$
La 0.2%+Cu 10ppm	$0.48^{h} \pm 0.014$	0.44j ± 0.007	$0.38^{g} \pm 0.007$	$0.50^{h} \pm 0.007$
La 0.2%+Cu 20ppm	$0.28^{i} \pm 0.028$	$0.35^{k} \pm 0.014$	$0.28^{h} \pm 0.007$	$0.36^{i} \pm 0.028$
La 0.2%+Cu 40ppm	$0.11^{j} \pm 0.028$	$0.26^{l} \pm 0.021$	$0.17^{a} \pm 0.007$	$0.26^{j} \pm 0.021$
La 0.25%+Cu 5ppm	$0.07^{j} \pm 0.007$	$0.10^{mn} \pm 0.007$	$0.78^{a} \pm 0.007$	$0.10^{k} \pm 0.007$
La 0.25%+Cu 10ppm	$0.06^{j} \pm 0.007$	$0.08^{mno} \pm 0.007$	$0.78^{a} \pm 0.007$	$0.08^{kl} \pm 0.007$
La 0.25%+Cu 20ppm	$0.05^{j} \pm 0.000$	$0.05^{j} \pm 0.000$	$0.78^{a} \pm 0.007$	$0.05^{l} \pm 0.007$
La 0.25%+Cu 40ppm	$0.05^{j} \pm 0.000$	$0.05^{j} \pm 0.007$	$0.78^{a} \pm 0.000$	$0.04^{l} \pm 0.007$

Means (± standard deviation) within the same column followed by different letters are significantly different ($p < 0.05$).

supplemented with lactic acid 0.1% enhanced the inhibition of bacterial growth slightly more than the lactic acid alone or the control sample (Table 4.1). Similarly, slightly lower turbidity readings were observed when lactic acid 0.2% was added with copper 5 and 10 ppm. However, the presence of lactic acid 0.2% with copper 20 ppm or higher significantly inhibited the growth of all bacterial strains ($p < 0.05$). Further addition of lactic acid 0.25% with copper 5 and 10 ppm showed a significant growth inhibition for all tested strains compared to the control and copper alone samples. However, the growth of *E. coli* O157:H7 was not significantly inhibited ($p > 0.05$) as compared to the lactic acid alone sample. When a combination of copper (20 and 40 ppm) and 0.25% of lactic acid was used, a significant growth inhibition of *E. coli* O157:H7 (Table 4.1) was observed as compared to the control and copper alone samples. The optical density (O.D. 610 nm) readings observed by the turbidity of *E. coli* O157:H7 show the same pattern of growth for all tested strains. The results show a significant effect with combinations of copper (20 and 40 ppm) and lactic acid (0.2%) when compared to the control or lactic acid only samples, indicating a synergistic inhibitory effect on the microbial growth. The results also indicate that lactic acid at a concentration of 0.2% is antimicrobial and sufficient to produce synergistic effect when combined with copper 20 ppm or higher concentrations.

4.2 Experiment 2: Colony Forming Units in Laboratory Medium

Figures 4.5-4.8 show the effects of different concentrations of copper and lactic acid, alone or in combination, on the survival and growth of 4 strains of *E. coli* O157:H7 grown in BHI broth during incubation at 37 ºC for 8 hours. In the control samples, the

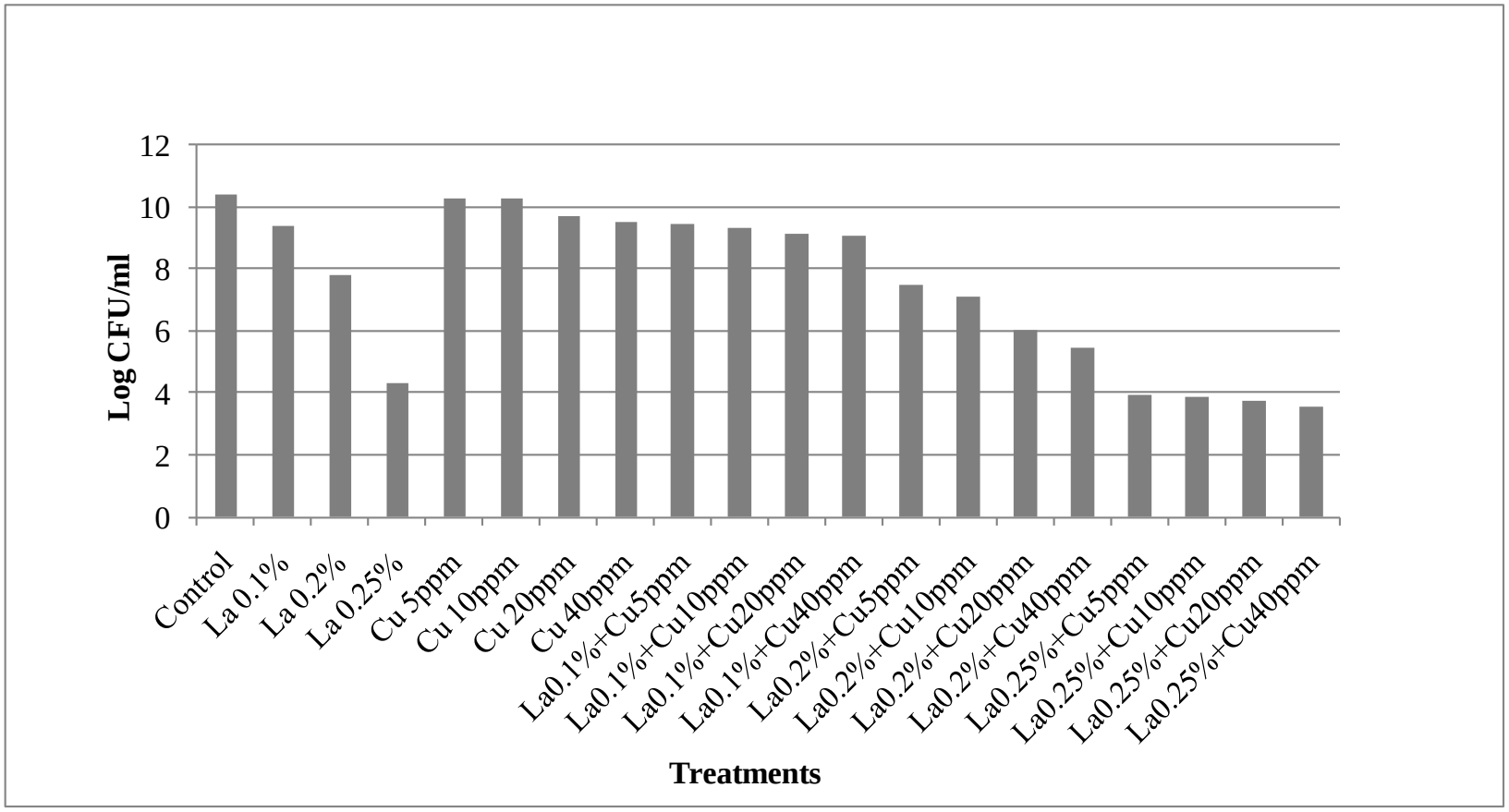

Figure 4.5. Populations of *E. coli* O157:H7 (Strain H1730) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

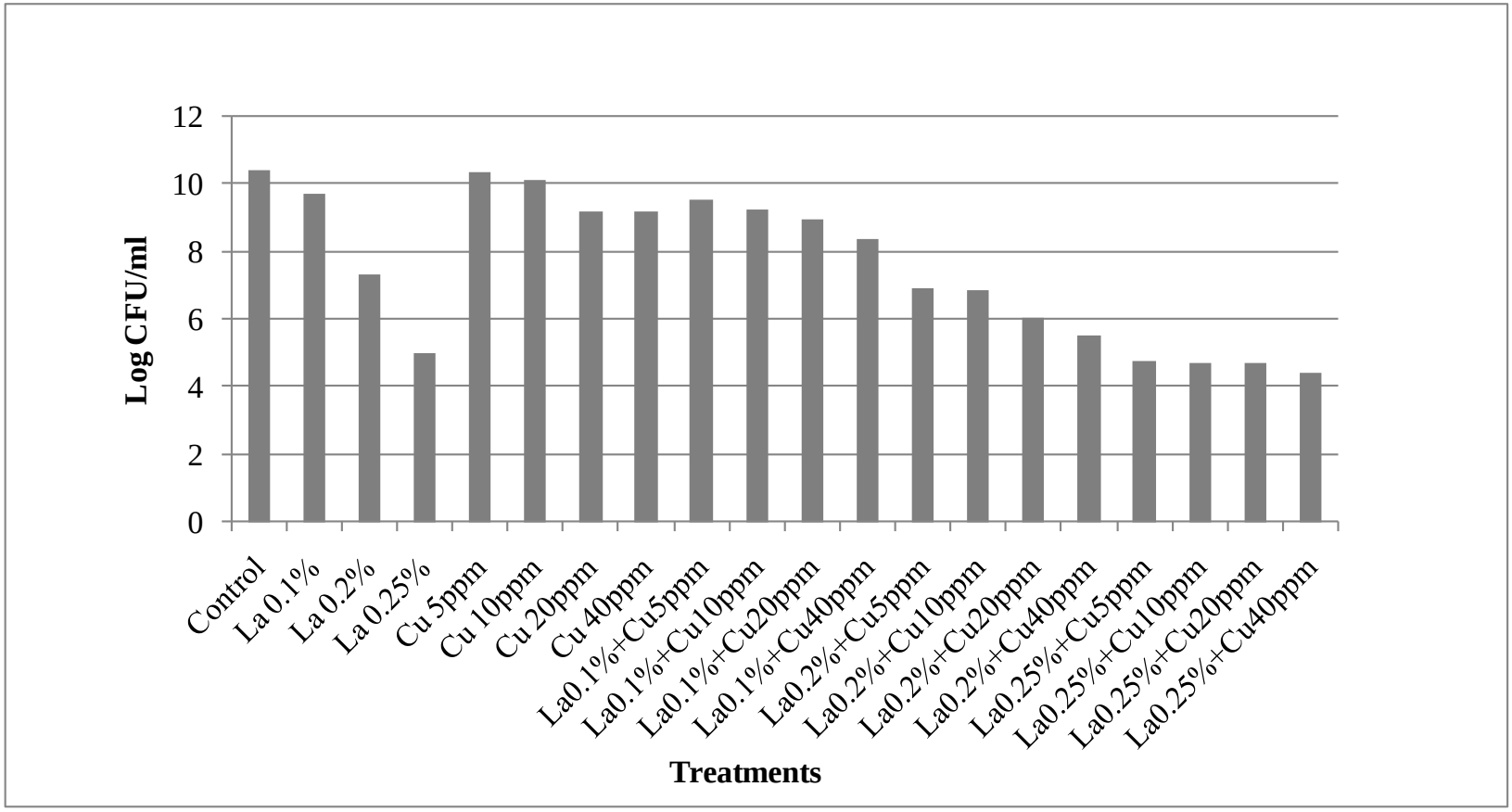

Figure 4.6. Populations of *E. coli* O157:H7 (Strain 43895+) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

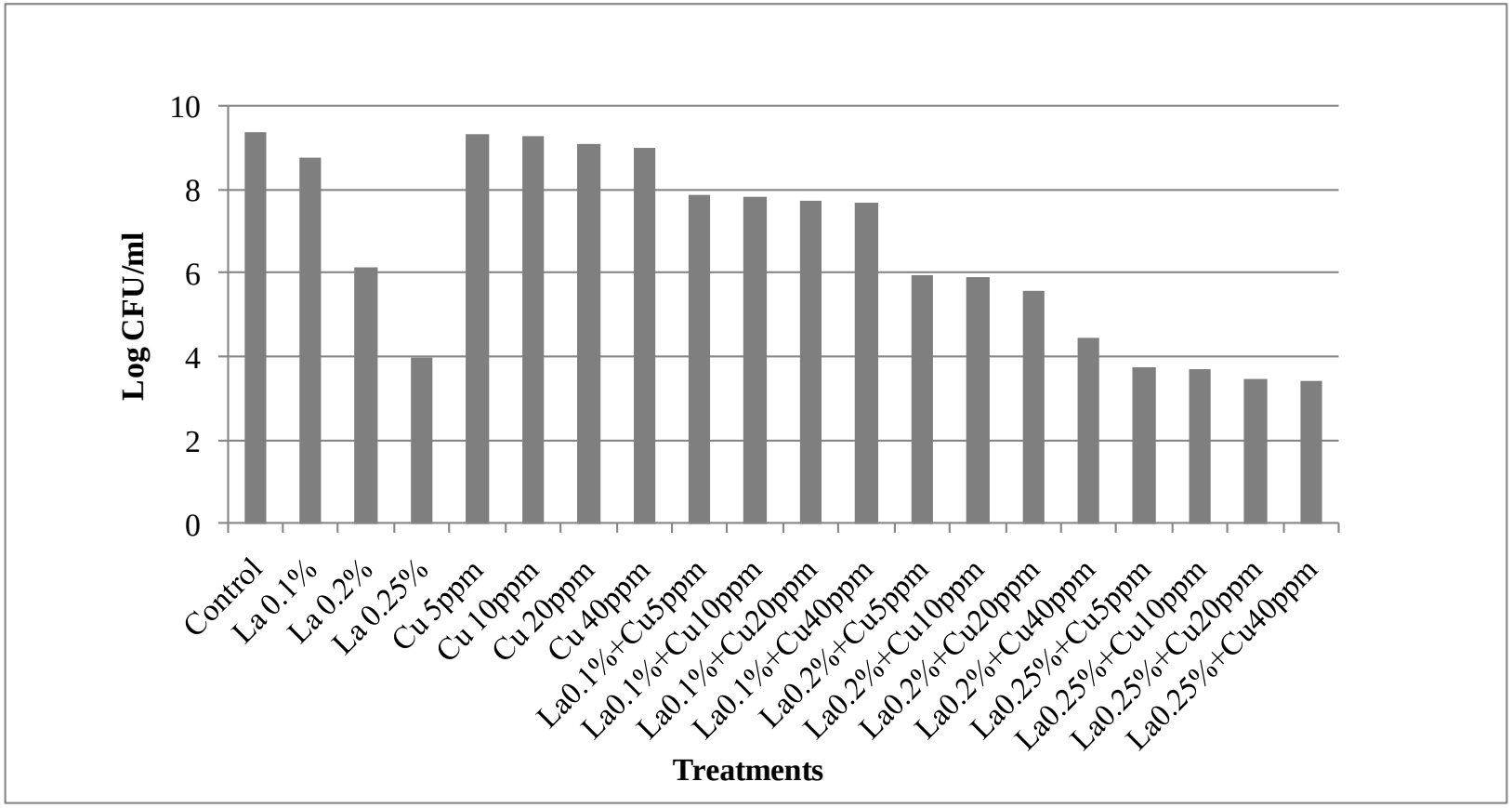

Figure 4.7. Populations of *E. coli* O157:H7 (Strain 43895-) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

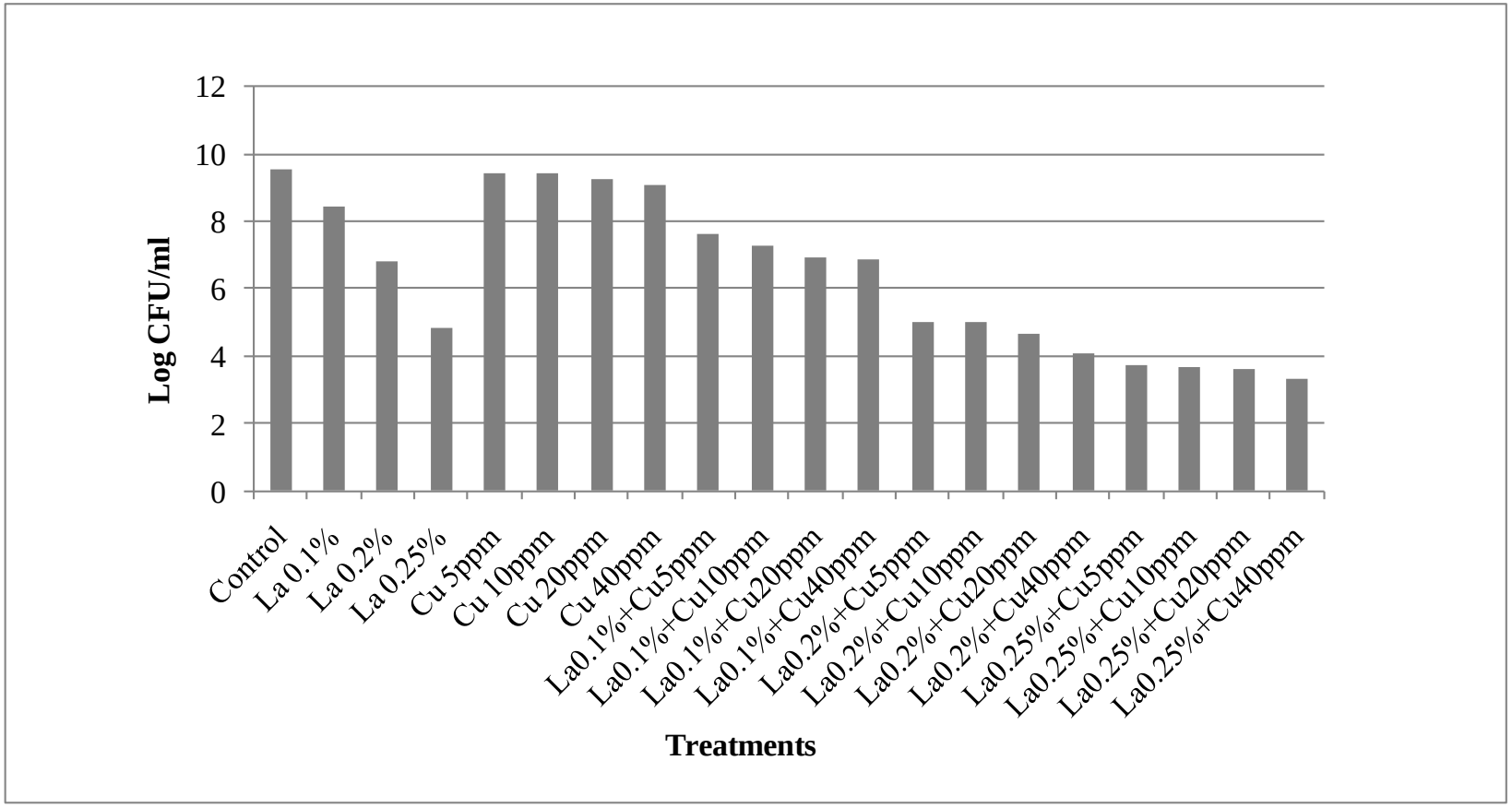

Figure 4.8. Populations of *E. coli* O157:H7 (Strain 86.24) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

number of *E. coli* strains increased from an initial population of 3 log CFU/ml and reached an average of 9.93 log CFU/ml. With the addition of copper at different concentrations, the growth of *E. coli* O157:H7 was not significantly inhibited ($p \leq 0.05$) (Table 4.2). Only 0.74 log CFU/ml was achieved when BHI broth was supplemented with copper 40 ppm. Therefore, the concentration of copper even at 40 ppm was not a significant bactericidal for these tested strains. When *E. coli* was grown in BHI broth containing 0.1% lactic acid, slight growth inhibition was observed in bacterial populations compared to the control.

A combination of copper at four different concentrations (5, 10, 20 and 40 ppm) and 0.1% lactic acid was found to significantly retard the growth of *E. coli* compared to the control and copper alone treatments. A 1.08 log reduction was achieved when 40 ppm copper was supplemented with 0.1% lactic acid when compared to the lactic acid only treatment. However, increasing the concentration of lactic acid to 0.2% caused significant inhibition ($p \leq 0.05$). An average of 3 log CFU/ml reduction of *E. coli* population was observed as compared to the control and copper only samples. The addition of 20 and 40 ppm of copper with 0.2% lactic acid caused further reduction of *E. coli* growth. A combination of 20 ppm copper with 0.2% lactic acid resulted in log reductions of 4.37, 3.74, and 1.46 when compared to the control, copper alone and lactic acid alone samples respectively. The addition of 40 ppm copper to 0.2% lactic acid, resulted in log reductions of 5.03, 4.29, and 2.12 CFU/ml when compared to the control, copper alone, and lactic acid alone samples respectively (Table 4.2). These results were

Table 4.2. Populations of *E. coli* O157:H7 Strains (Log CFU/ml) Grown in BHI Broth Containing Different Concentrations of Copper (Cu) and Lactic Acid (La)

	***E. coli* O157:H7 Strains**				
Treatments	**H1730**	**43895+**	**43895-**	**86.24**	**Average**
Control	10.42	10.4	9.37	9.55	9.93^{a} ±0.553
La 0.1%	9.40	9.68	8.75	8.45	9.07^{abc}±0.568
La 0.2%	7.81	7.30	6.15	6.84	7.02^{de} ±0.705
La 0.25%	4.29	4.97	4.00	4.85	4.52^{ghi} ±0.460
Cu 5ppm	10.28	10.35	9.34	9.45	9.85^{a} ± 0.534
Cu 10ppm	10.25	10.12	9.29	9.42	9.77^{a} ± 0.485
Cu 20ppm	9.70	9.20	9.08	9.25	9.30^{ab} ±0.271
Cu 40ppm	9.51	9.17	8.98	9.10	9.19^{ab} ±0.227
La 0.1%+Cu 5ppm	9.45	9.55	7.86	7.60	8.61^{bc} ±1.028
La 0.1%+Cu 10ppm	9.31	9.25	7.82	7.30	8.42^{bc} ±1.016
La 0.1%+Cu 20ppm	9.15	8.93	7.72	6.95	8.18^{bc} ±1.037
La 0.1%+Cu 40ppm	9.10	8.34	7.66	6.87	7.99^{cd} ±0.952
La 0.2%+Cu 5ppm	7.49	6.90	5.95	5.02	6.34^{ef} ± 1.085
La 0.2%+Cu 10ppm	7.11	6.82	5.90	5.00	6.20^{ef} ± 0.956
La 0.2%+Cu 20ppm	6.03	6.02	5.55	4.66	5.56^{fg} ± 0.644
La 0.2%+Cu 40ppm	5.46	5.51	4.56	4.10	4.90^{gh} ±0.693
La 0.25%+Cu 5ppm	3.95	4.76	3.76	3.73	4.05^{hi} ± 0.483
La0.25%+Cu10ppm	3.87	4.69	3.71	3.70	3.99^{hi} ± 0.471
La0.25%+Cu20ppm	3.77	4.67	3.47	3.61	3.88^{hi} ± 0.541
La0.25%+Cu40ppm	3.53	4.39	3.42	3.35	3.67^{i} ± 0.484

Means (± standard deviation) within the same column followed by different letters are significantly different ($p < 0.05$).

similar to observations made by Ibrahim et al. (2008) showing a significant inhibitory effect of copper and lactic acid on *E. coli* O157:H7 in carrot juice. When a combination of 4 different concentrations of copper and 0.25% lactic acid was used, a significant growth inhibition of *E. coli* O157:H7 ($p < 0.05$) was observed as compared to the control and copper alone samples. However, combined treatments of copper (40 ppm) with 0.25% lactic acid were not effective in inhibiting the growth of *E. coli* O157:H7 when compared with the lactic acid only sample. A reduction of less than 1 log (0.85 log CFU/ml) was achieved compared to the lactic acid only sample. This indicates that the combination of lactic acid 0.2% with copper 20 and 40 ppm is significantly antimicrobial for *E. coli* O157:H7.

Hence, the use of copper in combination with lactic acid could be an effective method to inactivate pathogenic bacteria from the surfaces of produce. The efficacy of copper ion in killing microorganisms is greatly enhanced by the use of lactic acid. 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. It is generally believed that the antimicrobial species of organic acids are fully protonated species, which can freely cross cell membrane (Bjornsdottir, Breidt, & McFeeters, 2006). The antimicrobial activity occurs through the diffusion of lactic acid molecules into the cells, thereby inhibiting essential metabolic reactions and causing an accumulation of toxic anions and ultimate death of microbial cells (Brul & Coote, 1999). Therefore, a strong outer membrane disintegrating property of lactic acid could have helped the entry of copper ions into the cells, thus producing a

lethal effect. Results of this study clearly demonstrated that the combination of copper with lactic acid produces a synergistic inhibitory effect on the survival of *E. coli* O157:H7 strains grown in laboratory media.

4.3 Experiment 3: Determination of pH

The pH of the different concentrations of copper, lactic acid and their combinations ranges from 5.48-7.20, with the control of pH 7.22 (Table 4.3).

Table 4.3. Impact of Copper and Lactic Acid Treatments on pH Values

Treatments	pH
Control	7.22
Lactic acid 0.2%	5.74
Copper 20 ppm	7.20
Copper 40 ppm	7.10
Lactic acid 0.2% + Copper 20 ppm	5.69
Lactic acid 0.2% + Copper 40 ppm	5.48

Treatments containing lactic acid with copper 40 ppm had the lowest pH followed by lactic acid with copper 20 ppm. Lactic acid alone had the lowest pH (5.74) compared to copper alone at 20 and 40 ppm (7.20 and 7.10). Earlier studies have shown that most foodborne pathogens are susceptible to the lethal effect of low pH. However, there have

been several outbreaks even in acidified foods. Han and Linton (2004) reported that *E. coli* O157:H7 survived well at pH values of 3.4-6.8 and was implicated in several outbreaks in acidic fruits and juices. Results of the current study indicate that the pH (acidic property) is not the only factor influencing the survival activity of *E. coli* O157:H7. A pH higher than 5 indicated that there might be some other mechanism involved in the inhibition of bacterial growth other than the acidic medium. These results show that the effect of copper ion and lactic acid could be the key.

4.4 Experiment 4: Sanitizing Treatment and Bacterial Enumeration on the Surface of Lettuce and Tomatoes

Figures 4.9 and 4.10 show the populations of *E. coli* O157:H7 on the surface of lettuce and tomatoes samples treated with copper and lactic acid solution. The initial inoculum concentration of mixed strains of *E. coli* O157:H7 was approximately 9 log CFU/ml. The samples were submerged into the bacterial solution to mimic field or dumptank situations where the entire surface of lettuce and tomatoes may come in contact with a larger surface area compared with spray or spot methods, thereby increasing the attachment of larger number of cells (Lang, Harris, & Beuchat, 2004). When inoculated lettuce pieces and tomatoes were treated with peptone water (control), the microbial populations reached 8.31 log CFU/lettuce piece (Figure 4.9) and 7.01 log CFU/tomato (Figure 4.10). Rinsing inoculated lettuce piece and whole tomato with copper 20 ppm solution alone resulted in 0.66 log and 0.07 log reductions of *E. coli*

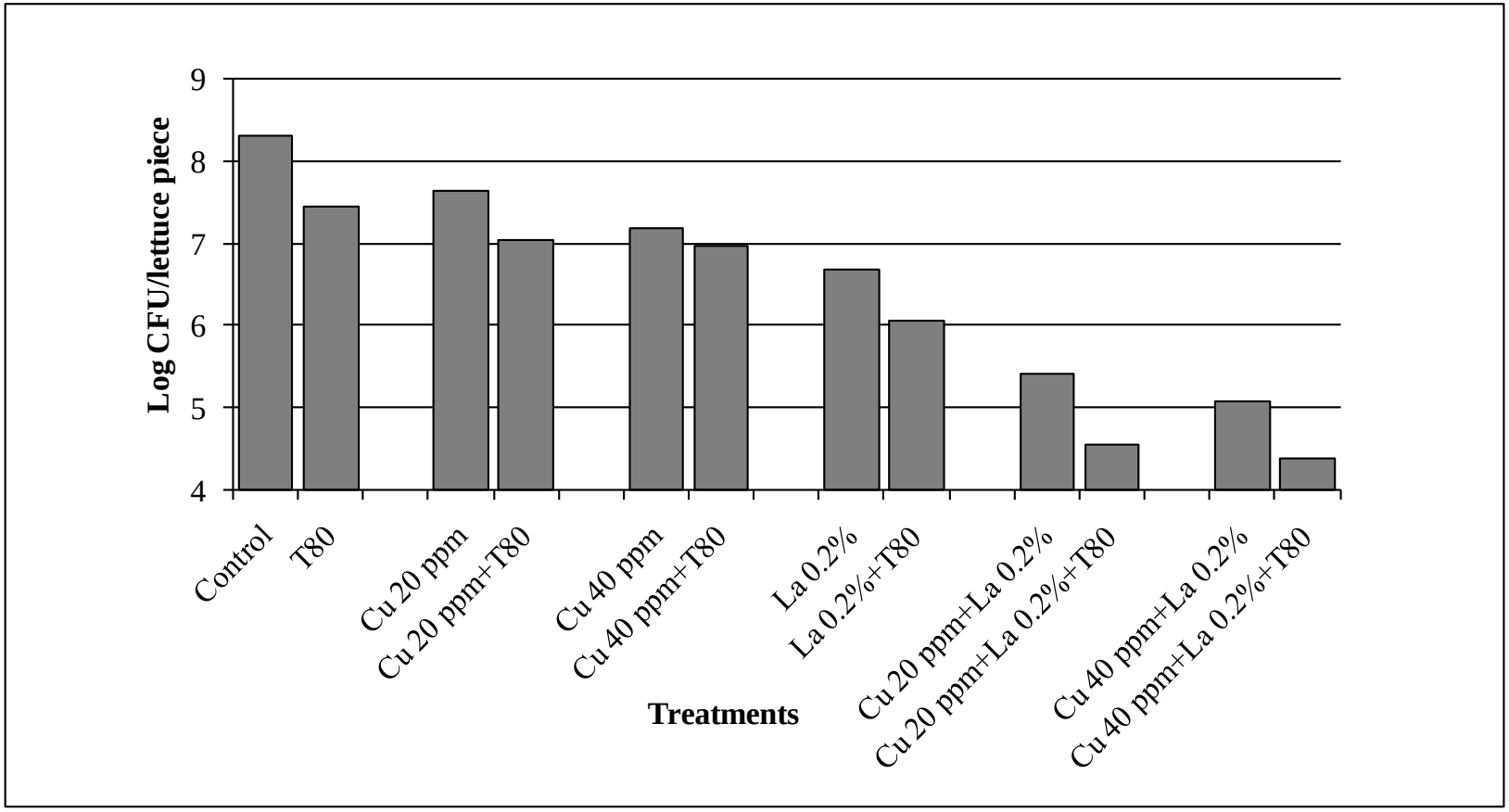

Figure 4.9. Effect of Copper (Cu) and Lactic Acid (La) Solutions Associated with and without Tween 80 (T80) on Mix 4 *E. coli* O157:H7 Strains Attached to the Surface of Lettuce

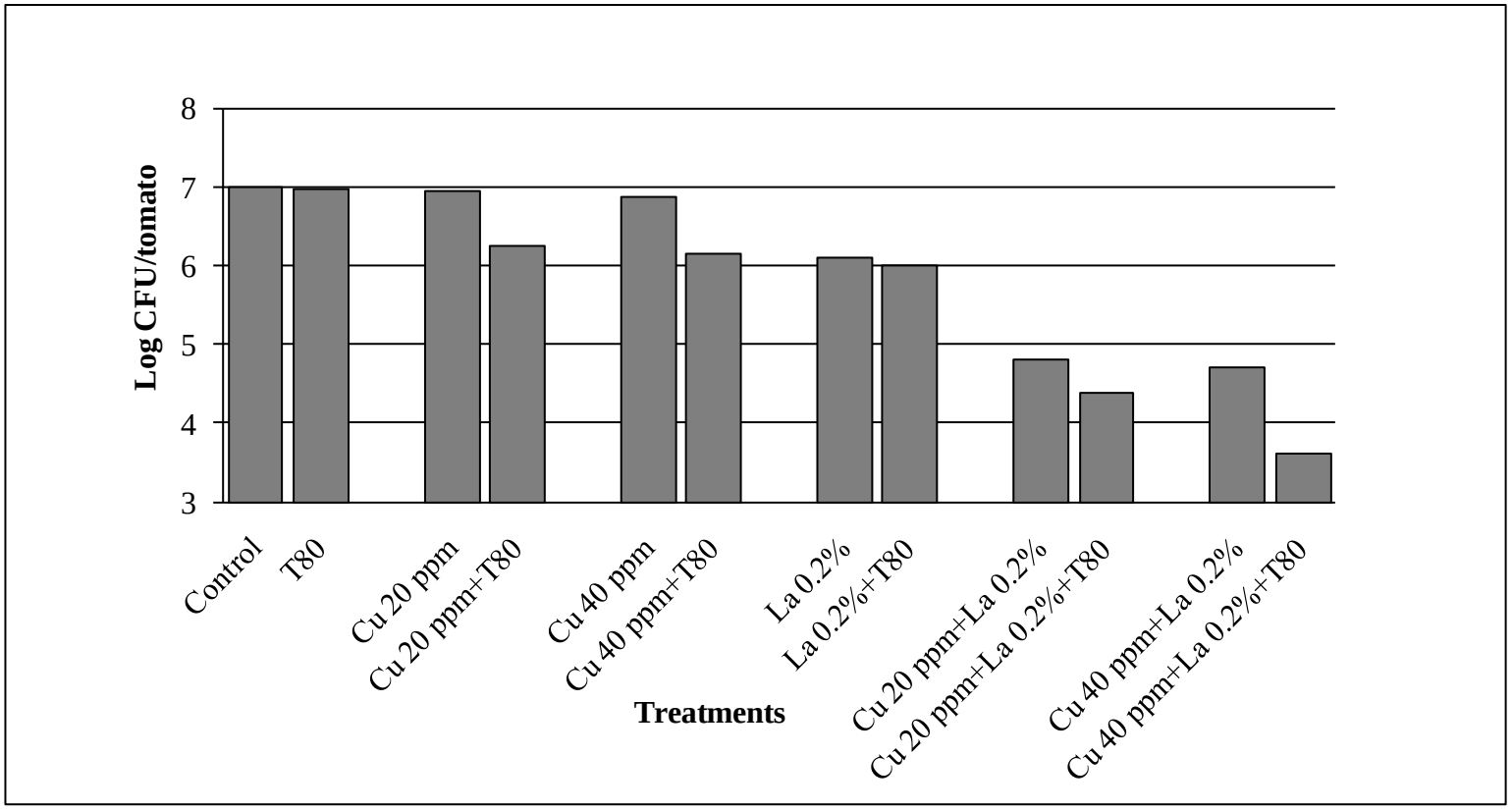

Figure 4.10. Effect of Copper (Cu) and Lactic Acid (La) Solutions Associated with and without Tween 80 (T80) on Mix 4 *E. coli* O157:H7 Strains Attached to the Surface of Tomato

O157:H7 respectively, while treatment with 40 ppm copper resulted in 1.12 log and 0.13 log reductions on lettuce and tomato surfaces respectively. However, 0.2% lactic acid produced a significant ($p < 0.05$) difference in microbial populations on both lettuce and tomato surfaces compared to the control.

When lettuce and tomato surfaces were treated with the combination of copper and lactic acid, reduction of higher populations of *E. coli* O157:H7 was achieved. When samples were rinsed with the combination of copper 20 ppm and 0.2% lactic acid, 2.89 log and 2.2 log reductions were achieved per lettuce piece and tomato respectively. Treatment with copper 40 ppm and 0.2% lactic acid resulted in 3.23 and 2.29 log reductions of *E. coli* O157:H7 from lettuce (Table 4.4) and tomato (Table 4.5) surfaces. The results also determined the effect of 0.1% Tween 80 in combination with copper and lactic acid on the removal of *E. coli* O157:H7 from the surfaces of lettuce and tomatoes. There was less than a 1 log reduction when the lettuce surface was treated with 0.1% Tween 80 alone, and there was no significant difference between the treated tomato surface and the control sample. Similarly, lettuce treated with copper 20 and 40 ppm with Tween 80 produced more than a log reduction. However, less than a log reduction was recovered from tomato surfaces than from lettuce undergoing the same treatment. With the addition of lactic acid (0.2%) to Tween 80 (0.1%), reductions of 2.25 log CFU/lettuce piece and one log CFU/tomato were observed. Figures 4.9 and 4.10 also show the number (< 1 log) of bacterial populations recovered from both lettuce and tomato samples rinsed with copper and lactic acid in the presence of Tween 80 as compared with individual treatments without Tween 80.

Table 4.4. Populations of Mix 4 *E. coli* O157:H7 Strains Recovered from Lettuce Surface after Treatment with Copper (Cu) and Lactic Acid (La) with and without Tween 80

	Populations (Log CFU/Lettuce Piece) of *E. coli* O157:H7	
Treatments	**Without Tween 80**	**With Tween 80 (0.1%)**
Control	$8.31^{a} \pm 0.014$	$7.46^{c} \pm 0.014$
La 0.2%	$6.67^{g} \pm 0.014$	$6.06^{h} \pm 0.007$
Cu 20 ppm	$7.65^{b} \pm 0.007$	$7.04^{e} \pm 0.014$
Cu 40 ppm	$7.19^{d} \pm 0.014$	$6.96^{f} \pm 0.014$
La 0.2%+Cu 20 ppm	$5.42^{i} \pm 0.014$	$4.54^{k} \pm 0.007$
La 0.2%+Cu 40 ppm	$5.08^{j} \pm 0.014$	$4.38^{l} \pm 0.007$

Means (± standard deviation) within the same column and row followed by different letters are significantly different ($p < 0.05$).

Table 4.5. Populations of Mix 4 *E. coli* O157:H7 Strains Recovered from Tomato Surface after Treatment with Copper (Cu) and Lactic Acid (La) with and without Tween 80

	Populations (Log CFU/tomato) of *E. coli* O157:H7	
Treatments	**Without Tween 80**	**With Tween 80 (0.1%)**
Control	$7.01^{a} \pm 0.014$	$6.98^{a} \pm 0.021$
La 0.2%	$6.11^{f} \pm 0.014$	$6.01^{g} \pm 0.021$
Cu 20 ppm	$6.94^{b} \pm 0.007$	$6.24^{d} \pm 0.014$
Cu 40 ppm	$6.88^{c} \pm 0.014$	$6.15^{e} \pm 0.014$
La 0.2%+Cu 20 ppm	$4.81^{h} \pm 0.021$	$4.38^{j} \pm 0.007$
La 0.2% +Cu 40 ppm	$4.72^{i} \pm 0.014$	$3.62^{k} \pm 0.021$

Means (± standard deviation) within the same column and row followed by different letters are significantly different ($p < 0.05$).

Results revealed that treatment with Tween 80 alone or in combination with copper or lactic acid did not substantially increase the bacterial reductions from the produce surfaces. However, the treatment of lettuce and tomatoes with combination of copper, lactic acid plus Tween 80 effectively reduced populations of *E. coli* O157:H7 from the produce samples. Lettuce and tomato treated with copper 20 ppm and 0.2% lactic acid with 0.1% Tween 80 reduced bacterial populations by 3.77 log and 2.63 log. When samples were treated with a higher concentration of copper (40 ppm) and both 0.2% lactic acid and Tween 80, higher microbial reductions on both produce surfaces were achieved. Rough lettuce surface with pores and folds would have provided higher attachment rates compared to the smooth surface of tomatoes. The populations on lettuce and tomato surfaces were reduced by 3.93 log CFU/lettuce piece and 3.39 log CFU/tomato respectively. The results thus indicate that copper 40 ppm with 0.2% lactic acid in the presence of Tween 80 was significantly more effective ($p < 0.05$) in removing pathogenic bacteria from the produce surfaces.

The overall results of this study indicated that a combination of copper and lactic acid was inhibitorier against *E. coli* O157:H7 than individual treatments of the two ingredients. Slightly larger populations of *E. coli* O157:H7 were recovered from lettuce samples than tomatoes, which may be due to the differences in their surface structure. When Tween 80 was added to the treatment solution, a higher recovery of bacterial populations was achieved from both produce types. Tween 80 is an ionic surfactant approved by the U.S Food and Drug Administration (FDA) and also generally recognized as a safe (GRAS) product. Addition of Tween 80 might have enhanced the lethality of

copper and lactic acid solution by increasing the surface contact of the solution with the microbes, thereby maximizing the release of pathogens from inoculated lettuce and tomato surfaces.

Results obtained from this study could improve the safety and establish an effective natural ingredient for the decontamination of fresh produce. The results showed that the combination of 40 ppm copper and 0.2% lactic acid can reduce populations of *E. coli* O157:H7 by an average of 5.03 log in laboratory medium. Similarly, when lettuce and tomato samples were treated with solution containing 40 ppm copper and 0.2% lactic acid with 0.1% Tween 80, reductions of 3.93 (Table 4.4) and 3.39 log (Table 4.5) were achieved respectively. Copper in combination with lactic acid may produce a synergy that reduces the number of pathogenic microorganisms, including *E. coli* O157:H7, on the surfaces of lettuce and tomatoes. This solution could be a potential decontaminant for fresh produce.

CHAPTER 5

CONCLUSIONS

In this project, four experiments were performed to determine: (a) the effect of copper and lactic acid on the survival and growth of *E. coli* O157:H7 in laboratory medium by measuring optical density, (b) the effect of copper and lactic acid on the survival and growth of *E. coli* O157:H7 in laboratory medium by counting colony forming units (CFU/ml), (c) the effect of pH on the survival and growth of *E. coli* O157:H7 in laboratory medium, and (d) the effect of combined copper and lactic acid solution to reduce *E. coli* O157:H7 on the surface of lettuce and tomatoes.

In the first experiment, lactic acid 0.2% and 0.25% were able to inhibit the population of *E. coli* O157:H7 after 8 hr of incubation at 37 ºC. Significant synergistic inhibition caused by the addition of 0.2% lactic acid to either 20 or 40 ppm of copper was observed for all *E. coli* strains tested. Results of O.D. readings due to the growth of *E. coli* were significantly lower for the combination treatment as compared to the control or individual treatment of copper and lactic acid. The second experiment was performed to enumerate bacterial population by counting CFU/ml after 8 hr of incubation at 37 ºC. The significant differences in microbial log reductions were observed in colony forming units with combination treatments of copper (20 and 40 ppm) and lactic acid (0.2%). The average log reduction of 5.03 was achieved by the addition of copper 40 ppm to 0.2% lactic acid when compared to the control (9.93) for all strains. However, in both experiments, 0.25% of lactic acid plus copper concentration (5, 10, 20 and 40 ppm) did

not have a larger effect as compared to the individual treatment. Less than 1 log reduction was obtained as compared to the lactic acid alone sample. Therefore, from these results, it can be inferred that 0.2% of lactic acid is sufficient to inhibit microorganisms with copper 20 and 40 ppm.

The pH levels of treatment solutions to determine the effect on bacterial survival and growth was also studied. The pH values observed were between 5.48 and 5.74, the lowest among all test samples was found for combination sample of lactic acid 0.2% plus 40 ppm copper. Previous studies have reported that *E. coli* O157:H7 survived well at pH values of 3.4 to 6.8 and have been implicated in several outbreaks in acidic fruits and juices. Therefore, results of the current study indicated that pH was not the only factor responsible for the bacterial inactivation in the media tested. The bacterial cells could have survived well in the range of pH higher than 5. The turbidity (O.D. 610 nm) readings and log CFU/ml observed for the combination treatment in the current study were very low. These findings clearly showed that there is a synergistic effect of copper and lactic acid for the inactivation of *E. coli* O157:H7 rather than the effect of pH and individual treatments. The mechanism involved with this is not well understood. However, it is believed that permeabilizer properties of lactic acid could have made it easier for copper ions to enter the cells and may have produced toxic effects.

The final experiment was conducted on the surface of lettuce and tomatoes. The effect of copper and lactic acid solution against mix *E. coli* O157:H7 strains present on produce surfaces was observed. Population reductions obtained in both inoculated samples surface rinsed with peptone water containing copper 40 ppm with 0.2% lactic

acid were higher followed by 20 ppm of copper plus 0.2% lactic acid. The higher number of bacterial attachments was observed in lettuce leaves than tomatoes due to its rough surface with folds and pores. It was also observed that when treatment solutions containing Tween 80 (0.1%) were used as surface rinse, higher bacterial populations were recovered from both lettuce and tomato surfaces. Log reduction of 3.39 and 3.93 were achieved with combination of copper 40 ppm, 0.2% lactic acid and Tween 80 (0.1%) on tomatoes and lettuce surfaces respectively. The results obtained from this study indicated that the copper and lactic acid solution was more effective with Tween 80 to reduce *E. coli* O157:H7 attached on produce surfaces. The efficacy of treatment could be the result of the detachment of the cells to the surfaces rather than antimicrobial effect of Tween 80.

This work showed that the use of natural ingredients such as copper and lactic acid could be used as sanitizers to reduce the bacterial population on fresh produce in order to provide consumers a product with higher microbiological safety. This study also showed that a combined treatment was more effective than individual treatments to inactivate *E. c*oli O157:H7. Additional study on the role of antimicrobial activity of copper and lactic acid to reduce gram-positive bacteria on produce surfaces is needed. Further studies can be done with combining other weak organic acids to see the effect of copper ion to inactivate pathogens. More studies are needed to explore the mechanisms of toxicity of copper ions and lactic acid to microorganisms. The cell morphology could be studied under confocal scanning laser microscopy to see the structural differences between treated and untreated cells. Results of these studies also suggest that copper and

lactic acid can be used in other food systems such as beverage and juice to inhibit or eliminate foodborne pathogens.

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