THE DEVELOPMENT OF ALTERNATIVE METHODS OF DISINFECTION

by

Enue Erdemely Sicairos Ruelas

A Dissertation Submitted to the Faculty of the

DEPARTMENT OF SOIL, WATER AND ENVIRONMENTAL SCIENCE

In Partial Fulfillment of the Requirements
For the Degree of

DOCTOR OF PHILOSOPHY

In the Graduate College

THE UNIVERSITY OF ARIZONA

2007

THE UNIVERSITY OF ARIZONA
GRADUATE COLLEGE

As members of the Dissertation Committee, we certify that we have read the dissertation prepared by Enue Erdemely Sicairos Ruelas

entitled THE DEVELOPMENT OF ALTERNATIVE METHODS OF DISINFECTION

and recommend that it be accepted as fulfilling the dissertation requirement for the Degree of Doctor of Philosophy

_____ Date: 05/31/07
Charles P. Gerba, Ph.D

_____ Date: 05/31/07
Allan D. Matthias, Ph.D

_____ Date: 05/31/07
Lynn A. Joens, Ph.D

_____ Date: 05/31/07
Kelly R. Bright, Ph.D

Final approval and acceptance of this dissertation is contingent upon the candidate's submission of the final copies of the dissertation to the Graduate College.

I hereby certify that I have read this dissertation prepared under my direction and recommend that it be accepted as fulfilling the dissertation requirement.

_____ Date: 05/31/07
Dissertation Director: Charles P. Gerba

STATEMENT BY THE AUTHOR

This dissertation has been submitted in partial fulfillment of the requirements for an advanced degree at the University of Arizona and is deposited in the University Library to be made available to borrowers under rules of the Library.

Brief quotations from this dissertation are allowable without special permission, provided that accurate acknowledgment of source is made. Request permission for extended quotation from or reproduction of this manuscript in whole or in part may be granted by the head of the major department or the Dean of the Graduate College when in his or her judgment the proposed use of the material is in the interests of scholarship. In all other instances, however, permission must be obtained from the author.

SIGNED: Enue Erdemely Sicairos Ruelas

ACKNOWLEDGMENTS

I would like to thank Dr. Charles P. Gerba for his guidance, support and the opportunity to obtain my Ph.D. in his laboratory. I would especially like to thank Dr. Kelly R. Bright for her helpful suggestions, support and continuous encouragement throughout this research. I thank my committee members Dr. Lynn A. Joens, Dr. Allan D. Matthias and Kevin M. Fitzsimmons for their valuable time. I would also like to thank all the members of the laboratory for making my work much more enjoyable.

DEDICATION

This dissertation is dedicated to my family (Mom, dad, brothers, sister, aunts, uncles and cousins) for their constant love, support, encouragement and understanding.

TABLE OF CONTENTS

TABLE OF CONTENTS – *Continued*

TABLE OF CONTENTS – *Continued*

ABSTRACT

Chlorine is the primary method for disinfection of drinking water in the United States; however, growing concerns about the potential hazards associated with carcinogenic chlorine disinfection by-products have resulted in increased efforts to develop alternative methods of water disinfection. In addition, it is sometimes difficult to maintain an adequate concentration of free chlorine throughout a drinking water distribution system due to intrusion events and the presence of biofilms.

Silver and copper are widely used as environmental biocides and as clinical antimicrobial agents. Copper has been extensively used as an algaecide for many years, and is reported to be one of the most toxic metals to heterotrophic bacteria in aquatic environments. In this study, silver and copper, both individually and in combination, were able to significantly reduce the numbers of the bacterial pathogens *Salmonella typhimurium, Escherichia coli, Listeria monocytogenes,* and *Mycobacterium fortuitum* in aqueous solutions. Silver and copper together could potentially be used as a secondary disinfectant in water treatment because their residual effect is long lasting and no harmful byproducts are generated.

Besides water, produce can also be a source of microbial pathogens. Fruits and vegetables are the third leading source in microbial foodborne outbreaks, accounting for more than 10,000 cases of foodborne illness in the U.S. between 1990 and 2001. Alternative produce sanitizers in addition to chlorine were evaluated against foodborne

pathogens inoculated onto lettuce. Overall, Fit® (citric acid, grapefruit oil extract) and chlorine yielded the greatest reductions of the study organisms.

A large number of compounds are available for use as disinfectants; however, some are highly toxic, corrosive and produce harmful by-products. Natural antimicrobial products are another possible alternative. The efficacy of a natural peptide-based antimicrobial, Absolute Fx, was assessed against bacteria and viruses. Absolute Fx effectively inactivated the study organisms.

INTRODUCTION

Problem Definition:

Filtration and chlorination of drinking water have dramatically decreased waterborne disease risks in the United States, especially typhoid fever mortality. Nevertheless, outbreaks associated with contaminated drinking water still occur. In addition, a substantial portion of waterborne illnesses may not be reported. Between the years of 1920 and 2002, at least 1,870 outbreaks were associated with drinking water, an average of 23 per year. This translates into approximately 900,000 reported illnesses (Craun *et al.* 2006). Outbreaks were associated with inadequately treated water and the contamination of distribution systems. Bacterial growth, as well as the presence of microorganisms of health concern (e.g. *Legionella pneumophila*) have been reported in chlorinated distribution systems (Kuchta *et al.* 1983; LeChevallier *et al.* 1987). Furthermore, the residual disinfectant (generally 0.2 mg/L chlorine) is severely reduced to nearly non-detectable levels as a result of the reaction with organic matter, biofilms, and tubercles formed on the pipes (Al-Jasser 2007; Ndiongue *et al.* 2005). This results in a vulnerable distribution system that may be further contaminated with microbial pathogens thorough intrusion events during pipe repair, illegal cross-connections, and the loss of pressure in the system. Water supply systems, water storage systems, and swimming pools are also some of the most attractive and susceptible targets for bioterrorism. Therefore, there is an

urgent need to evaluate alternative disinfectants that can provide a long-lasting residual effect by significantly reducing the number of human pathogens in water.

Fruits and vegetables are the third leading cause associated with foodborne outbreaks, accounting for more than 10,000 cases of foodborne illness from 1990 to 2001 (CSPI 2001). Recently, an outbreak of enterohemorrhagic *Escherichia coli* O157:H7 affected more 200 people, of which 30% developed a life-threatening complication, hemolytic uremic syndrome (HUS), and three people died (Anonymous 2006). The outbreak was associated with bagged spinach. *Listeria monocytogenes* has been linked to infection following the consumption of contaminated prepackaged salads, cabbage, lettuce, celery, and tomatoes (Berrang *et al.* 1989). *L. monocytogenes* is a major public health concern due to its high mortality rate (~20%) and its ability to grow at refrigeration temperatures (Gandhi and Chikindas 2007; Mead *et al.* 1999). Another group of microorganisms of health concern are viruses, which have emerged as the leading cause of foodborne illness in the United States (Allwood *et al.* 2005). A proper sanitizer treatment is needed at the point of consumption to reduce the risk of contracting a foodborne illness.

Literature Review:

Foodborne pathogens

The food supply in the United States is considered one of the safest in the world (Johnston *et al.* 2006). In fact, the estimated incidence of foodborne diseases in the U.S., including those caused by *Escherichia coli* 0157:H7, *Campylobacter* and *Cryptosporidium*, declined between 1996 and 2001 (CDC 2003). Nevertheless, in the United States alone, it is estimated that 76 million people contract foodborne illness each year, with an associated 325,000 hospitalizations and 5000 deaths (Mead *et al.* 1999). This means than one in four Americans contracts a foodborne illness, and greater than 1 in 1000 is hospitalized each year (Tauxe 2002). The approximate annual cost of such foodborne illnesses is $5 billion in direct medical expenses and lost productivity (Vij *et al.* 2006). Bacteria account for the highest number of deaths attributable to foodborne transmission (72%), followed by parasites (21%) and viruses (7%). *Salmonella* is responsible for highest number of deaths (31%), followed by *Listeria* (28%), *Toxoplasma* (21%), Noroviruses (7%), *Campylobacter* (5%), and *Escherichia coli* O157:H7 (3%) (Mead *et al.* 1999). Extrapolating the U.S. data to the rest of the world would mean that up to one-third of the population in developed countries are affected by microbiological foodborne disease each year, while the problem is likely to be even more widespread in developing countries (Schlundt 2002). Food safety is one of the top eleven priorities of the World Health Organization, which has called for systematic and more aggressive

steps to reduce the risk of foodborne diseases due to microbial contamination (WHO 2000).

The occurrence and epidemiology of foodborne disease in a population is the result of complex interactions among environmental, cultural and socioeconomic factors (Johnston *et al.* 2006). As new food sources become available as a result of the globalization of the market and changes in food consumption trends, new opportunities arise for the transmission of foodborne disease (Harris *et al.* 2001; Stine *et al.* 2005). Changes in human demographics and behavior, as well as the development of new virulence factors by microorganisms, may also contribute to the increase of the risk of foodborne illness (Beuchat 1998; Beuchat *et al.* 2001).

Fruits and vegetables have been associated with numerous foodborne outbreaks with an increasing number of outbreaks in recent years (Guan *et al.* 2005; Yaun *et al.* 2004). In the United States, the consumption of vegetables per capita per year was 153 kg in 1980, 193 kg in 2000, and 188 kg in 2001. Fresh vegetables accounted for 45% of the total vegetable consumption in 1980, 47% in 2000, and 48% in 2001. During 2001, the most commonly consumed fresh vegetables were lettuce, tomatoes, carrots, potatoes, and onions (Sturchler 2006). Fruits and vegetables are the third leading vehicle of microbial pathogens in foodborne outbreaks, accounting for more than 10,000 cases of foodborne illness from 1990 to 2001 (CSPI 2001). Cantaloupes, sprouts, melons, lettuce and berries were most frequently involved in the outbreaks (Matthews 2006).

The majority of pathogens implicated in produce-related outbreaks are transmitted via the fecal-oral route (Johnston *et al.* 2006). Microbial contamination of fruits and

vegetables can occur during plant growth, harvesting, transport, processing, distribution and marketing, or in the home (Beuchat 1998). Produce contaminated with pathogens cannot be completely disinfected by washing or rinsing the product in an aqueous solution (Rodgers and Ryser 2004; Shigenobu *et al.* 2003; Smith *et al.* 2003). The prevention of produce contamination with human pathogens is the only practical and effective means of ensuring safe produce for human consumption (Gorny 2006). There is therefore a need for effective sanitizer treatments to reduce the number of microbial pathogens on produce to safer levels.

Escherichia coli

Enterohemorrhagic *E. coli* (EHEC) was first recognized as a human pathogen in 1982 when it was identified as the cause of two outbreaks of hemorrhagic colitis associated with undercooked hamburgers served at fast food restaurants (Smith 2005). Since then, sporadic infections and outbreaks have been reported in many parts of the world, including North America, Australia, Canada, Western Europe, and Africa (Honish *et al.* 2007; Mannix *et al.* 2005; Raji *et al.* 2003; Rangel *et al.* 2005). *E. coli* O157:H7 is the EHEC serotype responsible for the greatest proportion of disease cases (Smith 2005). *E. coli* O157:H7 is the most common serotype found in North America (Torres *et al.* 2006). It is estimated that the O157:H7 serotype is responsible for more than 73,000 cases of illness and 61 deaths each year in the United States, whereas non-O157 EHEC cause approximately 36,000 cases and 30 deaths each year (Mead *et al.* 1999). *E. coli* O157:H7 infections lead to a mild non-bloody diarrhea or an acute grossly bloody diarrhea termed

hemorrhagic colitis. In about 8% of patients, primarily children and the eldery, *E. coli* O157:H7 infection can progress to hemolytic uremic syndrome (HUS), a severe post-diarrheal systemic complication. HUS is the leading cause of renal failure in children in the United States (Li *et al.* 2000). Undercooked ground beef has been implicated in most of the documented EHEC outbreaks; however, *E. coli* O157:H7 has been isolated from other products including fresh fruits and vegetables (Griffin and Tauxe 1991; Smith *et al.* 2003). Very few surveys have been conducted on the presence of the organism in raw produce (Harris *et al*. 2003). Produce related outbreaks are often associated with restaurants and other food establishments. Restaurants accounted for 40% of the *E. coli* O157:H7 outbreaks associated with produce, and approximately 47% were the result of cross-contamination during food preparation (Rangel *et al.* 2005).

Some studies have quantified the survival of *E. coli* O157:H7 on growing crops under both laboratory and greenhouse conditions (Cooley *et al.* 2006; Lang *et al.* 2004b; Solomon *et al.* 2003). Stine and coworkers (2005) found that *E. coli* O157:H7 survived on cantaloupe surfaces for more than two weeks under greenhouse conditions. They also found that the survival of the pathogen was enhanced under high humidity conditions. *E. coli* O157:H7 was found to survive for 12 days at 4°C on lettuce, swedes, bean sprouts and dry coleslaw (Francis and O'Beirne 2001). *E. coli* O157:H7 inoculated on lettuce increased significantly in number when it was kept at 12°C for 14 days. Packaging under modified atmosphere had little or no effect on the survival or growth of *E. coli* O157:H7 (Abdul-Raouf *et al.* 1993).

In Mexico *E. coli* O157:H7 was isolated on at least 20% of cabbage, cilantro, and coriander involved in one study (Zepeda-Lopez *et al.* 1995). Samadpour *et al.* (2006) isolated *E. coli* O157:H7 from 1.5% of sprout samples (Samadpour *et al.* 2006). On the other hand, several studies have reported the absence of *E. coli* O157:H7 on produce (FDA 2001; Johannessen *et al.* 2002; Johnston *et al.* 2006). It should be noted that in the studies in which *E. coli* O157:H7 was not detected, sample sizes ranged from 466 to over 1000 produce items. Nevertheless, outbreaks of *E. coli* O157:H7 have been associated with lettuce (Ackers *et al.* 1998; Hilborn *et al.* 1999), radish sprouts (Michino *et al.* 1999), apple cider (Cody *et al.* 1999; Hilborn *et al.* 2000), and alfafa sprouts (Ferguson *et al.* 2005). One recent outbreak was linked to contaminated spinach in which 200 people were affected, more than half of which were hospitalized, and three died (Anonymous 2006). This was at least the 26th reported outbreak of *E. coli* O157:H7 infection that has been traced to contaminated leafy green vegetables since 1993 (Maki 2006). The world's largest reported *E. coli* O157:H7 outbreak occurred in Japan in 1996 and was linked to the consumption of white radish sprouts. Approximately 6,000 school children were infected and 17 died (Michino *et al.* 1999). In the following year, white radish sprouts were once again implicated in another *E. coli* O157:H7 outbreak in Japan, affecting 126 people and resulting in one death (Taormina *et al.* 1999).

Salmonella

Salmonella is the most common cause of bacterial foodborne disease outbreaks of known etiology in the United States (CDC 2000). Non-typhoidal *Salmonellae* are also the most

common cause of death from foodborne illness associated with parasites, viruses or bacteria in the U.S., causing more than 500 deaths each year (Mead *et al.* 1999). *Salmonella* infections generate a $2.3 billion annual cost for medical care and loss of worker productivity (Mead *et al.* 1999). Typhoid fever and bacteremia are rare in the U.S.; however, they are still a significant cause of morbidity in Africa, Asia and South America (Andrews *et al.* 2005).

Mammals, amphibians, birds, and reptiles are natural reservoirs of *Salmonella* (Dobbin *et al.* 2005; Hidalgo-Vila *et al.* 2007). Poultry, other meat products, eggs, and dairy products are the most commonly implicated sources in salmonellosis outbreaks. Fresh fruits and vegetables are implicated less frequently, though outbreaks involving these commodities have been documented (Harris *et al.* 2003). Several surveys have reported the presence of various *Salmonella* serotypes on certain produce items. In 2001, the Food and Drug Administration conducted a survey of more than 1,000 imported produce from 21 countries of which *Salmonella* was detected in 35 samples (FDA 2001). The contaminated produce included celery, cilantro, lettuce, cantaloupe, scallions, parsley, and strawberries (FDA 2001). In an Italian study, *Salmonella* was found in 68.3% of lettuce and 71.9% of fennel samples (Ercolani 1976). In contrast, in another study that included U.S. and Mexican fresh produce, *Salmonella* was not isolated from broccoli, cabbage, cilantro, parsley, cantaloupe, collards, and kale with a total of 466 samples (Johnston *et al.* 2006). In the United States, several outbreaks of salmonellosis have been linked with produce, particulary cantaloupe. A large outbreak in 1990 involved cantaloupe served in salad bars that affected more than 200 people in 30 states (Ries *et*

al. 1990). In 1997, another *Salmonella* outbreak was reported in California due to the consumption of imported cantaloupe (Mohle-Boetani *et al.* 1999). Thereafter, three high profile multistate outbreaks with *Salmonella enterica* serovar Poona associated with eating cantaloupe imported from Mexico occurred during the spring of consecutive years between 2000 and 2002 (CDC 2002). Outbreaks of salmonellosis have also been linked to tomatoes (Anonymous 2005; Gupta *et al.* 2007), alfalfa sprouts (Proctor *et al.* 2001; Van Beneden *et al.* 1999), and bean sprouts (O'Mahony *et al.* 1990). In one study, *Salmonella* was able to survive for six days on tomato leaves under 100% relatively humidity condition (Rathinasabapathi 2004). Das *et al.* (2006) demonstrated that *Salmonella enteritis* can survive at 7°C for 10 days on inoculated cherry tomatoes (Das *et al.* 2006). Acidic foods such as orange juice which were once thought to be unlikely vehicles of *Salmonella* have recently been associated with outbreaks (Buxton *et al.* 1999; Krause *et al.* 2001).

Listeria monocytogenes

Listeria was originally isolated from laboratory rabbits in 1926 (Paoli *et al.* 2005). Although the organism has been used for decades as a model for the study of intracellular parasitism, *Listeria monocytogenes* was not considered a significant pathogen until the early 1980's, when it was recognized as a major foodborne human pathogen (Ramaswamy *et al.* 2007; Vazquez-Boland *et al.* 2001). *Listeria* was identified as an emergent pathogen after an outbreak of listeriosis in Novia Scotia, Canada in 1981 that was traced back to the consumption of contaminated coleslaw (Schlech *et al.* 1983).

Listeria is now a major public health concern because of its severe manifestations. In humans, this microorganism has the ability to cross the intestinal, placental and blood brain barriers, leading to gastroenteritis, maternofetal infections, and meningoencephalitis, respectively (Lecuit 2005; Nassif *et al.* 2002; Ramaswamy *et al.* 2007). *Listeria* is the leading cause of death among foodborne bacteria in the United States, with a very high mortality rate (~20%) (Mead *et al.* 1999). *Listeria* is widely distributed in the environment. It is found in soil and water, on decaying plant material growing as a saprophyte, and in the intestinal tract of animals and humans. Its presence on fruits and vegetables is therefore not surprising (Beuchat 1998; Budu-Amoako *et al.* 1992). *Listeria monocytogenes* was isolated from potatoes, radishes, cabbage, and cucumbers during a survey of 10 different types of produce that included 1000 samples at the retail level (Heisick *et al.* 1989). In another survey in Spain, *L. monocytogenes* was isolated from 7.8% of vegetables samples (De Simon *et al.* 1992). Harvey and Gilmour (1993) reported that 7 out of 66 sample salad vegetables and prepared salads in Northern Ireland were contaminated by this pathogen (Harvey and Gilmour 1993). In a study conducted in Malaysia, *L. monocytogenes* was detected in 22% of the leafy vegetable samples, 85% of the beans sprouts, and 80% of cucumbers (Arumugaswamy *et al.* 2005). In Taiwan, *L. monocytogenes* was detected in 12% of vegetables included in a study conducted by Wong and coworkers (1990).

In addition to being a deadly pathogen, *L. monocytogenes* is well known for its growth and survival in foods. The bacterium is able to survive and even grow at low temperatures (Gandhi Megha and Chikindas 2007; Le Marc *et al.* 2002), and is able to

tolerate high salt and bile concentrations (Pearson and Marth 1990) as well as a low pH (Davis *et al.* 1996). *L. monocytogenes* is able to reproduce on cauliflower, broccoli, and asparagus in storage at 4°C (Berrang *et al.* 1989). Growth of *L. monocytogenes* has also been observed on lettuce at 5°C (Koseki and Isobe 2005; Steinbrugge *et al.* 1988) and on whole tomatoes held at 21°C for 10 days, but not at 10°C (Beuchat and Brackett 1991). Survival of *L. monocytogenes* has been reported in frozen strawberries (Flessa *et al.* 2005) and peeled Hamlin oranges at 4°C (Pao *et al.* 1998). Interestingly, carrot juice has been reported to have an inhibitory effect on *L. monocytogenes* growth (Beuchat and Brackett 1990; Beuchat and Doyle 1995; Nguyen-The and Lund 1991).

Produce Sanitizers

Microorganisms can be present in raw or minimally processed fruits and vegetables at populations ranging from 10^3 to 10^9 CFU/g (Francis *et al.* 1999; Harris *et al.* 2001). Washing with tap water is a currently recommended means for reducing microbial contamination on produce; however, though washing fruits and vegetables in water may remove some soil and other debris, it cannot be relied upon to completely remove pathogenic microorganisms and can result in the cross-contamination of food preparation surfaces, utensils, and other foods items (Beuchat *et al.* 2001; Koseki and Isobe 2005). Fruits and vegetables may be exposed to soil, insects, animals, water, and/or humans during growing or harvesting and in the processing plants (Ukuku 2006). Additionally, crops may be fertilized with the manure from domestic animals (Ingham *et al.* 2005). Consequently, fruits and vegetables intended for raw consumption may be exposed to pathogenic bacteria, parasites, and viruses from animal excreta (birds, insects, rodents, and reptiles), from water (irrigation and rain), from infected workers, from manure, and from food processing facilities with poor sanitation (Guo *et al.* 2002). Therefore, methods that effectively and reliably reduce or eliminate pathogenic bacteria on fruits and vegetables are needed. Traditional methods of reducing microbial populations on produce involve chemical and physical treatments. The control of contamination requires that these treatments be applied to equipment and packinghouse facilities as well as to produce (Beuchat 1998).

Chlorine

Chlorine is the most commonly used sanitizing agent. It is used at a concentration 50 to 200 parts per million (ppm) with a contact time between 1 and 2 minutes (Adams *et al.* 1989; Behrsing *et al.* 2000; Beuchat 1998). Three forms have been approved for use: calcium hypochlorite ($CaClO_2$), sodium hypochlorite (NaOCl), and chlorine gas (Cl_2) (Stopforth *et al.* 2007). The antimicrobial activity of chlorine depends on the amount of free available chlorine in the solution, the temperature, the pH, and the amount of organic matter present (Landeen *et al.* 1989; Yahya *et al.* 1990). Low pH of internal produce tissue and high load of organic matter in the sanitizing solution significantly reduce the effectiveness of chlorine (Stopforth *et al.* 2007). Dipping produce in chlorine is broadly used because of its ease of implementation and low cost, but appears to have limited efficacy, resulting in only a 1 to 2 $\log_{10}$ reduction in the microbial load (Koseki and Isobe 2005; Smith *et al.* 2003; Yuk *et al.* 2006). A standard procedure for washing leaves in tap water reduced populations of microflora by 92%; the addition of 100 ppm of chlorine at pH 9 reduced the microbial count by 97.8%, indicating that this concentration of chlorine was only slightly more effective than tap water alone (Adams *et al.* 1989). No significant difference in the inactivation of *Salmonella* inoculated on the surface of tomatoes was observed when the concentration of chlorine was increased from 100 ppm to 320 ppm (Zhuang *et al.* 1995). Treatment of produce with even higher concentrations of chlorine (> 800 ppm) have also been studied (Alvarado-Casillas *et al.* 2007). Beuchat et al. (1998) demonstrated that the maximum reduction in human pathogen population in lettuce, tomatoes and apples was 2.3-$\log_{10}$ CFU/cm^2 after dipping in solutions of 2000 ppm

chlorine for 1 min (Beuchat 1998). Only a 90% reduction in *Salmonella* inoculated on fresh-cut cantaloupe was achieved after treatment with 2000 ppm of chlorine (Beuchat and Ryu 1997). Chlorine treatments may reduce the initial bacterial population; however, Li *et al.* (2001a) reported that after a few days, no significant difference was found in *E. coli* O157:H7 populations on chlorine washed and water washed lettuce (Li *et al.* 2001a). Similarly, the number of yeasts, molds, *Enterobacteriaceae,* and mesophilic aerobic microorganisms after treatment with 20 ppm at 50°C did not differ from the control over a storage period of 18 days (Li *et al.* 2001b). The addition of a surfactant may enhance the lethality of chlorine by increasing the surface contact of the sanitizer with the microbe (Weissinger and Beuchat 2000). The addition of 0.1% of the surfactant Tergitol to a dip solution of 100 ppm chlorine yielded a 2.73-$\log_{10}$ reduction in CFU/g of *Yersinia enterocolitica* on the surface of shredded lettuce. Adding 0.5% lactic acid to the same solution resulted in more than a 6-$\log_{10}$ CFU/g reduction (Escudero *et al.* 1999). Nevertheless, the use of surfactants or other agents in combination with chlorine can adversely affect the sensory qualities (e.g. taste, appearance) of the produce (Beuchat 1998). Because chlorine reacts with organic matter, components leaching from tissues of cut produce surfaces may neutralize some of the chlorine before it reaches microbial cells, thereby reducing its effectiveness. Crevices, cracks and small fissures in produce tissue, along with the hydrophobic nature of the waxy cuticle on the surfaces of many fruits and vegetables, may reduce the inactivation of microorganisms by chlorine and other disinfectants (Burnett *et al.* 2004; Lang *et al.* 2004a; Ukuku and Fett 2006).

Safety concerns about the formation of carcinogenic disinfection by-products due to the reaction of chlorine with organic matter and their impact on the environment have increased in recent years (Guzzella *et al.* 2005; Villanueva *et al.* 2007; Zwiener *et al.* 2007). For this reason, chlorine treatment of minimally processed vegetables is prohibited in several European countries, including Belgium, Germany, Switzerland, and The Netherlands (Gil and Selma 2006).

Chlorine dioxide (ClO_2)

Chlorine dioxide (ClO_2) is another produce sanitizing agent for produce washing that has been evaluated as a post-harvest treatment (Kreske *et al.* 2006; Sy *et al.* 2005). It has received attention due to new technologies that facilitate the shipment and development of simple, on-site generation equipment. Chlorine dioxide is more effective against many classes of microorganisms at lower concentrations than chlorine (Gil and Selma 2006). The major advantages of chlorine dioxide over chlorine include the following: there is a reduced reactivity with organic matter, it does not react with ammonia to form chloramines, and it maintains its efficacy over a wide pH range (Huang *et al.* 1997; Wondergem and Van Dijk-Looijaard 1991). It produces fewer disinfection by-products than chlorine (Hua and Reckhow 2007; Stevens 1988). The oxidizing power of chlorine dioxide has been reported to be 2.5 times greater than chlorine (Benarde *et al.* 1967). On the other hand, chlorine dioxide is a relatively unstable gas and can be explosive when concentrated. In addition, chlorine dioxide decomposes at temperatures greater than 30°C when exposed to light (Beuchat 1998). In the United States, chlorine dioxide is approved

for sanitation of fruit and vegetable processing equipment at a maximum concentration of 200 ppm (Beuchat 1998) and a maximum of 3 ppm for use on uncut produce. Only a 1 ppm maximum is permitted for peeled potatoes. A potable rinse, blanching, cooking, or canning must follow treatment with chlorine dioxide (FDA 1977c). Information on the effectiveness of chlorine dioxide on produce is limited. The concentration of the bacterium *Enterobacter sakazakii* after exposure to 10 ppm of chlorine dioxide for 1 min was reduced by 3.38-$\log_{10}$ CFU on a whole apple (Kim *et al.* 2006). Treatment with 4 ppm chlorine dioxide of fresh-cut lettuce, cabbage, and carrots inoculated with *Salmonella*, *E. coli* O157:H7, and *L. monocytogenes* resulted in significantly higher log reductions in comparison to a wash using tap water (Sy *et al.* 2005). *E. coli* O157:H7 and *L. monocytogenes* inoculated onto strawberries at approximately 7-$\log_{10}$ CFU/fruit were inactivated by exposure to 3 ppm chlorine dioxide for 10 min (Han *et al.* 2004). Costilow *et al.* (1984) reported that 2.5 ppm ClO_2 was effective against microorganisms in waters used to handle and wash cucumbers; however, a concentration up to 105 ppm did not reduce the microflora on the cucumbers (Costilow *et al.* 1984). Similar results were reported by Reina and coworkers (1995). Immersion of oranges inoculated with non-pathogenic *E. coli* in a solution with 100 ppm ClO_2 resulted in a 1-$\log_{10}$ higher reduction compared to immersion in deionized water only (Pao and Davis 1999).

Ozone

Ozone is one of the newest sanitizers used in postharvest management systems (Kim *et al.* 1999; Kim *et al.* 2003a). In 2001, the FDA approved the use of ozone for the treatment, storage, and processing of foods in both the gas and aqueous phase in direct contact with foods, including raw and minimally processed fruits and vegetables (FDA 2001). As with ClO_2, ozone has to be generated on site. Corona discharge is one of the most commonly used methods to produce ozone. In corona discharge, ozone is generated when an electrical discharge occurs between two conductors separated by a discharge gap (wire and cylinder) and fed with oxygen flowing between the electrodes (Chand *et al.* in Press; Yanallah *et al.* 2006). Ozone is highly unstable in water and decomposes to oxygen in a very short time. The half-life of ozone in distilled water at 20°C is generally considered to be 20 to 30 min (Khadre *et al.* 2001). Nevertheless, depending on the quality of the water, the half-life of ozone can range from seconds to hours (Von Gunten 2003). Water used in food processing or beverages generally contains readily oxidizable organic and inorganic substances. These substances may react rapidly with ozone, considerably decreasing its half-life (Kim *et al.* 2003b). Ozone is a very strong oxidizer; unfortunately this oxidizing power has the negative effect of causing deterioration and corrosion on metal and other types of surfaces (Glaze 1986). One of the major advantages of ozone is that it does not directly form trihalomethanes, potentially human carcinogenic substances, although it may indirectly form undesirable by-products if bromide is present in the washwater (Von Gunten *et al.* 2001). Ozone is not generally affected by pH within a range of 6-8, but its decomposition increases above pH 8. Disinfection, however, stills

occur at high pH, because the biocidal activity of the compound is relatively fast (Bryant *et al*. 1992). Levels of 0.5 to 4 mg/L are recommended for washwater and 1 ppm for flume water (Gil and Selma 2006). The use of ozone as an antimicrobial agent in the food processing industry has been reviewed by Kim *et al.* (1999) and Guzel-Seydim *et al.* (2004) (Guzel-Seydim *et al.* 2004; Kim *et al.* 1999). However, little has been reported about inactivation of pathogens on produce. Treatment with ozonated water can extend the shelf-life of grapes, pears, apples, oranges, raspberries, strawberries, and lettuce by reducing the microbial population and by oxidation of ethylene to retard ripening (Beltran *et al.* 2005; Beuchat 1998; Sarig *et al.* 1996). Fungal deterioration of blackberries and grapes was decreased by ozonation of the fruits (Beuchat 1992). Mold and bacterial counts were greatly decreased without any change in chemical composition and sensory quality in onions treated with ozone gas during storage (Song *et al.* 2000). Shredded lettuce that was submerged in water bubbled with ozone had decreased bacterial counts (Kim *et al.* 1999). Similar to chlorine, the degree of microbial control by ozone cannot be predicted based on antibacterial activity on water. Although bacteria spores are inactivated very quickly in ozonated water (Driedger *et al.* 2001; Facile *et al.* 2000), vegetative cells inoculated into wounds of fruits survived exposure to 1.5 ppm of ozone for 5 minutes (Smilanick *et al.* 1999). The use of ozone gas could be another alternative to reduce the microbial populations on produce prior cutting. Ozone gas showed reduction on fungal counts and increased shelf life of grapes and apples during storage (Gil and Selma 2006). Fungal growth during storage of blackberries was also inhibited by 0.1-0.3 ppm ozone (Barth *et al.* 1995). The shelf life of raspberries, grapes and

strawberries could be doubled when 2 to 3 ppm of ozone was applied continuously for a few hours per day (Richard *et al.* 2006). Garcia *et al.* (2003) reported the effects of ozone treatment in the microbiological and sensory quality of lettuce. A higher microbial inactivation and longer shelf life was observed with ozone treatment than with chlorine treatment.

Depending on the application, the efficacy of ozone varies considerably. Ozone at a concentration of 3 ppm was more effective than chlorine dioxide (3 to 5 ppm), chlorinated trisodium phosphate (100 to 200 ppm), and peroxyacetic acid (80 ppm) at reducing the microbial count of *E. coli* O175:H7 and *L. monocytogenes* in an aqueous model system. When lettuce, strawberries, apples, and cantaloupe were inoculated with the aforementioned pathogens and exposed to ozone and chlorine dioxide, a > 5-$\log_{10}$ reduction was observed (Rodgers *et al.* 2004). However, its been reported that ozonated water generally reduces only 3 log CFU/g of bacteria populations in produce (Cherry 1999; Khadre *et al.* 2001; Kim *et al.* 1999). Ozone was less effective than acidic electrolyzed water in reducing levels of coliforms and aerobic mesophiles in cucumbers (Koseki *et al.* 2004). A combination of sonication and high speed stirring has been reported to enhance the efficacy of ozone (Burleson *et al.* 1975; Kim *et al.* 1999).

Organic acids

Most fruits naturally possess significant concentration of organic acids such as acetic, sorbic, citric, malic, succinic, acetic and tartaric acid (Albertini *et al.* 2006; Silva *et al.* 2002). Organic acids such as lactic and acetic acids are effective antibacterial agents and

are classified by the FDA as generally recognized as safe (GRAS) (FDA 1977a; FDA 1977b). Lactic acid dips and sprays are used commercially to decontaminate animal carcasses containing *E. coli* O157:H7, *L. monocytogenes* and *Salmonella* (Castillo *et al.* 2001). Lactic acid might have a potential application for sanitation of fresh fruits and vegetables. Total and fecal coliform were reduced about 2 and 1 log/g, respectively, on mixed salad vegetables treated with 1% lactic acid (Torriani *et al.* 1997). In a separate study, shredded carrots were inoculated with *Aeromonas* and subject to treatment with 2% lactic acid. A 2.5-$\log_{10}$ reduction of *Aeromonas* was achieved immediately after decontamination, and the growth of *Aeromonas* was controlled throughout the shelf life. Also, the psychrotrophic indigenous flora was significantly reduced in comparison to the control sample and remained at lower levels throughout the shelf life. Organoleptic properties of the acid-treated shredded carrots were satisfactory (Uyttendaele *et al.* 2004). A 5% acetic acid wash was able to reduce the population of *E. coli* O157:H7 on inoculated apples by approximately 3 logs (Wright *et al.* 2000). *Yesinia enterocolitica* inoculated onto parsley leaves were reduced by more than 7 logs after washing for 15 min in a solution of 2% acetic acid or 40% vinegar (Karapinar and Gonul 1992). Various combinations of lactic acid, acetic acid and chlorine were observed to reduce the population of *L. monocytogenes* on lettuce (Zhang and Farber 1996). A 2 min dip in 5% acetic acid at room temperature was the most effective treatment among several investigated for reducing *E. coli* O157:H7 inoculated onto apples surfaces (Wright *et al.* 2000).

Treatment with citric acid in the form of lemon juice has shown to reduce *Salmonella typhi* inoculated onto papaya and jicama cubes (Fernandez- Escartin *et al.* 1988). In another study, acidification of sweet corn (to pH 5), particularly with citric acid, slowed *L. monocytogenes* and *L. innocua* growth (Thomas *et al.* 1999). Organic acids, in particular vinegar and lemon juice, have potential as inexpensive, simple produce sanitizers at the household level; however, it is not clear whether or not these treatements would produce off flavors in treated produce.

Hydrogen peroxide (H_2O_2)

The bactericidal activity of hydrogen peroxide is well known (Juven and Pierson 1996; Szymanska 2006). Hydrogen peroxide decomposes rapidly in water and oxygen through the action of catalase, an enzyme commonly found in plants, leaving no residual toxicity (Vlasits *et al.* 2007). The FDA specifies approved uses of hydrogen peroxide such as treatment of milk used for cheese, preparation of modified whey, and production of thermophile-free starch (Ukuku and Sapers 2006). Nevertheless, it has not yet been approved by the FDA for washing fruits and vegetables (Gil and Selma 2006). Juven and Pierson (1996) reviewed the antimicrobial activity of hydrogen peroxide and its use in the food industry (Juven and Pierson 1996). The use of H_2O_2 on whole and fresh cut produce has been recently studied. The antimicrobial activity of H_2O_2 as a preservative for salads, berries, and fresh cut melons has been reported (Forney *et al.* 1991; Sapers and Simmons 1998). Ukuku (2004) reported a 3.0 log CFU/cm^2 reduction in *Salmonella spp*. inoculated on the surface of fresh-cut melon and a 3.0 log CFU/cm^2 reduction of the indigenous

microflora at the surface of whole melon after treatment with 5% hydrogen peroxide for 5 minutes (Ukuku 2004). Treatment of cantaloupes with 5% hydrogen peroxide at 70°C for 1 minute resulted in a 5-$\log_{10}$ reduction of total mesophilic aerobes, a 3-$\log_{10}$ reduction of yeast and molds, and a 3.8-$\log_{10}$ reduction on inoculated *Salmonella* (Ukuku *et al.* 2004). Treatment of lettuce with 2% hydrogen peroxide at 50°C for 60 seconds was recommended as an effective means to reduce populations of *E. coli* O157:H7, *Salmonella enterica* and *L. monocytogenes*. The combination of lactic acid and hydrogen peroxide reduced the levels of the target pathogens by more than 3 logs; however, the sensory quality of the lettuce was negatively affected (Lin *et al.* 2002). Vapor treatments have been used to reduce postharvest decay in some fruits and vegetables. Microbial populations on prunes, whole cantaloupes, grapes, walnuts, raisins, and pistachios were significally reduced upon treatment with H_2O_2 vapor (Forney *et al.* 1991; Sapers and Simmons 1998; Ukuku *et al.* 2001; Ukuku and Sapers 2001). However, hydrogen peroxide is phytotoxic to some commodities, causing browning in lettuce and mushrooms and anthocyanin bleaching in raspberries and strawberries (Sapers and Simmons 1998). It has also been reported that hydrogen peroxide causes browning of apple peels (Sapers *et al.* 1999). Further studies on the effectiveness of H_2O_2 in inactivating pathogens on a wide range of raw fruits and vegetables are needed.

Waterborne pathogens

Mycobacterium avium Complex.

The *Mycobacterium avium* complex (MAC) consists of genetically similar and slowly growing environmental bacteria that include *M. avium* subsp. *avium*, *M. avium* subsp. *paratuberculosis*, *M. avium* subsp. *silvaticum*, *M. avium* subsp. *hominis*, and *M. intracellulare* (St Amand *et al.* 2005). The cell wall of these bacteria are unusual in that they contain high levels of lipid (waxy material) (Rezwan *et al.* 2007). They are rod-shaped bacteria with dimensions ranging from 0.2 to 0.6 by 1.0 to 10 μm (Tortoli 2003). Older literature frequently includes *M. scrofulaceun* in the group, referring to it as MAIS (Fry *et al.* 1986). *Mycobacterium avium* complex (MAC) pathogens are included on the U.S. Environmental Protection Agency's Drinking Water Contaminant Candidate List (Gerba *et al.* 2003).

MAC organisms are opportunistic pathogens that have been isolated from many environmental sources including drinking water, soil, rivers, plants, dairy products, marine environments, animals, and bioaerosols (Covert *et al.* 1999; Falkinham 2003; Fischeder *et al.* 1991; Grange *et al.* 1990; Heifets 2004; Konuk *et al.* 2007). MAC usually causes several different types of disease in humans including pulmonary disease, disseminated MAC, and lymphoadenitis in immunocompromised as well as immunocompetent hosts (Perez de Pedro *et al.* 2007; Rubin and Rahal 1994; Wolinsky 1979). Clinically, MAC pulmonary disease is more frequently seen in patients with underlying lung disease such as bronchiectasis, pneumoconiosis, and prior tuberclulosis,

but approximately 24% to 59% occur in immunocompetent persons without evident predisposing factors (Huang *et al.* 1998; Prince *et al.* 1989). Transmission probably occurs primarily through fine particles of aerosolization and secondarily through ingestion (Damsker and Bottone 1985; Bermudez *et al.* 1997). Disseminated MAC may begin in the lungs and then spread to the gastrointestinal tract or colonize the gastrointestinal tract after ingestion of contaminated water and food (Bermudez *et al.* 1992; Miltner *et al.* 2005).

There is increasing evidence which suggests that tap water is the vehicle by which mycobacteria infect and colonize the human body (Bolan *et al.* 1985; Campagnaro *et al.* 1994). In a number of cases, mycobacterial species (*M. kansasii*, *M. fortuitum, M. chelonae, M. avium*, and *M. xenopi*) have been recovered from tap water for some time after they were recovered from patients (Vaerewijck *et al.* 2005; Wallace *et al.* 1998). Furthermore, mycobacteria are capable of biofilm formation (*M. fortuitum*, *M. chelonae*, and MAC) and thus, mycobacterial populations can persist in the water distribution system in spite of their slow growth (Hall-Stoodley and Lappin-Scott 1998; Yamazaki *et al.* 2006). Falkinham III *et al.* (2001) conducted a study to measure the number of *M. avium* and *M. intracellulare* in raw source waters before and after treatment and within the distribution system over a period of 18 months. They found that the numbers of mycobacteria ranged from $1x10^1$ to $7x10^5$ CFU/L. Mycobacterial numbers were substantially higher in the distribution system samples (average of 25,000-fold) than in those collected immediately downstream from the treatment facilities. It was concluded that mycobacteria grow in the distribution system. The number of *M. avium* in raw waters

was correlated with turbidity present in raw water sources (Falkinham *et al.* 2001). Carson *et al.* (1978) and George *et al.* (1980) have also reported the ability of *M. avium* complex organisms to grow in water (Carson *et al.* 1978; George *et al.* 1980).

Mycobacteria also have an extraordinary ability to survive under starvation conditions, persisting despite of a lack of nutrients in tap water (Smeulders *et al.* 1999; Nyka 1974). In one study, members of the *Mycobacterium avium* complex were able to survive in deionized water, losing less than 90% viability after 2 years (Archuleta *et al.* 2005). It has been reported that *M. avium* can infect *Acanthamoeba castellanii* (an environmental amoeba) and grow intracellularly at both 37°C and 30°C. In addition, it was found that amoeba-grown *M. avium* was capable of invading intestinal mucosal cells with significantly increased efficiency compared with that of *M. avium* grown on agar media (Cirillo *et al.* 1997). Therefore, *M. avium* may survive the process of drinking water disinfection by intracellular colonization inside free-living amoebae. Disinfection may also contribute, in part, to the persistence of *M. avium* and *M. intracellulare* in drinking water distribution systems (Falkinham *et al.* 2001). *M. avium* and *M. intracellulare* are many times more resistant to chlorine, chloramine, chlorine dioxide, and ozone than are other water-borne microorganisms (Carson *et al.* 1978; Le Dantec *et al.* 2002). As a result, disinfection of drinking water allows for the selection of mycobacteria. In the absence of competitors, even the slowly growing mycobacteria are able to grow in the distribution system (Vaerewijck *et al.* 2005). In addition, mycobacteria can tolerate extremes in temperature (Schulze-Robbecke and Buchholtz

1992), resulting in the contamination of ice machines (Falkinham 2002), hot tap water, and spas (Embil *et al.* 1997; Labombardi *et al.* 2002; Lumb *et al.* 2004).

Food can be a source of human exposure to mycobacteria. MAC has been isolated from beef, pork, chicken, turkey, oysters, and milk (Konuk *et al.* 2007; Embrey 2002). Mycobacteria were present in 25 of 121 food samples (20%) in one study, and certain isolates of *M. avium* showed genetic homology to clinical isolates (Yoder *et al.* 1999). *M. avium* may also tolerate an acidic environment. Bodmer *et al.* (2000) demonstrated that the microorganism was able to reach the stationary phase when it was exposed to a solution of pH 2.2 for 24 hours. They also demonstrated that *M. avium* incubated with acid in the presence of water was significantly more resistant to pH 2.2 than *M. avium* in the presence of a buffer. Finally, they concluded that pre-adaptation under conditions similar to the conditions where *M. avium* is found in the environment results in increased acid resistance (Bodmer *et al.* 2000).

Synergy between disinfectants

The use of disinfectants in water and food has significally improved human welfare; nonetheless, today, the use of these disinfectants is facing new challenges: (a) emergent pathogens resistant to disinfection; (b) harmful disinfection by-products; (c) hazards associated with on-site storage of chemicals; (d) development of microbial disinfection resistance; (e) compliance of strict disinfection regulations; and (f) bacterial regrowth in water distribution systems. Therefore, there is a need for new disinfection approaches that target the aforementioned issues. Alternative disinfectants have been proposed, (e.g. ozone, silver, hydrogen peroxide) but none of them are ideal. As a result, researchers have started looking into other approaches, such as the combination of disinfectants. Several studies have reported a synergistic effect or an enhanced disinfection efficacy when two or more disinfectants are combined. A synergistic effect is observed when the combined disinfectants exhibit higher inactivation performance than the sum of the inactivation levels of the separate disinfectants (Pedahzur *et al.* 2000). Butkus *et al.* (2004) found a synergistic effect between silver ions and UV-C irradiation against the coliphage MS-2 in a phosphate buffer solution. In another study, the inactivation of *Legionella pneumophila* by a combination of copper and silver was shown to be relatively slow when compared to that of free chlorine. Nevertheless, when silver and copper were combined with low levels of free chlorine, the inactivation rates of bacterial indicator organisms were greater than those for free chlorine alone (Landeen *et al.* 1989; Yahya *et al.* 1990). Straub *et al.* (1995) reported that the inactivation of MS-2 and *E. coli*

in well water by the combined use of copper and chloramines was greatly enhanced compared to either disinfectant alone. Beer *et al.* (1999) found that electrolytically generated copper and silver ions used in swimming pool water along with lower levels of chlorine provided control of total coliform and heterotrophic bacteria equivalent to the control provided by high levels of chlorine. Yahya *et al.* (1991) reported an enhanced inactivation of coliphage MS-2 and poliovirus by the addition of 0.2 mg/L of free chlorine to water containing 400 μg/L of copper.

Escherichia coli, Enterococous faecalis, Salmonella enteritis and coliphage MS-2 were exposed to a combination of peracetic acid (0.5-3 mg/L) and UV (8-38 $mW*s/cm^2$) in a synthetic wastewater-like solution. The disinfection efficacy was well beyond that predicted by the additive effect of each individual disinfectant (Koivunen and Heinonen-Tanski 2005). In a separate study, Pyle *et al.* (1992) reported that copper (100 ppb) and silver (11 ppb) ions enhanced the antimicrobial activity of iodine against *Pseudomonas cepacia* in a phosphate-buffered water solution. When silver and copper ions were used with or without iodine, regrowth was prevented. Yahya *et al.* (1990) demonstrated that adding 400 ppb copper and 40 ppb silver to water systems containing contaminants similar to those in swimming pools allowed the concentration of free chlorine to be reduced at least three-fold (from 0.1 to 0.3 ppm). Enhanced inactivation rates for *E. coli, S. aureus, L. pneumophila*, *S. faecalis* (Landeen *et al.* 1989; Yahya *et al.* 1990) and *P. aeruginosa* (Landeen *et al.* 1989) were also obtained when water was treated with 400 ppb copper, 40 ppb silver and 0.2 ppm free chlorine. These studies suggest a synergistic

effect upon microorganisms subjected to copper or silver ions in the presence of low levels of chlorine.

Combinations of 1:1000 silver to hydrogen peroxide (w) exhibited a higher inactivation performance in comparison to each disinfectant alone against non-pathogenic *E. coli* in a phosphate buffer at pH 6.8 (Pedahzur *et al.* 2000).

Antimicrobial peptides

Antimicrobial peptides are host defense effector molecules that are produced by invertebrates, vertebrates, plants, and microorganisms (Bevins 1994). In the past half-century, many peptide antibiotics have been described. These may either be non-ribosomally synthesized peptides (e.g., gramicidins and polymyxins) or ribosomally synthesized (natural) peptides. The former are often drastically modified and are largely produced by bacteria, whereas the latter are produced by all species as a major component of the natural host defense system (Montesinos 2007). On the basis of their structure, antimicrobial peptides have been generally classified into three groups: linear peptides with a propensity for amphiphilic alpha-helical structures, peptides with beta or alpha-beta structures stabilized by different numbers of disulfide bridges, and peptides with an over-representation of certain amino acids or unusual structures (Sitaram 2006).

Several bacterial antimicrobial peptides (known as bacteriocins) have been identified and characterized (Cotter *et al.* 2005). Bacteriocins have been used successfully as food preservatives (Hakovirta *et al.* 2006). In addition to bacteria, fungi also produce antimicrobial peptides such as plectasin, the first fungus-derived defensin recently isolated from the saprophytic ascomycete, *Pseudoplectania nigrella* (Mygind *et al.* 2005).

In contrast to vertebrates, plants only employ an innate immune system that largely depends on the production of antimicrobial peptides. Several plant antimicrobial peptides appear to be particularly effective as antifungal agents (Thomma *et al.* 2003).

Such antimicrobial peptides produced by plants are also referred to as “plant defensins” because their chemical structure is similar to the defensins found in other types of organisms, including humans (Thomma *et al.* 2002). They are small peptides that have a characteristic three-dimensional folding pattern that is stabilized by eight disulfide-linked cysteines (Hill *et al.* 1991).

The activity of most antimicrobial peptides appears to correlate with membrane destabilization. The exposed surface of microbial membranes mainly consists of phospholipids with negatively charged head groups (Thomma *et al.* 2003). One of model describing the activity of antimicrobial peptides suggests that, after initial electrostatic binding of positively charged antimicrobial peptides to negatively charged microbial cell surface, these peptides insert into the plasma membrane and form multimeric ion-permeable channels in a voltage dependent manner allowing ions and larger molecules to cross the membrane (Thomma *et al.* 2003).

Dissertation format:

The appendices of this dissertation report the findings of 3 separate studies undertaken by the candidate: 1) Microbiological Evaluation of the Residual Disinfectant Efficacy of Copper and Silver in aqueous solution. 2) Efficacy of Produce Sanitizers against *Escherichia coli, Listeria monocytogenes* and MS-2 Coliphage on Lettuce. 3) A Natural Peptide-Based Antimicrobial with Broad-Spectrum Activity. A literature review related to the candidate's research is also appended. This dissertation format offers the advantage of including material ready for submission to peer-reviewed scientific journals (Appendices A, B and C), and material already submitted to a peer-reviewed journal (Appendix D).

The dissertation author was responsible for all the research presented in the manuscripts in appendices A, B and C; the author also actively participated in the writing process of the manuscript in appendix D.

PRESENT STUDY

The objective of the present study was to evaluate the efficacy of several alternative disinfectants/sanitizers against microbial pathogens. These included the evaluation of the residual efficacy of silver and copper, both individually and in combination, against bacterial and viral pathogens, the evaluation of alternative produce sanitizers against microorganisms inoculated on lettuce, and the assessment of a natural peptide-based product against bacteria and viruses.

The methods, results and conclusions of this study are presented in the manuscripts appended to this dissertation. The following is a summary of the most important findings.

The manuscript entitled, Microbiological Evaluation of the Residual Disinfectant Efficacy of Copper and Silver in aqueous solution" is found in Appendix A. This study was conducted to evaluate copper and silver as alternative residual disinfectants in drinking water distribution systems. Different bacterial pathogens including *Salmonella, Listeria monocytogenes, Escherichia coli,* and *Mycobacterium fortuitum*, were exposed to these metals in a model system at both room temperature (25°C) and 4°C. All of the bacterial species tested were significantly reduced after a 1-hour exposure to silver (100 μg/L) at room temperature. A synergistic effect was observed in all the bacterial species after one hour of exposure to Ag/Cu (100/400 μg/L) at 25°C. Copper (400 μg/L) alone performed poorly, with no significant difference observed after 7 hours of exposure against *Salmonella* and *E. coli* at 25°C. The inactivation of the study microorganisms was

adversely affected by low temperature. Longer exposure times were needed at 4°C in order to achieve a significant reduction.

The manuscript, "Efficacy of Produce Sanitizers against *Escherichia coli, Listeria monocytogenes* and MS-2 Coliphage on Lettuce" is found in Appendix B. The aim of this study was to evaluate the antimicrobial activity of three alternative produce sanitizers, Microdyn™ (colloidal silver), Gadicin® (10% stabilized chlorine dioxide) and Fit® (citric acid, grapefruit oil extract), against the foodborne pathogens *Listeria monocytogenes* and *Escherichia coli*, as well as the coliphage MS-2. Treatment with chlorine and a control (dechlorinated tap water only) were also included. Microdyn™ and Gadicin® were not effective. Fit® removed 99.5% of *Escherichia coli*, 99.9% of *Listeria monocytogenes*, and 99.5% of MS-2 coliphage inoculated on lettuce after 10 minutes contact time. Chlorine (150 ppm) removed 97.6% of *Escherichia coli*, 99.7% of *Listeria monocytogenes*, and 96.4% of MS-2 coliphage. These results suggest that some alternative treatments could represent a viable option to further disinfect fresh fruits and vegetables in the household and thus reduce the risk of foodborne illness.

The manuscript, "A Natural Peptide-Based Antimicrobial with Broad-Spectrum Activity" is found in Appendix C. A quantitative suspension test was used to assess the efficacy of a new natural peptide-based antimicrobial, Absolute Fx, against bacteria and viruses. It was found that Absolute Fx significally inactivated all of the bacteria and viruses tested.

The manuscript entitled, "Silver as a Disinfectant" may be found in Appendix D and is an extensive review of the various applications for silver as a disinfectant, its

mechanisms of antimicrobial action, its interaction with other disinfectants, and microbial resistance to silver.

REFERENCES

Anonymous. (2005) Outbreaks of *Salmonella* infections associated with eating Roma tomatoes--United States and Canada, 2004. *Can Commun Dis Rep* **31**, 225-228.

Anonymous. (2006) Leafy, green, healthful--but contaminated. The *E. coli* outbreak from bagged spinach is a reminder that fruit and vegetables can harbor harmful bacteria. *Harv Health Lett* **32**, 4.

Abbaszadegan M. and Gerba C.P. (1992) Coliphage MS-2 as UV light efficacy test surrogate for bacterial and viral pathogens. In: Proceedings Water Quality Technology Conference. Water Works Association; Denver CO, pp 219-235.

Abdul-Raouf, U.M., Beuchat, L.R. and Ammar, M.S. (1993) Survival and growth of *Escherichia coli* O157:H7 on salad vegetables. *Appl Environ Microbiol* **59**, 1999-2006.

Ackers, M.L., Mahon, B.E., Leahy, E., Goode, B., Damrow, T., Hayes, P.S., Bibb, W.F., Rice, D.H., Barrett, T.J., Hutwagner, L., Griffin, P.M. and Slutsker, L. (1998) An outbreak of *Escherichia coli* O157:H7 infections associated with leaf lettuce consumption. *J Infect Dis* **177**, 1588-1593.

Adams, M. R., Hartley, A. D. and Cox, L. J. (1989) Factors affecting the efficacy of washing procedures used in the production of prepared salads. *Food Microbiol* **6,** 69-77.

Adrian, T., Wigand, R. and Richter, J. (1987) Gastroenteritis in infants, associated with a genome type of adenovirus 31 and with combined rotavirus and adenovirus 31 infection. *Eur J Pediatr* **146**, 38-40.

Al-Jasser, A. O. (2007) Chlorine decay in drinking-water transmission and distribution systems: Pipe service age effect. *Wat Res* **41,** 387-396.

Alam, M.Z. and Ohgaki, S. (2002) Role of hydrogen peroxide and hydroxyl radical in producing the residual effect of ultraviolet radiation. *Water Environ Res* **74**, 248-255.

Albertini, M.V., Carcouet, E., Pailly, O., Gambotti, C., Luro, F. and Berti, L. (2006) Changes in organic acids and sugars during early stages of development of acidic and acidless citrus fruit. *J Agric Food Chem* **54**, 8335-8339.

Albinana-Gimenez, N., Clemente-Casares, P., Bofill-Mas, S., Hundesa, A., Ribas, F. and Girones, R. (2006) Distribution of human polyomaviruses, adenoviruses, and hepatitis E virus in the environment and in a drinking-water treatment plant. *Environ Sci Technol* **40**, 7416-7422.

Alter, T. and Scherer, K. (2006) Stress response of *Campylobacter spp*. and its role in food processing. *J Vet Med B Infect Dis Vet Public Health* **53**, 351-357.

Alvarado-Casillas, S., Ibarra-Sanchez, S., Rodriguez-Garcia, O., Martinez-Gonzales, N. and Castillo, A. (2007) Comparison of rinsing and sanitizing procedures for reducing bacterial pathogens on fresh cantaloupes and bell peppers. *J Food Prot* **70**, 655-660.

Allwood, P.B., Malik, Y.S., Maherchandani, S., Hedberg, C.W. and Goyal, S.M. (2005) Effect of temperature on the survival of F-specific RNA coliphage, feline calicivirus, and *Escherichia coli* in chlorinated water. *Int J Environ Res Public Health* **2**, 442-446.

Andrews H.L. and Baumler A.J. (2005) *Salmonella* species**.** In Fratamico PM, Bhunia AK, Smith JL eds. Foodborne pathogens microbiology and molecular biology. Great Britain: Caister Academic Press, pp. 329-330.

Aragao, B.R., Araujo, A.C. and Gomes, R.A. (1980) Simplified fluctuation test to distinguish liquid holding recovery from cell multiplication in ultraviolet-irradiated *Escherichia coli*. *J Bacteriol* **144**, 1176-1178.

Aragao, B.R., Caldeira-de-Araujo, A., De Mattos, J.C., Favre, A. and Alcantara-Gomes, R. (1990) Near-ultraviolet illumination inhibits the liquid holding recovery in *Escherichia coli* B**.** *Braz J Med Biol Res* **23**, 1263-1266.

Archuleta, R.J., Yvonne Hoppes, P. and Primm, T.P. (2005) *Mycobacterium avium* enters a state of metabolic dormancy in response to starvation. *Tuberculosis (Edinb)* **85**, 147-158.

Ariga, T., Shimada, Y., Shiratori, K., Ohgami, K., Yamazaki, S., Tagawa, Y., Kikuchi, M., Miyakita, Y., Fujita, K., Ishiko, H., Aoki, K. and Ohno, S. (2005) Five new genome types of adenovirus type 37 caused epidemic keratoconjunctivitis in Sapporo, Japan, for more than 10 years. *J Clin Microbiol* **43**, 726-732.

Arumugaswamy, R.K., Ali, G.R. and Abd Hamid, S.N. (1994) Prevalence of *Listeria monocytogenes* in foods in Malaysia. *Int J Food Microbiol* **23**, 117-121.

Bagdasar'ian, G.A. and Abieva, R.M. (1971) Survival of entero- and adenoviruses in water. *Gig Sanit* **36**, 10-14.

Bancroft, K., Chrostowski, P., Wright, R. L. and Suffet, I. H. (1984) Ozonation and oxidation competition values: Relationship to disinfection and microorganisms regrowth. *Wat Res* **18,** 473-478.

Barth, M.M., Zhou, C., Mercier, J. and Payne, F. A. (1995) Ozone Storage Effects on Anthocyanin Content and Fungal Growth in Blackberries. *J Food Sci* **60,** 1286-1288.

Bean N.H. and Griffin P.M (1990) Foodborne disease outbreak in the United States, 1973-1987: pathogens, vehicle and trends. *J Food Prot* **53**, 804-817.

Beer, C. W., Guilmartin, L. E., McLoughlin, T. F. and White, T. J. (1999) Swimming pool disinfection: efficacy of copper/silver ions with reduced chlorine levels. *J Environ Health:* **61,** 9-13.

Behrsing, J., Winkler, S., Franz, P. and Premier, R. (2000) Efficacy of chlorine for inactivation of *Escherichia coli* on vegetables. *Postharvest Biol and Technol* **19,** 187-192.

Beltran, D., Selma, M.V., Marin, A. and Gil, M.I. (2005) Ozonated water extends the shelf life of fresh-cut lettuce. *J Agric Food Chem* **53**, 5654-5663.

Benarde, M.A., Snow, W.B., Olivieri, V.P. and Davidson, B. (1967) Kinetics and mechanism of bacterial disinfection by chlorine dioxide. *Appl Microbiol* **15**, 257-265.

Berg, G. and Berman, D. (1980) Destruction by anaerobic mesophilic and thermophilic digestion of viruses and indicator bacteria indigenous to domestic sludges. *Appl Environ Microbiol* **39**, 361-368.

Bermudez, L.E., Petrofsky, M. and Goodman, J. (1997) Exposure to low oxygen tension and increased osmolarity enhance the ability of *Mycobacterium avium* to enter intestinal epithelial (HT-29) cells. *Infect Immun* **65**, 3768-3773.

Bermudez, L.E., Petrofsky, M., Kolonoski, P. and Young, L.S. (1992) An animal model of *Mycobacterium avium* complex disseminated infection after colonization of the intestinal tract. *J Infect Dis* **165**, 75-79.

Berrang M. E, Brackett R.E and Beuchat L. R. (1989) Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. *J Food Prot* **52**, 702-705.

Beuchat L.R. (1996) Pathogenic microorganisms associated with fresh produce. *J Food Prot* **59**, 204–216.

Beuchat, L.R. and Brackett, R.E. (1990) Inhibitory effects of raw carrots on *Listeria monocytogenes*. *Appl Environ Microbiol* **56**, 1734-1742.

Beuchat, L.R. and Brackett, R.E. (1991) Behavior of *Listeria monocytogenes* inoculated into raw tomatoes and processed tomato products. *Appl Environ Microbiol* **57**, 1367-1371.

Beuchat L.R. and Doyle M.P. (1995) Survival and growth of *Listeria monocytogenes* in foods treated or supplemented with carrot juice .*J Food Microbiol* **12**, 73-80.

Beuchat L. R. (1998) Surface decontamination of fruits and vegetables eaten raw: a review. Food Safety Issues. World Health Organization, Geneva.WHO/FSF/FOS/ 98.2.

Beuchat, L.R., Harris, L.J., Ward, T.E. and Kajs, T.M. (2001) Development of a proposed standard method for assessing the efficacy of fresh produce sanitizers. *J Food Prot* **64**, 1103-1109.

Beuchat, L.R. and Ryu, J.H. (1997) Produce handling and processing practices. *Emerg Infect Dis* **3**, 459-465.

Beuchat L.R. (1992) Surface disinfection of raw produce. *Dairy, Food Environ Sanit* **12**, 6-9.

Bevins C.L. (1994) Antimicrobial peptides as agents of mucosal immunity. In: Ciba Foundation symposium antimicrobial peptides. John Wiley and Sons.Baffins: New York, pp. 250-251.

Blatchley, E.R. 3rd, Gong, W.L., Alleman, J.E., Rose, J.B., Huffman, D.E., Otaki, M. and Lisle, J.T. (2007) Effects of wastewater disinfection on waterborne bacteria and viruses. *Wat Environ Res* **79**, 81-92.

Bryant E.A., Fulton G.P. and Budd G.C. (1992) Ozone. In Disinfection alternatives for safe drinking water. New York: Van Nostrand Reinhold, pp. 201-202.

Bodmer, T., Miltner, E. and Bermudez, L.E. (2000) *Mycobacterium avium* resists exposure to the acidic conditions of the stomach. *FEMS Microbiol Lett* **182**, 45-49.

Bolan, G., Reingold, A.L., Carson, L.A., Silcox, V.A., Woodley, C.L., Hayes, P.S., Hightower, A.W., McFarland, L., Brown, J.W. 3rd, Petersen, N.J. and et al. (1985) Infections with *Mycobacterium chelonei* in patients receiving dialysis and using processed hemodialyzers. *J Infect Dis* **152**, 1013-1019.

Budu-Amoako, E., Toora, S., Ablett, R.F. and Smith, J. (1992) Evaluation of the ability of primary selective enrichment to resuscitate heat-injured and freeze-injured *Listeria monocytogenes* cells. *Appl Environ Microbiol* **58**, 3177-3179.

Bull, R.J., Birnbaum, L.S., Cantor, K.P., Rose, J.B., Butterworth, B.E., Pegram, R. and Tuomisto, J. (1995) Water chlorination: essential process or cancer hazard? *Fundam Appl Toxicol* **28**, 155-166.

Burleson, G.R., Murray, T.M. and Pollard, M. (1975) Inactivation of viruses and bacteria by ozone, with and without sonication. *Appl Microbiol* **29**, 340-344.

Burnett, A.B., Iturriaga, M.H., Escartin, E.F., Pettigrew, C.A. and Beuchat, L.R. (2004) Influence of variations in methodology on populations of *Listeria monocytogenes* recovered from lettuce treated with sanitizers. *J Food Prot* **67**, 742-750.

Butkus, M.A., Labare, M.P., Starke, J.A., Moon, K. and Talbot, M. (2004) Use of aqueous silver to enhance inactivation of coliphage MS-2 by UV disinfection. *Appl Environ Microbiol* **70**, 2848-2853.

Buxton, J., Fyfe, M., King, A., Paccagnella, A., Campbell, K., Ellis, A. and Hockin, J. (1999) Outbreak of *Salmonella* serotype Muenchen infection in the United States and Canada associated with unpasteurized orange juice--the British Columbia experience. *Can Commun Dis Rep* **25**, 161-164.

Camel, V. and Bermond, A. (1998) The use of ozone and associated oxidation processes in drinking water treatment. *Wat Res* **32,** 3208-3222.

Campagnaro, R.L., Teichtahl, H. and Dwyer, B. (1994) A pseudoepidemic of *Mycobacterium chelonae*: contamination of a bronchoscope and autocleaner. *Aust N Z J Med* **24**, 693-695.

Carson, L.A., Petersen, N.J., Favero, M.S. and Aguero, S.M. (1978) Growth characteristics of atypical mycobacteria in water and their comparative resistance to disinfectants. *Appl Environ Microbiol* **36**, 839-846.

Castillo, A., Lucia, L.M., Roberson, D.B., Stevenson, T.H., Mercado, I. and Acuff, G.R. (2001) Lactic acid sprays reduce bacterial pathogens on cold beef carcass surfaces and in subsequently produced ground beef. *J Food Prot* **64**, 58-62.

Center for Science in the Public Interest. (2001) Closing the gaps in our federal food-safety net. Outbreak alert. Washington, D.C.

Centers for Disease Control and Prevention (1998) Outbreak of *Campylobacter enteritis* associated with cross-contamination of food--Oklahoma, 1996. *MMWR Morb Mortal Wkly Rep* **47**, 129-131.

Centers for Disease Control and Prevention. (1996).Surveillance for foodborne-disease outbreaks -- United States, 1988-1992. *MMWR* **45,** 1-55.

Centers for Disease Control and Prevention. (2000) Surveillance for foodborne disease outbreaks --United States, 1993-1997. *MMWR* **49**,1-51.

Centers for Disease Control and Prevention. (2003) Preliminary fodNet data on the incidence of foodborne illnesses - selected states, United States, 2002. *MMWR* 52,340-343.

Centers for Disease Control and Prevention (2002) Mutistate outbreaks of *Salmonella* serotype Poona infections associated with eating cantaloupe from Mexico--United States and Canada, 2000-2002. *MMWR* **51**, 1044-1047.

Chai, L.C., Robin, T., Ragavan, U.M., Gunsalam, J.W., Bakar, F.A., Ghazali, F.M., Radu, S. and Kumar, M.P. (2007) Thermophilic *Campylobacter spp*. in salad vegetables in Malaysia. *Int J Food Microbiol*. [Epub ahead of print]

Chand, R., Bremner, D. H., Namkung, K. C., Collier, P. J. and Gogate, P. R. Water disinfection using the novel approach of ozone and a liquid whistle reactor. *Biochem Eng J*. In Press, Corrected Proof.

Cherry J.P (1999) Improving the safety of fresh produce with antimicrobials. *Food Technology* **53**, 54-58.

Cho, M., Chung, H. and Yoon, J. (2003a) Disinfection of water containing natural organic matter by using ozone-initiated radical reactions. *Appl Environ Microbiol* **69**, 2284-2291.

Cho, M., Chung, H. and Yoon, J. (2003b) Quantitative evaluation of the synergistic sequential inactivation of *Bacillus subtilis* spores with ozone followed by chlorine. *Environ Sci Technol* **37**, 2134-2138.

Cirillo, J.D., Falkow, S., Tompkins, L.S. and Bermudez, L.E. (1997) Interaction of *Mycobacterium avium* with environmental amoebae enhances virulence. *Infect Immun* **65**, 3759-3767.

Cody, S.H., Glynn, M.K., Farrar, J.A., Cairns, K.L., Griffin, P.M., Kobayashi, J., Fyfe, M., Hoffman, R., King, A.S., Lewis, J.H., Swaminathan, B., Bryant, R.G. and Vugia, D.J. (1999) An outbreak of *Escherichia coli* O157:H7 infection from unpasteurized commercial apple juice. *Ann Intern Med* **130**, 202-209.

Cooley, M.B., Chao, D. and Mandrell, R.E. (2006) *Escherichia coli* O157:H7 survival and growth on lettuce is altered by the presence of epiphytic bacteria. *J Food Prot* **69**, 2329-2335.

Costilow, R. N., Uebersax, M.A. and Ward, P.J. (1984) Use of chlorine dioxide for controlling microorganisms during the handling and storage of fresh cucumbers. *J Food Sci* **49,** 396-401.

Cotter, P. D., Hill, C. and Ross, R. P. (2005) Bacteriocins: developing innate immunity for food. *Nat Rev Micro* **3,** 777-788.

Covert, T.C., Rodgers, M.R., Reyes, A.L. and Stelma, G.N. Jr. (1999) Occurrence of nontuberculous mycobacteria in environmental samples. *Appl Environ Microbiol* **65**, 2492-2496.

Crabill, M.B. and Lyman, E.D. (1963) Eye irritation associated with swimming. *Nebr State Med J* **48**, 454-455.

Craun, G.F., Calderon, R.L. and Wade, T.J. (2006) Assessing waterborne risks: an introduction. *J Water Health* **4**, 3-18.

Dahl, E. (1976) Physicochemical aspects of disinfection of water by means of ultrasound and ozone. *Wat Res* **10**, 677-684.

Damsker, B. and Bottone, E.J. (1985) *Mycobacterium avium-Mycobacterium intracellulare* from the intestinal tracts of patients with the acquired immunodeficiency syndrome: concepts regarding acquisition and pathogenesis. *J Infect Dis* **151**, 179-181.

Daniel, F.B., Meier, J.R. and Deangelo, A.B. (1993) Advances in research on carcinogenic and genotoxic by-products of chlorine disinfection: chlorinated hydroxyfuranones and chlorinated acetic acids. *Ann Ist Super Sanita* **29**, 279-291.

Das, E., Gurakan, G.C. and Bayindirli, A. (2006) Effect of controlled atmosphere storage, modified atmosphere packaging and gaseous ozone treatment on the survival of *Salmonella enteritidis* on cherry tomatoes. *Food Microbiol* **23**, 430-438.

Davis, M.J., Coote, P.J. and O'Byrne, C.P. (1996) Acid tolerance in *Listeria monocytogenes*: the adaptive acid tolerance response (ATR) and growth-phase-dependent acid resistance. *Microbiol* **142**, 2975-2982.

De Simon, M., Tarrago, C. and Ferrer, M.D. (1992) Incidence of *Listeria monocytogenes* in fresh foods in Barcelona (Spain). *Int J Food Microbiol* **16**, 153-156.

Dhokane, V.S., Hajare, S., Shashidhar, R., Sharma, A. and Bandekar, J.R. (2006) Radiation processing to ensure safety of minimally processed carrot (*Daucus carota*) and cucumber (*Cucumis sativus*): optimization of dose for the elimination of *Salmonella typhimurium* and *Listeria monocytogenes*. *J Food Prot* **69**, 444-448.

Dobbin, G., Hariharan, H., Daoust, P.Y., Hariharan, S., Heaney, S., Coles, M., Price, L. and Anne Muckle, C. (2005) Bacterial flora of free-living double-crested cormorant (*Phalacrocorax auritus*) chicks on Prince Edward Island, Canada, with reference to enteric bacteria and antibiotic resistance. *Comp Immunol Microbiol Infect Dis* **28**, 71-82.

Driedger, A., Staub, E., Pinkernell, U., Marinas, B., Koster, W. and Gunten, U.V. (2001) Inactivation of *Bacillus subtilis* spores and formation of bromate during ozonation. *Wat Res* **35,** 2950-2960.

Duffy, L. and Dykes, G.A. (2006) Growth temperature of four *Campylobacter jejuni* strains influences their subsequent survival in food and water. *Lett Appl Microbiol* **43**, 596-601.

Dziuban, E.J., Liang, J.L., Craun, G.F., Hill, V., Yu, P.A., Painter, J., Moore, M.R., Calderon, R.L., Roy, S.L. and Beach, M.J. (2006) Surveillance for waterborne disease and outbreaks associated with recreational water--United States, 2003-2004. *MMWR Surveill Summ* **55**, 1-30.

Embil, J., Warren, P., Yakrus, M., Stark, R., Corne, S., Forrest, D. and Hershfield, E. (1997) Pulmonary illness associated with exposure to *Mycobacterium-avium* complex in hot tub water. Hypersensitivity pneumonitis or infection?. *Chest* **111**, 813-816.

Embrey M.A. (2002) *Mycobacterium avium* Complex in Drinking Water. In: Hanbook of CCL Microbes in Drinking Water. Denver, CO: America Water Works Association, pp. 352-353.

Enriquez, C. E., Hurst, C. J. and Gerba, C. P. (1995) Survival of the enteric adenoviruses 40 and 41 in tap, sea, and waste water. *Wat Res* **29**, 2548-2553.

Environmental Protection Agency (2005). Green Book-8-Hour Ozone Area http://www.epa.gov/air/oaqps/greenbk/gnsum.html. Retrieved April 10, 2007.

Environmental Protection Agency. (1998) National primary drinking water regulations: disinfectants and disinfection by-products; final rule. Fed. Reg. 63 (241), 69390–69476.

Ercolani, G.L. (1976) Bacteriological quality assessment of fresh marketed lettuce and fennel. *Appl Environ Microbiol* **31**, 847-852.

Escudero, M.E., Velazquez, L., Di Genaro, M.S. and de Guzman, A.M. (1999) Effectiveness of various disinfectants in the elimination of *Yersinia enterocolitica* on fresh lettuce. *J Food Prot* **62**, 665-669.

Evans, A.S. (1958) Latent adenovirus infections of the human respiratory tract. *Am J Hyg* **67**, 256-266.

Fabian, I. (1995) Mechanistic aspects of ozone decomposition in aqueous solution: Proceedings of the international symposium on global environment and nuclear energy systems. *Progress in Nuclear Energy* **29**,167-174.

Falkinham, J.O. 3rd.(2002) Nontuberculous mycobacteria in the environment. *Clin Chest Med* **23**, 529-551.

Falkinham, J.O. 3rd. (2003) Mycobacterial aerosols and respiratory disease. *Emerg Infect Dis* **9**, 763-776.

Falkinham, J.O. 3rd, Norton, C.D. and LeChevallier, M.W. (2001) Factors influencing numbers of *Mycobacterium avium*, *Mycobacterium intracellulare*, and other mycobacteria in drinking water distribution systems. *Appl Environ Microbiol* **67**, 1225-1231.

Favier, A.-L., Burmeister, W. P. and Chroboczek, J. (2004) Unique physicochemical properties of human enteric Ad41 responsible for its survival and replication in the gastrointestinal tract. *Virol* **322**, 93-104.

Ferguson, D.D., Scheftel, J., Cronquist, A., Smith, K., Woo-Ming, A., Anderson, E., Knutsen, J., De, A.K. and Gershman, K. (2005) Temporally distinct *Escherichia coli* 0157 outbreaks associated with alfalfa sprouts linked to a common seed source--Colorado and Minnesota, 2003. *Epidemiol Infect* **133**, 439-447.

Fernandez -Escartin E. F., Castillo-Ayala A. and Saldana Lozano J. (1989) Survival and growth of *Salmonella* and *Shigella* on sliced fresh fruit. *J Food Prot* **57**, 471-472.

Fischeder, R., Schulze-Robbecke, R. and Weber, A. (1991) Occurrence of mycobacteria in drinking water samples. *Zentralbl Hyg Umweltmed* **192**, 154-158.

Fisher, C.W., Lee, D., Dodge, B.A., Hamman, K.M., Robbins, J.B. and Martin, S.E. (2000) Influence of catalase and superoxide dismutase on ozone inactivation of *Listeria monocytogenes*. *Appl Environ Microbiol* **66**, 1405-1409.

Flessa, S., Lusk, D.M. and Harris, L.J. (2005) Survival of *Listeria monocytogenes* on fresh and frozen strawberries. *Int J Food Microbiol* **101**, 255-262.

Food and Drug Adminstration. (1977a) Direct food substances affirmed as a generally recognized as safe, listing of specific substances affirmed as GRAS, acetic acid. Code of Federal Regulation, title 21, part 184 subpart B, section 184.1005.

Food and Drug Adminstration. (1977b) Direct food substances affirmed as a generally recognized as safe, listing of specific substances affirmed as GRAS, lactic acid. Code of Federal Regulation, title 21, part 184 subpart B, section 184.1061.

Food and Drug Administration. (1977c) Secondary direct additives permitted in food for human consumption: chlorine dioxide. Code of federal title 21, section173.100.

Food and Drug Administration. (2001) Secondary direct food additives, permitted in food for human consumption. Reg 66 (123): 33829-33830.

Food and Drug Administration. (2001) Survey of imported fresh produce. http://www.cfsan.fda.gov/~dms/prodsur6.html. Retreived April 11, 2007.

Forney C. F, Fij R.E., Denis-Arrue R. and Smilanick J. L. (1991) Vapor phase hydrogen peroxide inhibits postharvest decay of table grapes. *HortScience* **26,** 1512-1514.

Francis, G.A. and O'Beirne, D. (2001) Effects of vegetable type, package atmosphere and storage temperature on growth and survival of *Escherichia coli* O157:H7 and *Listeria monocytogenes*. *J Ind Microbiol Biotechnol* **27**, 111-116.

Francis, G. A., Thomas, C. and O'beirne, D. (1999) The microbiological safety of minimally processed vegetables. *Int J Food Sci & Technol* **34**, 1-22.

Fry, K.L., Meissner, P.S. and Falkinham, J.O. 3rd. (1986) Epidemiology of infection by nontuberculous mycobacteria. VI. Identification and use of epidemiologic markers for studies of *M. avium, M. intracellulare*, and *M. scrofulaceum*. *Am Rev Respir Dis* **134**, 39-43.

Gandhi, M. and Chikindas, M.L. (2007) *Listeria*: A foodborne pathogen that knows how to survive. *Int J Food Microbiol* **113**, 1-15.

George, K.L., Parker, B.C., Gruft, H. and Falkinham, J.O. 3rd. (1980) Epidemiology of infection by nontuberculous mycobacteria. II. Growth and survival in natural waters. *Am Rev Respir Dis* **122**, 89-94.

Gerba C.P. (2000) Drinking Water Treatment and Distribution. In: Environmental Microbiology. San Diego CA: Academic Press, pp. 550-551.

Gerba C.P., Nwachuku N. and Riley K.R. (2003) Disinfection resistance of waterborne pathogens on the United States Environmental Protection Agency's Contaminant Candidate List (CCL). *J Water SRT - Aqua* **52**, 81-94.

Gil M.I and Selma M.V. (2006) Overview of Hazards in Fresh-Cut Produce Production: Control and Management of Food Safety Hazards. James J ed. Microbial Hazard Identification in Fresh Fruits and Vegetables, New Jersey: John Wiley and Sons, pp. 182-189.

Gorny, J. (2006) Microbial contamination of fresh produce and vegetables. In: Sapers GM, Gorny J, Yousef AE, eds. Microbiology of fruits and vegetables. Boca Raton, Florida: CRC press, pp. 4-5.

Glaze, W.H. (1986) Reaction products of ozone: a review. *Environ Health Perspect* **69**, 151-157.

Grange, J.M., Yates, M.D. and Boughton, E. (1990) The avian tubercle *bacillus* and its relatives. *J Appl Bacteriol* **68**, 411-431.

Griffin, P.M. and Tauxe, R.V. (1991) The epidemiology of infections caused by *Escherichia coli* O157:H7, other enterohemorrhagic *E. coli*, and the associated hemolytic uremic syndrome. *Epidemiol Rev* **13**, 60-98.

Guan, T.T., Blank, G. and Holley, R.A. (2005) Survival of pathogenic bacteria in pesticide solutions and on treated tomato plants. *J Food Prot* **68**, 296-304.

Guo, X., Chen, J., Brackett, R.E. and Beuchat, L.R. (2002) Survival of *Salmonella* on tomatoes stored at high relative humidity, in soil, and on tomatoes in contact with soil. *J Food Prot* **65**, 274-279.

Gupta, S.K., Nalluswami, K., Snider, C., Perch, M., Balasegaram, M., Burmeister, D., Lockett, J., Sandt, C., Hoekstra, R.M. and Montgomery, S. (2007) Outbreak of *Salmonella* Braenderup infections associated with roma tomatoes, northeastern United States, 2004: a useful method for subtyping exposures in field investigations. *Epidemiol Infect* 1-9.

Guzel-Seydim, Z. B., Greene, A. K. and Seydim, A. C. (2004) Use of ozone in the food industry. *Lebensmittel-Wissenschaft und-Technologie* **37**, 453-460.

Guzzella, L., Monarca, S., Zani, C., Feretti, D., Zerbini, I., Buschini, A., Poli, P., Rossi, C. and Richardson, S.D. (2004) In vitro potential genotoxic effects of surface drinking water treated with chlorine and alternative disinfectants. *Mutat Res* **564**, 179-193.

Hakovirta, J., Reunanen, J. and Saris, P.E. (2006) Bioassay for nisin in milk, processed cheese, salad dressings, canned tomatoes, and liquid egg products. *Appl Environ Microbiol* **72**, 1001-1005.

Hall-Stoodley, L. and Lappin-Scott, H. (1998) Biofilm formation by the rapidly growing mycobacterial species *Mycobacterium fortuitum. FEMS Microbiol Lett* **168**, 77-84.

Hammes, F., Salhi, E., Koster, O., Kaiser, H.-P., Egli, T. and von Gunten, U. (2006) Mechanistic and kinetic evaluation of organic disinfection by-product and assimilable organic carbon (AOC) formation during the ozonation of drinking water. *Wat Res* **40,** 2275-2286.

Han, Y., Selby, T.L., Schultze, K.K., Nelson, P.E. and Linton, R.H. (2004) Decontamination of strawberries using batch and continuous chlorine dioxide gas treatments. *J Food Prot* **67**, 2450-2455.

Harris, L.J., Beuchat, L.R., Kajs, T.M., Ward, T.E. and Taylor, C.H. (2001) Efficacy and reproducibility of a produce wash in killing *Salmonella* on the surface of tomatoes assessed with a proposed standard method for produce sanitizers. *J Food Prot* **64**, 1477-1482.

Harris L.J., Farber J.N., Beuchat L.R., Parish, M.E. Suslow T.V., Garrett E.H. and Busta F.F. (2003). Outbreaks associated with fresh produce: incidence, growth, and survival of pathogens in fresh and fresh-cut produce. *Comp. Rev. Food Sci. Food Safety* **2**, 78-141.

Harvey, J. and Gilmour, A. (1993) Occurrence and characteristics of *Listeria* in foods produced in Northern Ireland. *Int J Food Microbiol* **19**, 193-205.

Hayes, E.B., Matte, T.D., O'Brien, T.R., McKinley, T.W., Logsdon, G.S., Rose, J.B., Heifets, L. (2004) Mycobacterial infections caused by nontuberculous mycobacteria. *Semin Respir Crit Care Med* **25**, 283-295.

Heisick, J.E., Wagner, D.E., Nierman, M.L. and Peeler, J.T. (1989) *Listeria spp*. found on fresh market produce. *Appl Environ Microbiol* **55**, 1925-1927.

Hidalgo-Vila, J., Diaz-Paniagua, C., de Frutos-Escobar, C., Jimenez-Martinez, C. and Perez-Santigosa, N. (2007) *Salmonella* in free living terrestrial and aquatic turtles. *Vet Microbiol* **119**, 311-315.

Hilborn, E.D., Mermin, J.H., Mshar, P.A., Hadler, J.L., Voetsch, A., Wojtkunski, C., Swartz, M., Mshar, R., Lambert-Fair, M.A., Farrar, J.A., Glynn, M.K. and Slutsker, L. (1999) A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch Intern Med* **159**, 1758-1764.

Hilborn, E.D., Mshar, P.A., Fiorentino, T.R., Dembek, Z.F., Barrett, T.J., Howard, R.T. and Cartter, M.L. (2000) An outbreak of *Escherichia coli* O157:H7 infections and haemolytic uraemic syndrome associated with consumption of unpasteurized apple cider. *Epidemiol Infect* **124**, 31-36.

Hill, C.P., Yee, J., Selsted, M.E. and Eisenberg, D. (1991) Crystal structure of defensin HNP-3, an amphiphilic dimer: mechanisms of membrane permeabilization. *Science* **251**, 1481-1485.

Honish, L., Zazulak, I., Mahabeer, R., Krywiak, K., Leyland, R., Hislop, N. and Chui, L. (2007) Outbreak of *Escherichia coli* O157:H7 gastroenteritis associated with consumption of beef donairs, Edmonton, Alberta, May-June 2006. *Can Commun Dis Rep* **33**, 14-19.

Hua, G. and Reckhow, D.A. (2007) Comparison of disinfection byproduct formation from chlorine and alternative disinfectants. *Wat Res* **41**, 1667-1678.

Huang, J.H., Oefner, P.J., Adi, V., Ratnam, K., Ruoss, S.J., Trako, E. and Kao, P.N. (1998) Analyses of the NRAMP1 and IFN-gammaR1 genes in women with *Mycobacterium avium-intracellulare* pulmonary disease. *Am J Respir Crit Care Med* **157**, 377-381.

Huang, J., Wang, L., Ren, N., Ma, F. and Juli (1997) Disinfection effect of chlorine dioxide on bacteria in water. *Wat Res* **31**, 607-613.

Hundesa, A., Maluquer de Motes, C., Bofill-Mas, S., Albinana-Gimenez, N. and Girones, R. (2006) Identification of human and animal adenoviruses and polyomaviruses for determination of sources of fecal contamination in the environment. *Appl Environ Microbiol* **72**, 7886-7893.

Hunt, N. K. and Marinas, B. J. (1997) Kinetics of *Escherichia coli* inactivation with ozone. *Wat Res* **31**, 1355-1362.

Ingham, S.C., Fanslau, M.A., Engel, R.A., Breuer, J.R., Breuer, J.E., Wright, T.H., Reith-Rozelle, J.K. and Zhu, J. (2005) Evaluation of fertilization-to-planting and fertilization-to-harvest intervals for safe use of noncomposted bovine manure in Wisconsin vegetable production. *J Food Prot* **68**, 1134-1142.

Johannessen, G.S., Loncarevic, S. and Kruse, H. (2002) Bacteriological analysis of fresh produce in Norway. *Int J Food Microbiol* **77**, 199-204.

Johnston, L.M., Jaykus, L.A., Moll, D., Anciso, J., Mora, B. and Moe, C.L. (2006) A field study of the microbiological quality of fresh produce of domestic and Mexican origin. *Int J Food Microbiol* **112**, 83-95.

Johnston, M.L., Moe L.C., Moll D. and Jaykus L. (2006) The epidemiology of produce-associated outbreaks of foodborne disease. In: James J, ed. Microbial hazards identification in fresh fruits and vegetables. New Jersey: John Wiley and Sons, Inc., pp. 83-95.

Juven B. J. and Pierson M. D. (1996) Antibacterial effects of hydrogen peroxide and methods for its detection and quantification. *J Food Prot* **59**, 1233-1241.

Karapinar, M. and Gonul, S.A. (1992) Removal of *Yersinia enterocolitica* from fresh parsley by washing with acetic acid or vinegar. *Int J Food Microbiol* **16**, 261-264.

Kerwin L.R. (2005) Ozone in Drinking Water Treatment: Process Design, Operation, and Optimization. Denver CO: America Water Works Association, pp. 15-16.

Khadre, M.A., Yousef, A.E. and Kim, J.G. (2001) Microbiological aspects of ozone applications in food: a review. *J Food Sci* **66,** 1242-1252.

Kim, H., Ryu, J.H. and Beuchat, L.R. (2006) Survival of *Enterobacter sakazakii* on fresh produce as affected by temperature, and effectiveness of sanitizers for its elimination. *Int J Food Microbiol* **111**, 134-143.

Kim, J.G., Yousef, A.E. and Dave, S. (1999) Application of ozone for enhancing the microbiological safety and quality of foods: a review. *J Food Prot* **62**, 1071-1087.

Kim, J.G., Yousef, A.E. and Khadre, M.A. (2003) Ozone and its current and future application in the food industry. *Adv Food Nutr Res* **45**, 167-218.

Koivunen, J. and Heinonen-Tanski, H. (2005) Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. *Wat Res* **39**, 1519-1526.

Kolavic-Gray, S.A., Binn, L.N., Sanchez, J.L., Cersovsky, S.B., Polyak, C.S., Mitchell-Raymundo, F., Asher, L.V., Vaughn, D.W., Feighner, B.H. and Innis, B.L. (2002) Large epidemic of adenovirus type 4 infection among military trainees: epidemiological, clinical, and laboratory studies. *Clin Infect Dis* **35**, 808-818.

Komanapalli, I.R. and Lau, B.H. (1996) Ozone-induced damage of *Escherichia coli* K-12. *Appl Microbiol Biotechnol* **46**, 610-614.

Komanapalli, I.R., Mudd, J.B. and Lau, B.H. (1997) Effect of ozone on metabolic activities of *Escherichia coli* K-12. *Toxicol Lett* **90**, 61-66.

Konuk, M., Korcan, E., Dulgerbaki, S. and Altindis, M. (2007) Isolation and identification of mycobacteria from raw milk samples in Afyonkarahisar district of Turkey. *Int J Food Microbiol* **115**, 343-347.

Koseki, S. and Isobe, S. (2005) Growth of *Listeria monocytogene*s on iceberg lettuce and solid media. *Int J Food Microbiol* **101**, 217-225.

Koseki, S., Yoshida, K., Isobe, S. and Itoh, K. (2004) Efficacy of acidic electrolyzed water for microbial decontamination of cucumbers and strawberries. *J Food Prot* **67**, 1247-1251.

Krause, G., Terzagian, R. and Hammond, R. (2001) Outbreak of *Salmonella* serotype Anatum infection associated with unpasteurized orange juice. *South Med J* **94**, 1168-1172.

Kreske, A.C., Ryu, J.H. and Beuchat, L.R. (2006) Evaluation of chlorine, chlorine dioxide, and a peroxyacetic acid-based sanitizer for effectiveness in killing *Bacillus cereus* and *Bacillus thuringiensis* spores in suspensions, on the surface of stainless steel, and on apples. *J Food Prot* **69**, 1892-1903.

Kuchta, J.M., States, S.J., McNamara, A.M., Wadowsky, R.M. and Yee, R.B. (1983) Susceptibility of *Legionella pneumophila* to chlorine in tap water. *Appl Environ Microbiol* **46**, 1134-1139.

Kukkula, M., Arstila, P., Klossner, M.L., Maunula, L., Bonsdorff, C.H. and Jaatinen, P. (1997) Waterborne outbreak of viral gastroenteritis. *Scand J Infect Dis* **29**, 415-8.

Labombardi, V.J., O'brien, A.M. and Kislak, J.W. (2002) Pseudo-outbreak of *Mycobacterium fortuitum* due to contaminated ice machines. *Am J Infect Control* **30**, 184-186.

Lafortune, R., Caillet, S. and Lacroix, M. (2005) Combined effects of coating, modified atmosphere packaging, and gamma irradiation on quality maintenance of ready-to-use carrots (*Daucus carota*). *J Food Prot* **68**, 353-359.

Landeen, L.K., Yahya, M.T. and Gerba, C.P. (1989) Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila. Appl Environ Microbiol* **55**, 3045-3050.

Lang, M.M., Harris, L.J. and Beuchat, L.R. (2004a) Evaluation of inoculation method and inoculum drying time for their tffects on survival and efficiency of recovery of *Escherichia coli* O157: H7, *Salmonella*, and *Listeria monocytogenes* inoculated on the surface of tomatoes. *J Food Prot* **67**, 732-741.

Lang, M.M., Harris, L.J. and Beuchat, L.R. (2004b) Survival and recovery of *Escherichia coli* O157: H7, *Salmonella,* and *Listeria monocytogenes* on lettuce and parsley as affected by method of inoculation, time between inoculation and analysis, and treatment with chlorinated water. *J Food Prot* **67**, 1092-1103.

Langerak, D.I. (1978) The influence of irradiation and packaging on the quality of prepacked vegetables. *Ann Nutr Aliment* **32**, 569-86.

Lasobras, J., Dellunde, J., Jofre, J. and Lucena, F. (1999) Occurrence and levels of phages proposed as surrogate indicators of enteric viruses in different types of sludges. *Appl Environ Microbiol* **86,** 723-729.

Lawrence J. and Cappelli F.P. (1997) Ozone in drinking water treatment: a review. *Sci Total Environ* **7**, 99-108.

Le Dantec, C., Duguet, J.P., Montiel, A., Dumoutier, N., Dubrou, S. and Vincent, V. (2002) Chlorine disinfection of atypical mycobacteria isolated from a water distribution system. *Appl Environ Microbiol* **68**, 1025-1032.

Le Marc, Y., Huchet, V., Bourgeois, C.M., Guyonnet, J.P., Mafart, P. and Thuault, D. (2002) Modelling the growth kinetics of *Listeria* as a function of temperature, pH and organic acid concentration. *Int J Food Microbiol* **73**, 219-237.

LeChevallier, M.W., Babcock, T.M. and Lee, R.G. (1987) Examination and characterization of distribution system biofilms. *Appl Environ Microbiol* **53**, 2714-2724.

Lecuit, M. (2005) Understanding how *Listeria monocytogenes* targets and crosses host barriers. *Clin Microbiol Infect* **11**, 430-436.

Li Y., Brackett R.E., Chen J. and Beuchat L.R. (2001a) Survival and growth of *Escherichia coli* 0157:H7 inoculated onto cut lettuce before or after heating in chlorinated water followed by storage at 5 or 15 °C. *J Food Prot* **64**, 305-309.

Li, Y., Brackett, R.E., Shewfelt, R.L. and Beuchat, L.R. (2001b) Changes in appearance and natural microflora on iceberg lettuce treated in warm, chlorinated water and then stored at refrigeration temperature. *Food Microbiol* **18,** 299-308.

Li, Y., Frey, E., Mackenzie, A.M. and Finlay, B.B. (2000) Human response to *Escherichia coli* O157:H7 infection: antibodies to secreted virulence factors. *Infect Immun* **68**, 5090-5095.

Lin, C.M., Moon, S.S., Doyle, M.P. and McWatters, K.H. (2002) Inactivation of *Escherichia coli* O157:H7, *Salmonella enterica* serotype enteritidis, and *Listeria monocytogenes* on lettuce by hydrogen peroxide and lactic acid and by hydrogen peroxide with mild heat. *J Food Prot* **65**, 1215-1220.

Lin, Y.S., Stout, J.E., Yu, V.L. and Vidic, R.D. (1998) Disinfection of water distribution systems for *Legionella*. *Semin Respir Infect* **13**, 147-159.

Luber, P., Brynestad, S., Topsch, D., Scherer, K. and Bartelt, E. (2006) Quantification of *Campylobacter* species cross-contamination during handling of contaminated fresh chicken parts in kitchens. *Appl Environ Microbiol* **72**, 66-70.

Lumb, R., Stapledon, R., Scroop, A., Bond, P., Cunliffe, D., Goodwin, A., Doyle, R. and Bastian, I. (2004) Investigation of spa pools associated with lung disorders caused by *Mycobacterium avium* complex in immunocompetent adults. *Appl Environ Microbiol* **70**, 4906-4910.

Ma, J. and Graham, N. J. D. (2000) Degradation of atrazine by manganese-catalysed ozonation--influence of radical scavengers. *Wat Res* **34**, 3822-3828.

Maki, D.G. (2006) Don't eat the spinach--controlling foodborne infectious disease. *N Engl J Med* **355**, 1952-1955.

Mannix, M., O'Connell, N., McNamara, E., Fitzgerald, A., Prendiville, T., Norris, T., Greally, T., Fitzgerald, R., Whyte, D., Barron, D., Monaghan, R., Whelan, E., Carroll, A., Curtin, A., Collins, C., Quinn, J., O'Dea, F., O'Riordan, M., Buckley, J., McCarthy, J. and Mc Keown, P. (2005) Large *E. coli* O157 outbreak in Ireland, October-November 2005. *Euro Surveill* **10**, 1202-1223.

Matthews K.R. (2006) Microorganims associated with fresh produce. In: Matthews KR, ed. Microbiology of fresh produce. Washington, D.C.: ASM press, pp. 2-3.

Mathews, A.P., Panda, K.K., Ananthi, S. and Padmanabhan, K. (2004) Mass transfer and oxidation kinetics in an in situ ozone generator. *Water Sci Technol* **49**, 19-24.

McCready, S., Muller, J.A., Boubriak, I., Berquist, B.R., Ng, W.L. and Dassarma, S. (2005) UV irradiation induces homologous recombination genes in the model archaeon, *Halobacterium spp.* NRC-1. *Saline Systems* **1**, 3.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607-625.

Meunier, L., Canonica, S. and Von Gunten, U. (2006) Implications of sequential use of UV and ozone for drinking water quality. *Wat Res* **40**, 1864-1876.

Michino, H., Araki, K., Minami, S., Takaya, S., Sakai, N., Miyazaki, M., Ono, A. and Yanagawa, H. (1999) Massive outbreak of *Escherichia coli* O157:H7 infection in schoolchildren in Sakai City, Japan, associated with consumption of white radish sprouts. *Am J Epidemiol* **150**, 787-796.

Millard, P.S., Gensheimer, K.F., Addiss, D.G., Sosin, D.M., Beckett, G.A., Houck-Jankoski, A. and Hudson, A. (1994) An outbreak of cryptosporidiosis from fresh-pressed apple cider. *JAMA* **272**, 1592-1596.

Miltner, E., Daroogheh, K., Mehta, P.K., Cirillo, S.L., Cirillo, J.D. and Bermudez, L.E. (2005) Identification of *Mycobacterium avium* genes that affect invasion of the intestinal epithelium. *Infect Immun* **73**, 4214-4221.

Miltner, E.C. and Bermudez, L.E. (2000) *Mycobacterium avium* grown in *Acanthamoeba castellanii* is protected from the effects of antimicrobials. *Antimicrob Agents Chemother* **44**, 1990-1994.

Mohle-Boetani, J.C., Reporter, R., Werner, S.B., Abbott, S., Farrar, J., Waterman, S.H. and Vugia, D.J. (1999) An outbreak of *Salmonella* serogroup Saphra due to cantaloupes from Mexico. *J Infect Dis* **180**, 1361-1364.

Montesinos, E. (2007) Antimicrobial peptides and plant disease control. *FEMS Microbiol Lett*. **270**,1-11.

Mygind, P.H., Fischer, R.L., Schnorr, K.M., Hansen, M.T., Sonksen, C.P., Ludvigsen, S., Raventos, D., Buskov, S., Christensen, B., De Maria, L., Taboureau, O., Yaver, D., Elvig-Jorgensen, S.G., Sorensen, M.V., Christensen, B.E., Kjaerulff, S., Frimodt-Moller, N., Lehrer, R.I., Zasloff, M. and Kristensen, H.H. (2005) Plectasin is a peptide antibiotic with therapeutic potential from a saprophytic fungus. *Nature* **437**, 975-980.

Nachmkin I. and Guerry. (2005) *Campylobacter* infetions. In: Fratamico PM, Bhunia AK, Smith JL eds. Foodborne Pathogens Microbiology and Molecular Biology. Great Britain: Caister Academic Press, pp. 285-289.

Nassif, X., Bourdoulous, S., Eugene, E. and Couraud, P.O. (2002) How do extracellular pathogens cross the blood-brain barrier? *Trends Microbiol* **10**, 227-232.

Ndiongue, S., Huck, P. M. and Slawson, R. M. (2005) Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system. *Wat Res* **39,** 953-964.

Nebel, C., Gottschling, R.D., Hutchison, R.L., McBride, T.J., Taylor, D.M., Pavoni, J.L., Tittlebaum, M.E., Spencer, H.E. and Fleischman, M. (1973) Ozone disinfection of industrial-municipal secondary effluents. *J Water Pollut Control Fed* **45**, 2493-2507.

Nguyen-the, C. and Carlin, F. (1994) The microbiology of minimally processed fresh fruits and vegetables. *Crit Rev Food Sci Nutr* **34**, 371-401.

Nguyen-The, C. and Lund, B.M. (1991) The lethal effect of carrot on *Listeria* species. *J Appl Bacteriol* **70**, 479-488.

Nickols D and Varas A. J. (1992) Ozonation. In: Bryant EA, Fulton GP, Budd GC eds. Disinfection alternatives for safe drinking water. New York: Van Nostrand Reinhold, pp 196-197.

Nwachcuku, N. and Gerba, C.P. (2004) Emerging waterborne pathogens: can we kill them all? *Curr Opin Biotechnol* **15**, 175-180.

Nyka, W. (1974) Studies on the effect of starvation on mycobacteria. *Infect Immun* **9**, 843-850.

O'Brien, R.J. (1989) The epidemiology of nontuberculous mycobacterial disease. *Clin Chest Med* **10**, 407-418.

O'Mahony, M., Cowden, J., Smyth, B., Lynch, D., Hall, M., Rowe, B., Teare, E.L., Tettmar, R.E., Rampling, A.M., Coles, M. and et al. (1990) An outbreak of *Salmonella* saint-paul infection associated with beansprouts. *Epidemiol Infect* **104**, 229-235.

Ottoson, J., Hansen, A., Bjorlenius, B., Norder, H. and Stenstrom, T.A. (2006) Removal of viruses, parasitic protozoa and microbial indicators in conventional and membrane processes in a wastewater pilot plant. *Wat Res* **40**, 1449-1457.

Paoli G.C, Bhunia A.K. and Bayles D.O. (2005) *Listeria monocytogenes.* In: Fratamico PM, Bhunia AK, Smith JL eds. Foodborne pathogens microbiology and molecular biology. Great Britain: Caister Academic Press, pp. 295-296.

Pao S, Eldon G.E, Brown E and Schneider K.R. (1998) Challenge studies with selected pathogenic bacteria on freshly peeled hamlin orange. *J Food Sci* **63**, 359–362.

Pao, S. and Davis, C.L. (1999) Enhancing microbiological safety of fresh orange juice by fruit immersion in hot water and chemical sanitizers. *J Food Prot* **62**, 756-760.

Papapetropoulou, M. and Vantarakis, A.C. (1998) Detection of adenovirus outbreak at a municipal swimming pool by nested PCR amplification. *J Infect* **36**, 101-103.

Paraskeva, P. and Graham, N.J. (2002) Ozonation of municipal wastewater effluents. *Wat Environ Res* **74**, 569-581.

Park, C.E. and Sanders, G.W. (1992) Occurrence of thermotolerant *Campylobacters* in fresh vegetables sold at farmers' outdoor markets and supermarkets. *Can J Microbiol* **38**, 313-316.

Pearson, L.J. and Marth, E.H. (1990) *Listeria monocytogenes*--threat to a safe food supply: a review. *J Dairy Sci* **73**, 912-928.

Pedahzur, R., Katzenelson, D., Barnea, N., Lev, O., Shuval, H. and Ulitzur, S. (2000) The efficacy of long-lasting residual drinking water disinfectants based on hydrogen peroxide and silver. *Wat Sci Tech* **42**, 293-298.

Perez de Pedro, I., de la Quintana-Beltran, P. and Bermudez-Ruiz, P. (2007) Cervical lymphadenitis due to *Mycobacterium avium* in an immunocompetent adult. *Enferm Infecc Microbiol Clin* **25**, 159-161.

Pope, J.E., Krizova, A., Garg, A.X., Thiessen-Philbrook, H. and Ouimet, J.M. (2007) *Campylobacter* reactive arthritis: a systematic review. *Semin Arthritis Rheum*. [Epub ahead of print]

Prince, D.S., Peterson, D.D., Steiner, R.M., Gottlieb, J.E., Scott, R., Israel, H.L., Figueroa, W.G. and Fish, J.E. (1989) Infection with *Mycobacterium avium* complex in patients without predisposing conditions. *N Engl J Med* **321**, 863-868.

Proctor, M.E., Hamacher, M., Tortorello, M.L., Archer, J.R. and Davis, J.P. (2001) Multistate outbreak of *Salmonella* serovar Muenchen infections associated with alfalfa

sprouts grown from seeds pretreated with calcium hypochlorite. *J Clin Microbiol* **39**, 3461-3465.

Pyle, B.H., Broadaway, S.C. and McFeters, G.A. (1992) Efficacy of copper and silver ions with iodine in the inactivation of *Pseudomonas cepacia*. *J Appl Bacteriol* **72**, 71-79.

Raji, M.A., Jiwa, S.F., Minga, M.U. and Gwakisa, P.S. (2003) *Escherichia coli* 0157: H7 reservoir, transmission, diagnosis and the african situation: a review. *East Afr Med J* **80**, 271-276.

Ramaswamy, V., Cresence, V.M., Rejitha, J.S., Lekshmi, M.U., Dharsana, K.S., Prasad, S.P. and Vijila, H.M. (2007) *Listeria* - review of epidemiology and pathogenesis. *J Microbiol Immunol Infect* **40**, 4-13.

Rangel, J.M., Sparling, P.H., Crowe, C., Griffin, P.M. and Swerdlow, D.L. (2005) Epidemiology of *Escherichia coli* O157:H7 outbreaks, United States, 1982-2002. *Emerg Infect Dis* **11**, 603-609.

Rathinasabapathi, B. (2004) Survival of *Salmonella* Montevideo on tomato leaves and mature green tomatoes. *J Food Prot* **67**, 2277-2279.

Reeser, R.J., Medler, R.T., Billington, S.J., Jost, B.H. and Joens, L.A. (2007) Characterization of *Campylobacter jejuni* biofilms under defined growth conditions. *Appl Environ Microbiol* **73**, 1908-1913.

Reina L.D., Fleming H. P. and Humphries E.G. (1995) Microbiological control of cucumber hydrocooling water with chlorine dioxide. *J Food Prot* **58**, 541-546.

Rezwan, M., Laneelle, M.A., Sander, P. and Daffe, M. (2007) Breaking down the wall: fractionation of mycobacteria. *J Microbiol Methods* **68**, 32-39.

Rice R G, Robson C M, Miller G W. and Hill A. G. (1981) Uses of ozone in drinking water. *J Am Water Works Assoc* **73**, 44-56.

Richard H Linton, Yingchang Han, Selby T.L and Nelson P.E. (2006) Vapor-phase sanitation (decontamination) treaments. In: Sapers GM, Gorny JR, Yousef AE eds. Microbiology of fruits and vegetables. Boca Raton, Florida: CRC Press, pp. 421-422.

Ries A.A., Zaga S, Langkop C, Tauxe R.V. and Blake P.A. (1990) A multistate outbreak of *Salmonella* chester linked to imported cantaloupe. Intersciencee Conf. Antimicrob. Agent Chemother.American Society of Microbiology: Washington D.C, pp. 20-23.

Rodgers, S.L. and Ryser, E.T. (2004) Reduction of microbial pathogens during apple cider production using sodium hypochlorite, copper ion, and sonication. *J Food Prot* **67**, 766-771.

Rodgers, S.L., Cash, J.N., Siddiq, M. and Ryser, E.T. (2004) A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe. *J Food Prot* **67**, 721-731.

Rubin, D.S. and Rahal, J.J. (1994) *Mycobacterium-avium* complex. *Infect Dis Clin North Am* **8**, 413-426.

Rusin P., Enriquez C.E., Johnson D. and Gerba C.P. (2000) Environmental transmitted pathogens. In Environmental Microbiology. San Diego CA: Academic Press, pp. 474-475.

Samadpour, M., Barbour, M.W., Nguyen, T., Cao, T.M., Buck, F., Depavia, G.A., Mazengia, E., Yang, P., Alfi, D., Lopes, M. and Stopforth, J.D. (2006) Incidence of enterohemorrhagic *Escherichia coli*, *Escherichia coli* O157, *Salmonella*, and *Listeria monocytogenes* in retail fresh ground beef, sprouts, and mushrooms. *J Food Prot* **69**, 441-443.

Sanderson, W.T., Almaguer, D. and Kirk, L.H. 3rd. (1999) Ozone-induced respiratory illness during the repair of a portland cement kiln. *Scand J Work Environ Health* **25**, 227-232.

Sapers G.M., Miller R.L. and Matrazzo A.M. (1999) Effectiveness of sanitizing agents in inactivating *Escherichia coli* in golden delicious apples. *J Food Sci* **64**, 734-737.

Sapers GM and Simmons G. (1998) Hydrogen peroxide disinfection of minimally processed fruits and vegetables. *Food Technol* **52**, 48-53.

Sarig, P., Zahavi, T., Zutkhi, Y., Yannai, S., Lisker, N. and Ben-Arie, R. (1996) Ozone for control of post-harvest decay of table grapes caused byRhizopus stolonifer. *Physiol Mol Plant Path* **48**, 403-415.

Schlech, W.F. 3rd., Lavigne, P.M., Bortolussi, R.A., Allen, A.C., Haldane, E.V., Wort, A.J., Hightower, A.W., Johnson, S.E., King, S.H., Nicholls, E.S. and Broome, C.V. (1983) Epidemic listeriosis--evidence for transmission by food. *N Engl J Med* **308**, 203-206.

Schlundt, J. (2002) New directions in foodborne disease prevention. *Int J Food Microbiol* **78**, 3-17.

Schulze-Robbecke, R. and Buchholtz, K. (1992) Heat susceptibility of aquatic mycobacteria. *Appl Environ Microbiol* **58**, 1869-1873.

Shigenobu, K., Kyoichiro, Y., Yoshinori K. and Kazuhiko, I. (2003) Influence of inoculation method, spot inoculation site, and inoculation size on the efficacy of acidic electrolyzed water against pathogens on lettuce. *J Food Prot* **66**, 2010-2016.

Silva, B.M., Andrade, P.B., Mendes, G.C., Seabra, R.M. and Ferreira, M.A. (2002) Study of the organic acids composition of quince (Cydonia oblonga Miller) fruit and jam. *J Agric Food Chem* **50**, 2313-2317.

Sitaram, N. (2006) Antimicrobial peptides with unusual amino acid compositions and unusual structures. *Curr Med Chem* **13**, 679-696.

Smedbold, H.T., Ahlen, C., Norback, D. and Hilt, B. (2001) Sign of eye irritation in female hospital workers and the indoor environment. *Indoor Air* **11**, 223-231.

Smeulders, M.J., Keer, J., Speight, R.A. and Williams, H.D. (1999) Adaptation of *Mycobacterium smegmatis* to stationary phase. *J Bacteriol* **181**, 270-283.

Smilanick J.L., Crisosto C. and Mlikota F. (1999) Postharvest use of ozone on fresh fruit. *Perish Handling Quart*.10-15.

Smith J.L, and Fratamico P.M (2005) Diarrhea-inducing *Escherichia coli*. In: Fratamico PM, Bhunia AK, Smith JL, eds. Foodborne pathogens. Great Britain, UK: Caister Academic Press, pp. 358-359.

Smith, S., Dunbar, M., Tucker, D. and Schaffner, D.W. (2003) Efficacy of a commercial produce wash on bacterial contamination of lettuce in a food service setting. *J Food Prot* **66**, 2359-2361.

Solomon, E.B., Pang, H.J. and Matthews, K.R. (2003) Persistence of *Escherichia coli* O157:H7 on lettuce plants following spray irrigation with contaminated water. *J Food Prot* **66**, 2198-2202.

Song J, Fan L, Hildebrand P.D. and Forney, C.F. (2000) Biological effects of corona discharge on onions in a commercial storage facility. *Hort Technol* **10**, 608–612.

St Amand, A.L., Frank, D.N., De Groote, M.A. and Pace, N.R. (2005) Use of specific rRNA oligonucleotide probes for microscopic detection of *Mycobacterium avium* complex organisms in tissue. *J Clin Microbiol* **43**, 1505-1514.

Steffen, W., Mochmann, H., Kontny, I., Richter, U., Werner, U. and el Naeem, O. (1986) A food-borne infection caused by *Campylobacter jejuni* serotype Lauwers 19. *Zentralbl Bakteriol Mikrobiol Hyg* **183**, 28-35.

Steinbrugge E.D., Maxcy R.B. and Liewen M.B. (1988) Fate of *Listeria monocytogenes* on ready to eat serve lettuce. *J Food Prot* **51**, 596-599.

Stevens, A.A. (1982) Reaction products of chlorine dioxide. *Environ Health Perspect* **46**, 101-110.

Stevens, C., Khan, V. A., Lu, J. Y., Wilson, C. L., Pusey, P. L., Kabwe, M. K., Igwegbe, E. C. K., Chalutz, E. and Droby, S. (1998) The germicidal and hormetic effects of UV-C light on reducing brown rot disease and yeast microflora of peaches. *Crop Protect* **17**, 75-84.

Stine, S.W., Song, I.H., Choi, C.Y. and Gerba, C.P. (2005) Application of microbial risk assessment to the development of standards for enteric pathogens in water used to irrigate fresh produce. *J Food Prot* **68**, 913-918.

Stopforth, J.D., Skandamis, P.N., Geornaras, I. and Sofos, J.N. (2007) Acid tolerance of acid-adapted and nonacid-adapted *Escherichia coli* O157:H7 strains in beef decontamination runoff fluids or on beef tissue. *Food Microbiol* **24**, 530-538.

Straub, T.M., Gerba, C.P., Zhou, X., Price, R. and Yahya, M.T. (1995) Synergistic inactivaction of *Escherichia coli* and MS-2 coliphage by chloramine and cupric chloride. *Wat Res* **29**, 811-818.

Sturchler, D.A. (2006). Plant-Derived Food. In: Exposure a guide to sources of infection. Washington, D.C.: ASM press, pp.177-178.

Sy, K.V., Murray, M.B., Harrison, M.D. and Beuchat, L.R. (2005) Evaluation of gaseous chlorine dioxide as a sanitizer for killing *Salmonella*, *Escherichia coli* O157:H7, *Listeria monocytogenes,* and yeasts and molds on fresh and fresh-cut produce. *J Food Prot* **68**, 1176-1187.

Szymanska, J. (2006) Antifungal efficacy of hydrogen peroxide in dental unit waterline disinfection. *Ann Agric Environ Med* **13**, 313-317.

Tam, C.C., O'brien, S.J., Petersen, I., Islam, A., Hayward, A. and Rodrigues, L.C. (2007) Guillain-barre syndrome and preceding infection with *Campylobacter*, influenza and epstein-barr virus in the general practice research database. *PLoS ONE* **2**, e344.

Tanner, B.D., Kuwahara, S., Gerba, C.P. and Reynolds, K.A. (2004) Evaluation of electrochemically generated ozone for the disinfection of water and wastewater. *Wat Sci Technol* **50**, 19-25.

Taormina, P.J., Beuchat, L.R. and Slutsker, L. (1999) Infections associated with eating seed sprouts: an international concern. *Emerg Infect Dis* **5**, 626-634.

Tauxe, R.V. (2002) Emerging foodborne pathogens. *Int J Food Microbiol* **78**, 31-41.

Taylor, M.B., Marx, F.E. and Grabow, W.O. (1997) Rotavirus, astrovirus and adenovirus associated with an outbreak of gastroenteritis in a South African child care centre. *Epidemiol Infect* **119**, 227-230.

Thoma, F. (1999) Light and dark in chromatin repair: repair of UV-induced DNA lesions by photolyase and nucleotide excision repair. *EMBO J* **18**, 6585-6598.

Thomma, B.P., Cammue, B.P. and Thevissen, K. (2002) Plant defensins. *Planta* **216**, 193-202.

Thomma, B.P., Cammue, B.P. and Thevissen, K. (2003) Mode of action of plant defensins suggests therapeutic potential. *Curr Drug Targets Infect Disord* **3**, 1-8.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J. and Gerba, C.P. (2005) Inactivation of enteric adenovirus and feline calicivirus by ozone. *Water Res* **39**, 3650-3656.

Thurston-Enriquez, J.A., Haas, C.N., Jacangelo, J., Riley, K. and Gerba, C.P. (2003) Inactivation of feline calicivirus and adenovirus type 40 by UV radiation. *Appl Environ Microbiol* **69**, 577-582.

Torrence M.E. and Isaacson R.E. (2003) *Campylobacter spp.* In: Microbial Food Safety in Animal Agriculture. Iowa: Iowa State Press, pp. 175-176.

Torres, A.G., Li, Y., Tutt, C.B., Xin, L., Eaves-Pyles, T. and Soong, L. (2006) Outer membrane protein A of *Escherichia coli* O157:H7 stimulates dendritic cell activation. *Infect Immun* **74**, 2676-2685.

Torriani S, Orsi C and Vescovo. (1997) Potential of *Lactobacillus casei*, culture permeate, and lactic acid to control microorganims in ready-to-eat vegetables. *J Food Prot* **60**, 1564-1567.

Tortoli, E. (2003) Impact of genotypic studies on mycobacterial taxonomy: the new mycobacteria of the 1990s. *Clin Microbiol Rev* **16**, 319-354.

Ukuku, D.O. (2004) Effect of hydrogen peroxide treatment on microbial quality and appearance of whole and fresh-cut melons contaminated with *Salmonella spp. Int J Food Microbiol* **95**, 137-146.

Ukuku D. O, Pilizota V and Sapers G.M. (2001) Influence of washing treatment on native microflora and *Escherichia coli* 25922 populations of inoculated cantaloupes. *J Food Safety* **21**, 31-47.

Ukuku, D.O., Pilizota, V. and Sapers, G.M. (2004) Effect of hot water and hydrogen peroxide treatments on survival of *Salmonella* and microbial quality of whole and fresh-cut cantaloupe. *J Food Prot* **67**, 432-437.

Ukuku, D.O. and Sapers, G.M. (2001) Effect of sanitizer treatments on *Salmonella* Stanley attached to the surface of cantaloupe and cell transfer to fresh-cut tissues during cutting practices. *J Food Prot* **64**, 1286-1291.

Ukuku, D.O. (2006) Effect of sanitizing treatments on removal of bacteria from cantaloupe surface, and re-contamination with *Salmonella. Food Microbiol* **23**, 289-293.

Ukuku, D.O. and Fett, W.F. (2006) Effects of cell surface charge and hydrophobicity on attachment of 16 *Salmonella* serovars to cantaloupe rind and decontamination with sanitizers. *J Food Prot* **69**, 1835-1843.

Ukuku, D.O. and Sapers G.M. (2006) Microbiological safety issues of fresh melons. In: Sapers GM, Gorny JR, Yousef AE eds. Microbiology of fruits and vegetables. Boca Raton, Florida: CRC Press, pp. 29-30.

Uyttendaele, M., Neyts, K., Vanderswalmen, H., Notebaert, E. and Debevere, J. (2004) Control of *Aeromonas* on minimally processed vegetables by decontamination with lactic acid, chlorinated water, or thyme essential oil solution. *Int J Food Microbiol* **90**, 263-271.

Vaerewijck, M.J., Huys, G., Palomino, J.C., Swings, J. and Portaels, F. (2005) *Mycobacteria* in drinking water distribution systems: ecology and significance for human health. *FEMS Microbiol Rev* **29**, 911-934.

Van Beneden, C.A., Keene, W.E., Strang, R.A., Werker, D.H., King, A.S., Mahon, B., Hedberg, K., Bell, A., Kelly, M.T., Balan, V.K., Mac Kenzie, W.R. and Fleming, D. (1999) Multinational outbreak of *Salmonella* enterica serotype Newport infections due to contaminated alfalfa sprouts. *JAMA* **281**, 158-162.

Vazquez-Boland, J.A., Kuhn, M., Berche, P., Chakraborty, T., Dominguez-Bernal, G., Goebel, W., Gonzalez-Zorn, B., Wehland, J. and Kreft, J. (2001) *Listeria* pathogenesis and molecular virulence determinants. *Clin Microbiol Rev* **14**, 584-640.

Vij, V., Ailes, E., Wolyniak, C., Angulo, F.J. and Klontz, K.C. (2006) Recalls of spices due to bacterial contamination monitored by the U.S. Food and Drug Administration: the predominance of *Salmonellae*. *J Food Prot* **69**, 233-237.

Villanueva, C.M., Cantor, K.P., Grimalt, J.O., Malats, N., Silverman, D., Tardon, A., Garcia-Closas, R., Serra, C., Carrato, A., Castano-Vinyals, G., Marcos, R., Rothman, N., Real, F.X., Dosemeci, M. and Kogevinas, M. (2007) Bladder cancer and exposure to water disinfection by-products through ingestion, bathing, showering, and swimming in pools. *Am J Epidemiol* **165**, 148-156.

Vlasits, J., Jakopitsch, C., Schwanninger, M., Holubar, P. and Obinger, C. (2007) Hydrogen peroxide oxidation by catalase-peroxidase follows a non-scrambling mechanism. *FEBS Letters* **581**, 320-324.

Von Gunten, U. (2003a) Ozonation of drinking water: part I. Oxidation kinetics and product formation. *Water Res* **37**, 1443-1467.

Von Gunten, U. (2003b) Ozonation of drinking water: part II. Disinfection and by-product formation in presence of bromide, iodide or chlorine. *Water Res* **37**, 1469-1487.

Von Gunten, U., Driedger, A., Gallard, H. and Salhi, E. (2001) By-products formation during drinking water disinfection: a tool to assess disinfection efficiency? *Water Res* **35**, 2095-2099.

Wallace, R.J. Jr, Brown, B.A. and Griffith, D.E. (1998) Nosocomial outbreaks/pseudo-outbreaks caused by nontuberculous mycobacteria. *Ann Rev Microbiol* **52**, 453-490.

Watanabe-Akanuma, M., Inaba, Y. and Ohta, T. (2007) Mutagenicity of UV-irradiated maltol in *Salmonella typhimurium*. *Mutagenesis* **22**, 43-47.

Weissinger, W.R. and Beuchat, L.R. (2000) Comparison of aqueous chemical treatments to eliminate *Salmonella* on alfalfa seeds. *J Food Prot* **63**, 1475-1482.

Wert, E. C., Rosario-Ortiz, F. L., Drury, D. D. and Snyder, S. A. (2007) Formation of oxidation byproducts from ozonation of wastewater. *Wat Res* **41,** 1481-1490.

Wolinsky, E. (1979) Nontuberculous mycobacteria and associated diseases. *Am Rev Respir Dis* **119**, 107-159.

Wolinsky, E. (1995) Mycobacterial lymphadenitis in children: a prospective study of 105 nontuberculous cases with long-term follow-up. *Clin Infect Dis* **20**, 954-963.

Wondergem, E. and Van Dijk-Looijaard, A. M. (1991) Chlorine dioxide as a post-disinfectant for Dutch drinking water. *Sci Total Environ* **102**, 101-112.

Wong, H.C., Chao, W.L. and Lee, S.J. (1990) Incidence and characterization of *Listeria monocytogenes* in foods available in Taiwan. *Appl Environ Microbiol* **56**, 3101-3114.

World Health Organization. (2000) WHO, Resolution WHA 53.15 on Food Safety. Fifty-third Health Assembly, Geneva, Switzerland.

Wright, J.R., Sumner S.S, Hackney C.R, Pierson, M.D. and Zoecklein.W.B.(2000) Reduction of *Escherichia coli* O157:H7 on apples using wash and chemical sanitizers. *Dairy Food Environ Sanit* **20,** 120-126.

Xu, P., Janex, M.L., Savoye, P., Cockx, A. and Lazarova, V. (2002) Wastewater disinfection by ozone: main parameters for process design. *Wat Res* **36**, 1043-1055.

Yahya, M.T., Landeen, L.K., Messina, M.C., Kutz, S.M., Schulze, R. and Gerba, C.P. (1990) Disinfection of bacteria in water systems by using electrolytically generated copper: silver and reduced levels of free chlorine. *Can J Microbiol* **36**, 109-116.

Yahya, M.T., Straub T.M., Gerba C.P. and Margolin A.B. (1991) Inactivation of bacteriophage MS-2 and poliovirus in copper, galvanized and plastic domestic water pipes. *Int J Enviro health* **1**, 76-86.

Yamazaki, Y., Danelishvili, L., Wu, M., Hidaka, E., Katsuyama, T., Stang, B., Petrofsky, M., Bildfell, R. and Bermudez, L.E. (2006) The ability to form biofilm influences *Mycobacterium avium* invasion and translocation of bronchial epithelial cells. *Cell Microbiol* **8**, 806-814.

Yanallah, K., Hadj Ziane, S., Belasri, A. and Meslem, Y. (2006) Numerical modeling of ozone production in direct current corona discharge: Numerical Simulations in chemistry, after a symposium held in Algeria. *J Mol Struct: Theochem* **777**, 125-129.

Yaun, B.R., Sumner, S.S., Eifert, J.D. and Marcy, J.E. (2004) Inhibition of pathogens on fresh produce by ultraviolet energy. *Int J Food Microbiol* **90**, 1-8.

Yoder, S., Argueta, C., Holtzman, A., Aronson, T., Berlin, O.G., Tomasek, P., Glover, N., Froman, S. and Stelma, G. Jr. (1999) PCR comparison of *Mycobacterium avium* isolates obtained from patients and foods. *Appl Environ Microbiol* **65**, 2650-2653.

Yuk, H.G., Bartz, J. A. and Schneider, K. R. (2006) The effectiveness of sanitizer treatments in nactivation of *Salmonella* spp. from bell pepper, cucumber, and strawberry. *J Food Sci* **71**, 95-99.

Zepeda-Lopez H., Ortega-Rodriguez M, Quinonez-Ramirez E.I., and Vazquez-Salinas C. (1995). Isolation of *Esherichia coli* 0157:H7 from vegetables. *Annual Mtg America Society of Microbiology (abstracts)*, Washington, D. C.

Zhang, S. and Farber, J. M. (1996) The effects of various disinfectants against *Listeria monocytogenes* on fresh-cut vegetables. *Food Microbiol* **13**, 311-321.

Zhuang, R.Y., Beuchat, L.R. and Angulo, F.J. (1995) Fate of *Salmonella montevideo* on and in raw tomatoes as affected by temperature and treatment with chlorine. *Appl Environ Microbiol* **61**, 2127-2131.

Zwiener, C., Richardson, S.D., De Marini, D.M., Grummt, T., Glauner, T. and Frimmel, F.H. (2007) Drowning in disinfection byproducts? Assessing swimming pool water. *Environ Sci Technol* **41**, 363-372.

APPENDIX A:

MICROBIOLOGICAL EVALUATION OF THE RESIDUAL DISINFECTANT EFFICACY OF COPPER AND SILVER IN AQUEOUS SOLUTION

Enue E. Sicairos-Ruelas, Charles P. Gerba, and Kelly R. Bright*

The University of Arizona, Dept of Soil, Water & Environmental Science, Building 38, Room 429, Tucson, Arizona 85721

Running Title: Residual efficacy of copper and silver
Keywords: Silver, copper, disinfection, disinfectant residual, water distribution system

Author for correspondence:
* Dr. Kelly R. Bright
The University of Arizona
Dept. of Soil, Water & Environmental Science
Building 38, Room 429
Tucson, Arizona 85721
Tel: (520) 626-8094
Fax:(520) 621-6163
Email: bright@email.arizona.edu

Abstract

Chlorine is commonly used as a residual disinfectant in water distribution systems at a concentration of 0.2 mg/L; however, intrusion events (e.g., organic matter, microbial contamination) and the presence of biofilms can severely decrease the amount of free chlorine. Therefore, a longer lasting residual is needed. The efficacy of 100 μg/L silver and 400 μg/L copper, both individually and in combination, were evaluated against *Salmonella enterica* serovar Typhimurium*, Listeria monocytogenes, Escherichia coli,* and *Mycobacterium fortuitum* in an aqueous system at 24°C and 4°C. Silver caused a significant reduction in the Gram-negative species and also *L. monocytogenes* within one hour of exposure. There was a >5.0-$\log_{10}$ reduction in *E. coli* and *L. monocytogenes* within three hours and *Salmonella* serovar Typhimurium within seven hours with silver. Longer exposure times were required at 4°C to achieve significant reductions. *M. fortuitum* was the most resistant species to silver, with a 1.20-$\log_{10}$ reduction within 24 hours. Copper performed poorly against *Salmonella* serovar Typhimurium and *E. coli* at 24°C; nevertheless, a 2.80-$\log_{10}$ and a 2.56-$\log_{10}$ reduction were observed in the Gram-positive species, *L. monocytogenes* and *M. fortuitum*, respectively. A synergistic effect was observed against all species after one hour of exposure when silver and copper were combined at 24°C. In addition, silver was not adversely affected by the presence of organic matter at a concentration that completely inhibited 0.2 mg/L chlorine. Our results suggest that silver and copper show promise as secondary residual disinfectants against a wide variety of bacteria in drinking water distribution systems.

Introduction

Chlorine is the principal drinking water disinfectant used in the United States (50). In addition to primary treatment with chlorine, a residual disinfectant is required because of the potential for bacterial regrowth and contamination of water can be expected once the water enters the drinking water distribution system (1, 8, 29). In the United States, the Environmental Protection Agency's Surface Water Treatment Rule requires the maintenance of at least a 0.2 mg/L residual of free chlorine (4, 14); however, it is sometimes difficult to maintain an adequate concentration of free chlorine throughout the distribution system (20). Factors affecting the concentration of chlorine include the interaction of the chemical with organic matter in the water, the presence of biofilms, and tubercle formation on the pipes (1, 33). In addition, the intrusion of contaminated material during pipe repair, through illegal cross-connections, or through the loss of pressure in the distribution system may further promote disinfectant decay (29, 34). Some microorganisms of health concern such as *Legionella pneumophila* are resistant to chlorine at the concentrations routinely found in water distribution systems (26) and corrosion of plumbing systems is a concern with the extreme levels of chlorine required for *L. pneumophila* inactivation (44).

In addition to the aforementioned problems with chlorine disinfection of water, concern over the potential human health effects of trihalomethanes, haloacetic acids, and other chlorine disinfection by-products (formed as the result of the reaction of the

halogenated element with organic matter) have resulted in increased efforts to develop alternative methods of water disinfection (16, 19, 57).

The antimicrobial activity of silver (Ag) has been known since ancient times. Over the past several decades, silver has been used in water treatment, in dietary supplements, in medical applications, and to create antimicrobial coatings and products (5, 10, 21, 38, 42, 43). Copper (Cu) has also been extensively used as an algaecide for many years (9) and is reported to be one of the most toxic metals to heterotrophic bacteria in aquatic environments (56). Copper is known to have bactericidal and virucidal properties (50). Both copper and silver ions have been used in combination with low levels of chlorine to effectively control *L. pneumophila* in hospital water distribution systems (7).

It has been proposed that copper and silver inactivate bacteria by binding to the sulfhydryl (-SH) groups of enzymes, affecting important metabolic processes such as respiration (46, 51). These metals may also bind to nucleic acids, resulting in cross-linking, or they may catalyze the formation of radicals which cleave chemical bonds (13, 32).

The objective of the current study was to evaluate the residual disinfectant efficacy of silver and copper, both individually and in combination, for the inactivation of *Salmonella enterica* serovar Typhimurium, *Escherichia coli, Listeria monocytogenes* and *Mycobacterium fortuitum* in an aqueous model system. Although *L. monocytogenes* is not a waterborne pathogen, it was included to compare the disinfection efficacy against Gram-positive versus Gram-negative bacteria.

All experiments were conducted in demand-free 0.01 M phosphate buffer (3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4; pH 7.5) rather than tap water so that the test solutions could be standardized. This is a standard method for evaluating the efficacy of various water treatments [e.g., calculating concentration-time (Ct) values for disinfectants such as chlorine, chloramines, chlorine dioxide, ozone, etc.] against specific microorganisms (25, 52, 53). In addition, silver and copper have been shown to be highly effective against *Legionella pneumophila* in tap water in numerous studies over long periods of time (27, 37, 48).

Materials and methods

Glassware

All of the glassware were soaked overnight in a 10% nitric acid solution to reduce metal contamination and then rinsed with distilled water and autoclaved. Glassware used for the experiments involving chlorine were soaked for 24 hours in a 30% (v/v) chlorine solution then rinsed with distilled water and baked at 104°C for two hours to satisfy chlorine demand. Experiments were conducted in 250 ml sterile Erlenmeyer flasks.

Disinfectant preparation

Stock solutions of silver and copper were prepared in distilled water using $AgNO_3$ (J.T. Baker, Phillipsburg, NY) and $CuCl_2$* H_2O (J.T. Baker, Phillipsburg, NY) and then added to the test suspension at a final concentration of 100 μg/L silver and 400 μg/L copper. The silver and copper concentrations were confirmed at the beginning of each experiment by a colorimetric procedure using a Hach DR/2000 spectrophotometer (23).

Similarly, a chlorine stock solution was prepared in distilled water using 5.0% sodium hypochlorite (NaOCl; J.T. Baker, Phillipsburg, NJ) and then added to the test suspension at a final concentration of 0.2 mg/L free chlorine. The initial free chlorine concentration was determined by the *N, N,*-dimethyl-*p*-phenylenediamine method adapted from *Standard Methods for the Examination of Water and Wastewater* (2).

Humic acid (Aldrich Chemical Company, Inc., Milwaukee, WI) was used as a source of organic matter in order to achieve a concentration of 3 mg/L or 10 mg/L of total organic carbon (TOC). The final TOC concentrations were confirmed by combustion analysis using a TOC-VCSH instrument (Shimadzu, Columbia, MD).

Maintenance and preparation of bacterial isolates

All test bacteria in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Strains included *Escherichia coli* ATCC 25922, *Listeria monocytogenes* ATCC 19115, *Salmonella enterica* serovar Typhimurium ATCC 23564, and *Mycobacterium fortuitum* ATCC 6841. The bacteria were maintained on Tryptic Soy Agar (TSA; Difco, Sparks, MD). Erlenmeyer flasks containing 100 ml of Tryptic Soy

Broth (TSB; Difco, Sparks, MD) were inoculated and incubated on an orbital shaker (250 rpm) at 37°C overnight prior testing. After incubation, the bacteria were pelleted via centrifugation (15,300 × *g*, 10 min, 10°C, JA-14 rotor, Beckman J2-21 centrifuge; Beckman Coulter, Inc., Fullerton, CA). The pelleted cells were washed by resuspension in 100 ml of 0.01 M phosphate buffer (3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4; pH 7.5) followed by centrifugation as described previously. This step was repeated one additional time. The final pellet was resuspended in 20 ml of 0.01 M phosphate buffer. The test suspensions were then prepared by adding small volumes of the bacterial suspension to 10 ml of 0.01 M phosphate buffer, resulting in an optical turbidity (measured using a BIOLOG turbidimeter, Hayward, CA) equivalent to a McFarland number 0.5 optical density standard (1.5×10^8 CFU/ml). This solution was then diluted further in 0.01 M phosphate buffer to achieve the desired final test concentration (approximately 1.0×10^7 CFU/ml).

Experimental procedure

The disinfection treatments included the following: (*i*) 100 µg/L silver, (*ii*) 400 µg/L copper, (*iii*) 100 µg/L silver and 400 µg/L copper, *(iv)* 100 µg/L silver and 3 mg/L total organic carbon (TOC), *(v)* 100 µg/L silver and 10 mg/L TOC, *(vi)* 0.2 mg/L chlorine, *(vii)* 0.2 mg/L chlorine and 3 mg/L TOC, and *(viii)* 0.2 mg/L chlorine and 10 mg/L TOC. A control with bacteria but no added disinfectant and another with disinfectant but no bacteria were also included. Experiments were performed in triplicate at room

temperature (24°C) and at 4°C for the silver, the copper and the silver/copper treatments. Experiments conducted with added TOC and/or chlorine were performed at 24°C only.

Purified stocks of the bacteria were added separately to the disinfection systems and the flasks were placed on an orbital shaker (200 rpm) for the duration of the experiment. At predetermined time intervals, 1 ml samples were collected and neutralized with Dey Engley neutralizing broth (D/E; Difco, Sparks, MD) at a ratio of 1:1. Samples were assayed immediately.

Assay for bacteria

Bacterial samples were serially diluted in 0.01 M phosphate buffer and the surviving bacteria were enumerated in duplicate using the spread plate method on the following agar media: *E. coli,* mEndo (Difco, Sparks, MD); *L. monocytogenes, Listeria* selective agar (LSA; Oxford, Columbia, MD); *Salmonella* serovar Typhimurium, Hektoen (Difco, Sparks, MD); *M. fortuitum*, Tryptic Soy Agar (TSA; Difco, Sparks, MD). The plates were incubated at 37°C for 24 hours (*E. coli, Salmonella* serovar Typhimurium, and *L. monocytogenes*) or 72 hours (*M. fortuitum*).

Data analyses

Data were reported as logarithmic reduction $-\log_{10} N_t / N_0$ where N_t was the concentration of microorganisms at time *t* and N_0 was the concentration of microorganisms at time zero.

Synergy was evaluated by the following formula described by Koivunen and Heinonen-Tanski (24):

Synergy value = $\log_{10}$ reduction by a combination of treatments 1 and 2 – ($\log_{10}$ reduction by treatment 1 + $\log_{10}$ reduction by treatment 2)

If the value of the equation is positive, a synergistic effect is present, whereas a negative value represents an antagonistic effect. The value zero means that the efficiency of the combined treatments is the same as the sum of the two individual treatments.

A Student's t-test was used for the analysis of variance to determine if there were significant differences between the control and the disinfection treatments. Differences were considered significant if the resultant *P* value was ≤ 0.05.

Results

The impact of the metals on bacterial reductions is shown in Tables 1 through 8. There was a significant reduction in *Salmonella* serovar Typhimurium within one hour of exposure with both the silver (100 μg/L) and the silver/copper (100/400 μg/L) treatments in the experiments conducted at room temperature (Table 1). The observed reductions were also significant for these treatments for every sampling time thereafter. After three hours of exposure to silver and silver/copper at room temperature, *Salmonella* serovar

Typhimurium was reduced by 3.72-$\log_{10}$ and by 2.88-$\log_{10}$, respectively. After seven hours, a 5.51-$\log_{10}$ reduction was observed with silver and a 3.51-$\log_{10}$ reduction was observed with silver/copper. No significant reductions were found at any of the exposure times with the copper (400 μg/L) treatment.

Significant reductions of 2.44-$\log_{10}$ and 3.11-$\log_{10}$ of *E. coli* were achieved after one hour of exposure at room temperature to silver (100 μg/L) and silver/copper (100/400 μg/L), respectively (Table 3); after 3 hours of exposure and each subsequent sampling time, greater than a 5.51-$\log_{10}$ reduction was observed with both the silver and the silver/copper treatments. As with the *Salmonella* serovar Typhimurium experiment, no significant reductions in *E. coli* were observed at any of the exposure times with the copper (400 μg/L) treatment.

M. fortuitum was the most resistant bacterial species to silver (Table 5) with only a 1.20-$\log_{10}$ reduction observed after 24 hours of exposure. In contrast, a 2.56-$\log_{10}$ and a 2.96-$\log_{10}$ reduction were observed for the copper treatment after 7 hours and 24 hours of exposure, respectively. *M. fortuitum* was therefore much more sensitive to copper than were *Salmonella* serovar Typhimurium and *E. coli*. In addition, a 4.07-$\log_{10}$ reduction was observed for the silver/copper treatment after 24 hours. Although the overall reductions observed for *M. fortuitum* were lower than those observed for the other bacterial species in this study, they were, nevertheless, still statistically significant in comparison to the control.

Similar reductions to those found for *E. coli* were achieved with both the silver and the silver/copper treatments for *L. monocytogenes* (Table 7). Within three hours of

exposure, reductions greater than 5.03-$\log_{10}$ were observed for both the silver and the silver/copper treatments; within five hours, no bacteria were recovered with these treatments (>6.51-$\log_{10}$ reduction). Unlike *E. coli*, however, *L. monocytogenes* was also found to be sensitive to the 400 μg/L copper treatment, with a 2.43-$\log_{10}$ reduction after five hours and a 2.80-$\log_{10}$ reduction after seven hours of exposure.

A lower temperature adversely affected the inactivation of all the bacterial species tested. Longer exposure times were needed at 4°C in order to achieve significant reductions and these reductions were generally lower than those observed during the tests conducted at 24°C. A 0.14-$\log_{10}$ and a 2.10-$\log_{10}$ reduction were achieved for *M. fortuitum* after 24 hours of exposure to the copper and the silver/copper treatments at 4°C (Table 6). Likewise, only a 2.12-$\log_{10}$, a 0.71-$\log_{10}$, and a 1.37-$\log_{10}$ reduction were achieved for *L. monocytogenes* after seven hours of exposure to the silver, the copper, and the silver/copper treatments at 4°C (Table 8). These were significantly lower than the reductions achieved for these organisms with these treatments at room temperature (Tables 5 and 7), particularly for the copper treatment.

Salmonella serovar Typhimurium was reduced by only 0.26-$\log_{10}$ after seven hours of exposure to silver at 4°C (Table 2) in comparison to the 5.51-$\log_{10}$ reduction achieved at room temperature with this treatment. As with the experiments conducted at room temperature, the copper treatment did not yield any significant reductions within seven hours. Interestingly though, a 2.39-$\log_{10}$ reduction was observed after 7 hours of exposure to silver/copper (100/400 μg/L). This was only about 10-fold lower than the

reduction of 3.51-$\log_{10}$ achieved at room temperature. Thus, the lower temperature did not seem to have as great an effect on the two metals when used in combination.

The reductions in *E. coli* observed in the tests conducted at 4°C did not appear to be as affected by the lower temperature as the reductions in the other bacterial species studied (Table 4). It took five hours to achieve greater than a 4-$\log_{10}$ reduction and seven hours for greater than a 5-$\log_{10}$ reduction in *E. coli* at 4°C (versus three hours at room temperature) with both the silver and the silver/copper treatments; nonetheless, significant reductions were observed within one hour. Copper was still ineffective against *E. coli* at this temperature.

A synergistic effect with both metals present was observed for all of the bacterial species after one hour of exposure at 24°C. Nevertheless, at later exposure times, there appeared to be a small antagonistic effect between the two metal ions. In other words, the combined effect of the two metals was less than what would be expected by the addition of the two individual effects of the metals.

A similar early synergy (within 1 to 2 hours of exposure) was found for *Salmonella* serovar Typhimurium and *L. monocytogenes*, but not for the other bacterial species tested at 4°C. In addition, although synergy was not observed between the metals in the latter sampling times with the *Salmonella* serovar Typhimurium experiment conducted at room temperature, a significant synergistic effect was found throughout the course of the experiment at all exposure times at 4°C with *Salmonella* serovar Typhimurium.

The effect of organic matter on the antimicrobial activities of both silver and chlorine are presented in Tables 9 and 10. *E. coli* was chosen as a representative bacterial species because it is known to be sensitive to the effects of both disinfectants. The inactivation of *E. coli* by 100 μg/L of silver was not affected by the presence of either 3 mg/L or 10 mg/L of total organic carbon (Table 9). In contrast, a chlorine concentration of 0.2 mg/L (the concentration generally used for chlorine residual in distribution systems) was almost completely neutralized by the addition of 3 mg/L and 10 mg/L TOC (Table 10). The chlorine alone resulted in greater than a 5.70-$\log_{10}$ reduction within one hour of exposure; however with the addition of either 3 mg/L or 10 mg/L TOC to the chlorine, reductions of only 0.19-$\log_{10}$ and 0.18-$\log_{10}$ were achieved, respectively, after seven hours of exposure.

Discussion

Silver and copper ion concentrations (100 and 400 μg/L, respectively) remained constant in the controls with no added bacteria at both 24°C and 4°C throughout the course of all the experiments. Likewise, the copper concentrations remained unchanged in the treatment suspensions containing *E. coli* and *Salmonella* serovar Typhimurium throughout the experiments (seven hours) conducted at both 24°C and 4°C. In contrast, silver was not detected in any of the treatment supernatants after seven hours at 24°C and only 10 μg/L and 30 μg/L of silver were detected after seven hours at 4°C for *E. coli* and *Salmonella* serovar Typhimurium, respectively. This suggests that the silver was most

likely bound to cellular components (e.g. proteins), whereas copper was being prevented from entering or interacting with the microbial cells. Silver may gain access to the inside of the cell by an active uptake mechanism of a microbial transport system (45, 47). The temperature also affected the amount of unbound silver, with more detected at 4°C than at 24°C (10-30 μg/L versus none detected). At low temperatures, bacterial growth, and therefore their metabolism, is affected (18). Thus, low temperatures can adversely reduce the antimicrobial properties of copper and silver by limiting the numbers of ions that can be successfully transported inside the cell.

Unlike these Gram-negative species, *L. monocytogenes* was sensitive to both metals, although only moderately sensitive to copper. In the treatment suspensions containing *L. monocytogenes*, the silver levels dropped to 10 μg/L after seven hours at 24°C and to 40 μg/L at 4°C. The copper levels also dropped to 290 μg/L at 24°C and to 340 μg/L at 4°C. This suggests that both the silver and copper were interacting with the *L. monocytogenes* cells.

Copper and silver can react with the outer and inner bacterial cell components, disrupting normal bacterial metabolism. An early synergism was observed when copper and silver were used in combination against the bacterial species tested. The reasons for this enhanced effect are not known, but it could be due to different target sites for the two metals. The ability of the cell to recover is thus overwhelmed by the combined damage. Silver may also inactivate enzymes involved in the regulation of copper homeostasis.

Copper is considered safe to humans, as demonstrated by the widespread and prolonged use of copper intrauterine devices (9) and copper pipes/plumbing fixtures (3,

15). Both the Environmental Protection Agency (EPA) and the World Health Organization (WHO) also regard silver as safe for human consumption. In addition, silver has not been observed to have any detrimental effects on mammalian cells (6) at the low concentrations needed to inactivate bacteria.

Based on epidemiological and pharmacokinetic data, a lifetime limit of 10 grams of silver can be considered a No Observable Adverse Effect Level (NOAEL) for humans (54). In the United States, no primary standards exist for silver as a component in drinking water. The EPA recommends a secondary non-enforceable standard of 0.1 mg/L (100 μg/L) for silver and 1 mg/L (1000 μg/L) for copper (17). The World Health Organization has stated this amount of silver in water disinfection could easily be tolerated since the total absorbed dose would only be half of the NOAEL after 70 years (54).

There is considerable variation in the response of different microorganisms to antimicrobials. The underlying reasons are poorly understood, but the chemical composition of the outer cell layers is thought to be a key factor (31, 39). *L. monocytogenes* was used as a model for the inactivation of Gram-positive microorganisms by silver and copper. Treatment with Cu (400 μg/L) resulted in a 2.43-$\log_{10}$ reduction of *L. monocytogenes* after 5 hours at 24°C. In contrast, the Gram-negative organisms, *E. coli* and *Salmonella* serovar Typhimurium, were not significantly affected by copper. Gram-positive microorganisms are able to bind up to 30 times more Cu^{+2} than *E. coli* (11). It has also been reported that the Gram-negative species *Pseudomonas* and

Ferrobacillus can tolerate up to 300 mg/L copper before an inhibitory effect is observed (41). This is 750 times the concentration used in this study.

Mycobacteria, although they are Gram-positive, have an unusual cell wall that contains high levels of lipids (waxy material), making these microorganisms naturally resistant to many disinfectants by reducing their uptake (31, 36). Furthermore, the ability of some strains of mycobacteria to clump together confers extra protection against chemical agents. Mycobacteria have been found to be resistant to chlorine in water supplies and swimming pools. Thus, the residual chlorine in distribution systems does not eradicate this pathogen (35).

The results of the present study show a significant reduction of *M. fortuitum* after treatment with Ag/Cu (100/400 μg/L) and Cu (400 μg/L). It is interesting to note that the results for copper were quite similar for *M. fortuitum* and the other Gram-positive bacterium, *L. monocytogenes* (2.56-$\log_{10}$ and 2.80-$\log_{10}$ reductions after seven hours, respectively), whereas *M. fortuitum* was much more resistant to silver (1.20-$\log_{10}$ versus a > 6.51-$\log_{10}$ reduction for *L. monocytogenes* after seven hours). *M. fortuitum* was included in this study as a model for *Mycobacterium avium* Complex (MAC) inactivation. The MAC bacteria are currently on the EPA's contaminant candidate list (CCL) for possible future regulation in drinking water (22).

Silver (100 μg/L) caused a significant reduction in the Gram-negative species, *Salmonella* serovar Typhimurium and *E. coli*, and the Gram-positive species, *L. monocytogenes*, within one hour of exposure. The silver treatment resulted in a >5.0-$\log_{10}$ reduction in *E. coli* and *L. monocytogenes* within three hours of exposure and *Salmonella*

serovar Typhimurium within seven hours. *M. fortuitum* was far more resistant to silver than all of the other species tested, with only a 1.20-$\log_{10}$ reduction observed within 24 hours.

An early synergistic effect was observed within the first few hours of exposure when silver (100 μg/L) and copper (400 μg/L) were used in combination against all of the species tested. Although there was no such synergy found in the latter sampling times, a combination of silver and copper could still be preferable to the use of either metal individually to provide a disinfectant residual in water distribution systems (that have diverse microbial populations that include resistant organisms such as the mycobacteria).

One of the objections to using copper and silver in water distribution systems has been that these metals will concentrate on surfaces and this will limit the availability of ions in the liquid phase. It is unclear as to whether these metals would then become unavailable and no longer provide an antimicrobial effect. It is possible that the concentration of these metals on water distribution surfaces could inhibit the formation of biofilms or create a disinfectant residual reservoir with long-term usage. Silver has been used extensively as an additive in point-of-use water filtration devices to prevent such biofilm formation on the carbon filters (40, 55).

The residual disinfectant (usually chlorine) in distribution systems can be severely affected by intrusions events (e.g., organic matter and microbial contamination) and by the presence of biofilms that might react with the chemical (29). As demonstrated by the results of this study, organic matter does not adversely affect the antimicrobial action of

silver. In comparison, the activity of 0.2 mg/L chlorine was completely inhibited by the same levels of TOC. Similarly, in an earlier study, Butkus *et al*. (12) found silver to be unaffected by the presence of organic matter.

There have also been numerous studies that suggest that silver and copper can be utilized along with low concentrations of chlorine to effectively treat water. In one study, the inactivation of *Legionella pneumophila* by a combination of copper and silver was shown to be relatively slow when compared to that of free chlorine; nevertheless, when silver and copper were combined with low levels of free chlorine, the inactivation rates of bacterial indicator organisms were greater than those for free chlorine alone (28, 55). Beer *et al.* (5) found that electrolytically generated copper and silver ions used in swimming pool water along with lower levels of chlorine provided control of total coliforms and heterotrophic bacteria equivalent to the control provided by high levels of chlorine. Yahya *et al.* (55) demonstrated that adding 400 μg/L copper and 40 μg/L silver to water systems containing contaminants similar to those in swimming pools allowed the concentration of free chlorine to be reduced at least three-fold (from 0.3 to 0.1 mg/L). Enhanced inactivation rates for *E. coli, L. pneumophila*, *Staphylococcus aureus, Streptococcus faecalis* (28, 55), and *Pseudomonas aeruginosa* (28) were also obtained when water was treated with 400 μg/L copper, 40 μg/L silver, and 0.2 mg/L free chlorine.

The combination of silver and copper with chlorine may also be useful in preventing the development of microbial resistance. As demonstrated in the current study, a combination of silver and copper is not adversely affected by the presence of

organic matter (at concentrations that would neutralize the chlorine levels usually found in drinking water) and is effective against diverse species of bacteria. In addition, silver and copper do not produce any harmful disinfectant by-products (30) or cause corrosion of pipes (49). Silver and copper therefore show promise as secondary residual disinfectants for drinking water distribution systems. They may be used together or also in conjunction with a low level of chlorine or another disinfectant to provide an additional, long-lasting residual disinfectant in water distribution systems.

Acknowledgments

The authors would like to thank Deena M. Clark for her assistance in the performance of the experiments.

References

1. Al-Jasser, A. O. 2007. Chlorine decay in drinking-water transmission and distribution systems: Pipe service age effect. Water Res. **41:** 387-396.

2. American Public Health Association. 2005. Inorganic nonmetallic constituents, p. 4-67. *In* Eaton, A.D., V. S. Clesceri, E. W. Rice, and A. E. Greenberg (ed.), Standard methods for the examination of water and wastewater, 21st ed. American Public Health Association, Washington, D.C.

3. Assanta, M. A., D. Roy, M.J. Lemay, and D. Montpetit. 2002. Attachment of *Arcobacter butzleri*, a new waterborne pathogen, to water distribution pipe surfaces. J. Food Prot. **65:** 1240-1247.

4. Barwick, R. S., D. A. Levy, G. F. Craun, M. J. Beach, and R. L. Calderon. 2000. Surveillance for waterborne-disease outbreaks--United States, 1997-1998. MMWR CDC Surveill. Summ. **49:** 1-21.

5. Beer, C. W., L. E. Guilmartin, T. F. McLoughlin, and T. J. White. 1999. Swimming pool disinfection: efficacy of copper/silver ions with reduced chlorine levels. J. Environ. Health **61:** 9-13.

6. Berger, T. J., J. A. Spadaro, S. E. Chapin, and R. O. Becker. 1976. Electrically generated silver ions: quantitative effects on bacterial and mammalian cells. Antimicrob. Agents Chemother. **9**: 357-358.

7. Biurrun, A., L. Caballero, C. Pelaz, E. Leon, and A. Gago. 1999. Treatment of a *Legionella pneumophila*-colonized water distribution system using copper-silver ionization and continuous chlorination. Infect. Control Hosp. Epidemiol. **20**: 426-428.

8. Bois, F. Y., T. Fahmy, J. C. Block, and D. Gatel. 1997. Dynamic modeling of bacteria in a pilot drinking-water distribution system. Water Res. **31**: 3146-3156.

9. Borkow, G., and J. Gabbay. 2005. Copper as a biocidal tool. Curr. Med. Chem. **12**: 2163-2175.

10. Bright, K. R., C. P. Gerba, and P. A. Rusin. 2002. Rapid reduction of *Staphylococcus aureus* populations on stainless steel surfaces by zeolite ceramic coatings containing silver and zinc ions. J. Hosp. Infect. **52**: 307-309.

11. Bruins, M. R., S. Kapil, and F. W. Oehme. 2000. Microbial resistance to metals in the environment. Ecotoxicol. Environ. Saf. **45:** 198-207.

12. Butkus, M. A., M. Talbot, and M. P. Labare. 2005. Feasibility of the silver-UV process for drinking water disinfection. Water Res. **39**: 4925-4932.

13. Cervantes, C., and F. Gutierrez-Corona. 1994. Copper resistance mechanisms in bacteria and fungi. FEMS Microbiol. Rev. **14:** 121-137.

14. Clark, R. M., and M. Sivaganesan. 2002. Predicting chlorine residuals in drinking water: second order model. J. Water Resour. Manage. **128:** 152-162.

15. Domek, M. J., M. W. LeChevallier, S. C. Cameron, and G.A. McFeters. 1984. Evidence for the role of copper in the injury process of coliform bacteria in drinking water. Appl. Environ. Microbiol. **48:** 289-293.

16. Eish, M. Y., and M. J. Wells. 2006. Assessing the trihalomethane formation potential of aquatic fulvic and humic acids fractionated using thin-layer chromatography. J. Chromatogr. A **1116:** 272-276.

17. Environmental Protection Agency. 2002. National secondary drinking water regulations. http://www.epa.gov/safewater/mcl.html. Accessed April 19, 2007.

18. Felip, M., M. L. Pace, and J. J. Cole. 1996. Regulation of planktonic bacterial growth rates: the effects of temperature and resources. Microb. Ecol. **31:** 15–28.

19. Fiessinger, F., J. J. Rook, and J. P. Duguet. 1985. Alternative methods for chlorination: organic micropollutants in drinking water and health. Sci. Total Environ. **47**: 299-315.

20. Fliermans, C. B., G. E. Bettinger, and A. W. Fynsk. 1982. Treatment of cooling systems containing high levels of *Legionella pneumophila*. Water Res. **16:** 903-909.

21. George, N., J. Faoagali, and M. Muller. 1997. Silvazine (silver sulfadiazine and chlorhexidine) activity against 200 clinical isolates. Burns **23:** 493-495.

22. Gerba, C. P., N. Nwachuku, and K. R. Riley. 2003. Disinfection resistance of waterborne pathogens on the United States Environmental Protection Agency's Contaminant Candidate List (CCL). J. Water SRT - Aqua **52:** 81-94.

23. Hach Company. 1989. p. 160-161, *In* Hach Water Analysis Handbook. Loveland, CO.

24. Koivunen, J., and H. Heinonen-Tanski. 2005. Inactivation of enteric microorganisms with chemical disinfectants, UV irradiation and combined chemical/UV treatments. Water Res. **39:** 1519-1526.

25. Korich, D. G., J. R. Mead, M. S. Madore, N. A. Sinclair, and C. R. Sterling. 1990. Effects of ozone, chlorine dioxide, chlorine, and monochloramine on *Cryptosporidium parvum* oocyst viability. Appl. Environ. Microbiol. **56:** 1423-1428.

26. Kuchta, J. M., S. J. States, A. M. McNamara, R. M. Wadowsky, and R. B. Yee. 1983. Susceptibility of *Legionella pneumophila* to chlorine in tap water. Appl. Environ. Microbiol. **46:** 1134-1139.

27. Kusnetsov, J., Iivanainen, E., Elomaa, N., Zacheus, O., and Martikainen, P. J. 2001. Copper and silver ions more effective against legionellae than against mycobacteria in a hospital warm water system. Water Res. **35**: 4217-4225.

28. Landeen, L. K., M. T. Yahya, and C. P. Gerba. 1989. Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila.* Appl. Environ. Microbiol. **55:** 3045-3050.

29. LeChevallier, M. W., R. W. Gullick, M. R. Karim, M. Friedman, and J. E. Funk. 2003. The potential for health risks from intrusion of contaminants into the distribution system from pressure transients. J. Water Health **1:** 3-14.

30. Liu, Z., J. E. Stout, L. Tedesco, M. Boldin, C. Hwang, W. F. Diven, and V. L. Yu. 1994. Controlled evaluation of copper-silver ionization in eradicating *Legionella pneumophila* from a hospital water distribution system. J. Infect. Dis. **169:** 919-922.

31. McDonnell, G., and A. D. Russell. 1999. Antiseptics and disinfectants: activity, action, and resistance. Clin. Microbiol. Rev. **12:** 147-179.

32. Modak, S. M., L. Sampath, and C. L. Fox, Jr. 1988. Combined topical use of silver sulfadiazine and antibiotics as a possible solution to bacterial resistance in burn wounds. J. Burn Care Rehabil. **9:** 359-363.

33. Ndiongue, S., P. M. Huck, and R. M. Slawson. 2005. Effects of temperature and biodegradable organic matter on control of biofilms by free chlorine in a model drinking water distribution system. Water Res. **39:** 953-964.

34. Payment, P. 1999. Poor efficacy of residual chlorine disinfectant in drinking water to inactivate waterborne pathogens in distribution systems. Can. J. Microbiol. **45:** 709-715.

35. Pelletier, P. A., G. C. du Moulin, and K. D. Stottmeier. 1988. Mycobacteria in public water supplies: comparative resistance to chlorine. Microbiol. Sci. **5:** 147-148.

36. Rezwan, M., M. A. Laneelle, P. Sander, and M. Daffe. 2007. Breaking down the wall: fractionation of mycobacteria. J. Microbiol. Methods **68:** 32-39.

37. Rohr, U., M. Senger, F. Selenka, R. Turley, and M. Wilhelm. 1999. Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system. Clin. Infect. Dis. **29:** 1507-1511.

38. Rusin, P. A., K. R. Bright, and G. P. Gerba. 2003. Rapid reduction of *Legionella pneumophila* on stainless steel with zeolite coatings containing silver and zinc ions. Lett. Appl. Microbiol. **36**: 69-72.

39. Russell, A. D. 2003. Similarities and differences in the responses of microorganisms to biocides. J. Antimicrob. Chemother. **52:** 750-763.

40. Russell, A. D., and W. B. Hugo. 1994. Antimicrobial activity and action of silver. Prog. Med. Chem. **31:** 351-370.

41. Sadler, W. R., and P. A. Trudinger. 1967. The inhibition of microorganisms by heavy metals. Miner. Deposita. **2:** 158-168.

42. Silver, S., J. F. Lo, and A. Gupta. 1999. Silver cations as an antimicrobial agent: clinical uses and bacterial resistance. APUA Newsletter. **17:** 1-3.

43. Silvestry-Rodriguez, N., E. E. Sicairos-Ruelas, C. P. Gerba, and K. R. Bright. 2007. Silver as a disinfectant. Rev. Environ. Contam. Toxicol. **191:** 23-45.

44. Skaliy, P., T. A. Thompson, G. W. Gorman, G. K. Morris, H. V. McEachern, and D. C. Mackel. 1980. Laboratory studies of disinfectants against *Legionella pneumophila*. Appl. Environ. Microbiol. **40:** 697-700.

45. Slawson, R. M., H. Lee, and J. T. Trevors. 1990. Bacterial interactions with silver. Biol. Met. **3:** 151-154.

46. Slawson, R. M., M. I. Van Dyke, H. Lee, J. T. Trevors. 1992. Germanium and silver resistance, accumulation, and toxicity in microorganisms. Plasmid **27:** 72-79.

47. Solioz, M., and A. Odermatt. 1995. Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. J. Biol. Chem. **270:** 9217-9221.

48. Stout, J. E., Y. S. Lin, A. M. Goetz, and R. R. Muder. 1998. Controlling *Legionella* in hospital water systems: experience with the superheat-and-flush method and copper-silver ionization. Infect. Control Hosp. Epidemiol. **19:** 911-914.

49. Stout, J. E., and V. L. Yu. 1997. Legionellosis. N. Engl. J. Med. **337:** 682-687.

50. Straub, T. M., C. P. Gerba, X. Zhou, R. Price, and M. T. Yahya. 1995. Synergistic inactivation of *Escherichia coli* and MS-2 coliphage by chloramine and cupric chloride. Water Res. **29:** 811-818.

51. Thurman, R. B., and C. P. Gerba. 1989. The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. CRC Crit. Rev. Environ. Contr. **18:** 295-315.

52. Thurston-Enriquez, J. A., C. N. Haas, J. Jacangelo, and C. P. Gerba. 2005. Inactivation of enteric adenovirus and feline calicivirus by chlorine dioxide. Appl. Environ. Microbiol. **71:** 3100-3115.

53. Thurston-Enriquez, J. A., C. N. Haas, J. Jacangelo, and C. P. Gerba. 2005. Inactivation of enteric adenovirus and feline calicivirus by ozone. Water Res. **39:** 3650-3656.

54. World Health Organization. 2004. Chemical fact sheets, p. 434. *In* Guidelines for drinking-water quality, 3rd ed., vol. 1. Geneva, Switzerland.

55. Yahya, M. T., L. K. Landeen, M. C. Messina, S. M. Kutz, R. Schulze, and C. P. Gerba. 1990. Disinfection of bacteria in water systems by using electrolytically generated copper:silver and reduced levels of free chlorine. Can. J. Microbiol. **36:** 109-116.

56. Yahya, M. T., T. M. Straub, and C. P. Gerba. 1990. Inactivation of poliovirus and bacteriophage MS-2 in copper, galvanized, and plastic domestic water pipes. J. Environ. Health **1:** 76-86.

57. Zhou, H., X. J. Zhang, and Z. S. Wang. 2004. Occurrence of haloacetic acids in drinking water in certain cities of China. Biomed. Environ. Sci. **17:** 299-308.

Table 1. Reduction[a] of *Salmonella* serovar Typhimurium[b] after exposure to silver (100 μg/L), copper (400 μg/L), and silver/copper (100/400 μg/L) at 24°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	1.38×10^7	7.24×10^5	< 0.001	7.50×10^6	0.102	5.00×10^4	< 0.001
	1.48×10^7	6.84×10^5		1.40×10^7		5.30×10^4	
	1.43×10^7	1.10×10^6		1.08×10^7		6.70×10^4	
2	9.10×10^6	1.40×10^4	0.006	5.30×10^6	0.160	1.80×10^4	0.006
	1.36×10^7	7.80×10^4		1.07×10^7		1.50×10^4	
	1.15×10^7	2.20×10^4		8.00×10^6		2.70×10^4	
3	6.80×10^6	1.01×10^3	0.008	4.80×10^6	0.061	3.50×10^4	0.008
	1.08×10^7	2.56×10^3		7.40×10^6		1.47×10^4	
	8.80×10^6	6.52×10^3		6.10×10^6		1.80×10^4	
5	7.80×10^6	7.10×10^2	0.013	3.90×10^6	0.074	1.01×10^4	0.013
	1.41×10^7	7.40×10^2		9.40×10^6		1.40×10^4	
	1.10×10^7	5.90×10^2		6.60×10^6		2.20×10^4	
7	2.30×10^6	2.00×10^1	0.041	4.40×10^6	0.314	5.20×10^3	0.041
	7.50×10^6	9.00×10^1		7.30×10^6		5.10×10^3	
	5.00×10^6	9.00×10^1		5.80×10^6		6.04×10^3	

[a] CFU/ml
[b] Inoculum 1.77×10^7 CFU/ml
[c] Probability (comparison of treatment and control)

Table 2. Reduction[a] of *Salmonella* serovar Typhimurium[b] after exposure to silver (100 µg/L), copper (400 µg/L), and silver/copper (100/400 µg/L) at 4°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	7.70×10^6	3.80×10^6	0.062	9.40×10^6	0.413	6.60×10^4	0.003
	5.90×10^6	5.60×10^6		7.10×10^6		8.80×10^4	
	7.60×10^6	6.30×10^6		3.40×10^6		1.74×10^4	
2	4.70×10^6	4.10×10^6	0.483	7.90×10^6	0.351	2.70×10^4	0.005
	4.50×10^6	5.80×10^6		7.40×10^6		4.50×10^4	
	6.10×10^6	5.50×10^6		2.30×10^6		1.53×10^5	
3	7.50×10^6	6.30×10^6	0.173	8.20×10^6	0.306	4.90×10^4	0.006
	5.20×10^6	5.42×10^6		5.00×10^6		4.80×10^4	
	6.00×10^6	3.80×10^6		2.60×10^6		4.30×10^4	
5	8.00×10^6	7.20×10^6	0.049	9.10×10^6	0.176	3.20×10^4	0.001
	7.10×10^6	4.30×10^6		3.10×10^6		2.70×10^4	
	7.30×10^6	3.70×10^6		2.60×10^6		8.50×10^4	
7	9.00×10^6	5.80×10^6	0.010	1.06×10^7	0.118	4.30×10^4	0.006
	1.16×10^7	3.90×10^6		2.50×10^6		4.70×10^4	
	8.00×10^6	5.20×10^6		2.80×10^6		2.20×10^4	

[a] CFU/ml
[b] Inoculum 9.11×10^6 CFU/ml
[c] Probability (comparison of treatment and control)

Table 3. Reduction[a] of *Escherichia coli*[b] after exposure to silver (100 µg/L), copper (400 µg/L), and silver/copper (100/400 µg/L) at 24°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	5.50×10^6	5.50×10^4	0.019	6.30×10^6	0.483	5.00×10^3	0.019
	1.01×10^7	3.20×10^4		1.01×10^7		7.00×10^3	
	1.14×10^7	1.80×10^4		1.03×10^7		1.00×10^4	
2	9.30×10^6	1.04×10^3	< 0.001	7.80×10^6	0.221	1.50×10^2	0.019
	8.50×10^6	3.30×10^2		1.07×10^7		2.70×10^2	
	8.80×10^6	2.10×10^2		1.06×10^7		1.40×10^2	
3	4.20×10^6	2.00×10^1	0.014	9.40×10^6	0.153	1.00×10^1	0.014
	7.40×10^6	<10[d]		6.20×10^6		<10[d]	
	7.50×10^6	<10[d]		8.60×10^6		5.00×10^1	
5	8.10×10^6	<10[d]	0.005	6.60×10^6	0.300	<10[d]	0.010
	6.40×10^6	<10[d]		6.30×10^6		<10[d]	
	5.80×10^6	<10[d]		6.10×10^6		<10[d]	
7	6.20×10^6	<10[d]	0.016	5.50×10^6	0.087	<10[d]	0.016
	7.90×10^6	<10[d]		5.40×10^6		<10[d]	
	1.15×10^6	<10[d]		6.70×10^6		<10[d]	

[a] CFU/ml
[b] Inoculum 9.62×10^6 CFU/ml
[c] Probability (comparison of treatment and control)
[d] Detection limit 10 CFU/ml

Table 4. Reduction[a] of *Escherichia coli*[b] after exposure to silver (100 µg/L), copper (400 µg/L), and silver/copper (100/400 µg/L) at 4°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	2.30×10^6	1.25×10^5	< 0.001	1.60×10^6	0.048	1.82×10^5	< 0.001
	2.70×10^6	5.10×10^5		1.60×10^6		2.46×10^5	
	2.00×10^6	3.06×10^5		2.10×10^6		2.70×10^5	
2	5.90×10^6	1.11×10^5	< 0.001	1.20×10^6	0.440	4.00×10^3	0.119
	1.00×10^6	3.50×10^4		5.60×10^6		8.00×10^3	
	1.20×10^6	1.50×10^4		2.30×10^6		9.20×10^4	
3	2.90×10^6	1.30×10^4	0.002	1.60×10^6	0.009	1.60×10^4	0.002
	3.50×10^6	1.50×10^4		2.30×10^6		5.70×10^4	
	3.00×10^6	1.46×10^4		2.20×10^6		1.08×10^5	
5	1.50×10^6	5.80×10^1	0.013	4.00×10^6	0.274	4.90×10^2	0.013
	2.40×10^6	1.20×10^2		3.00×10^6		<10[d]	
	2.70×10^6	1.20×10^2		1.30×10^6		8.60×10^2	
7	5.90×10^6	4.00×10^0	0.024	2.70×10^6	0.422	<10[d]	0.024
	1.28×10^6	<10[d]		4.00×10^6		<10[d]	
	4.10×10^6	<10[d]		2.20×10^6		1.80×10^1	

[a] CFU/ml
[b] Inoculum 2.05×10^6 CFU/ml
[c] Probability (comparison of treatment and control)
[d] Detection limit 10 CFU/ml

Table 5. Reduction[a] of *Mycobacterium fortuitum*[b] after exposure to silver (100 μg/L), copper (400 μg/L), and silver/copper (100/400 μg/L) at 24°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	7.88×10^5	9.96×10^5	0.496	5.56×10^5	0.030	4.28×10^5	0.009
	1.11×10^6	9.08×10^5		6.12×10^5		4.62×10^5	
	9.49×10^5	9.40×10^5		5.80×10^5		4.42×10^5	
2	7.30×10^5	6.40×10^5	0.009	4.64×10^5	0.003	3.22×10^5	0.005
	8.16×10^5	5.38×10^5		5.72×10^5		3.31×10^5	
	9.02×10^5	5.90×10^5		5.01×10^5		3.19×10^5	
3	1.19×10^6	4.78×10^5	0.001	1.99×10^5	< 0.001	2.96×10^5	0.005
	9.28×10^5	2.08×10^5		3.66×10^5		3.22×10^5	
	1.19×10^6	3.12×10^5		2.12×10^5		3.13×10^5	
5	1.02×10^6	1.51×10^5	< 0.001	2.94×10^3	0.001	7.40×10^4	0.001
	9.60×10^5	1.57×10^5		2.05×10^3		1.03×10^5	
	1.08×10^6	1.53×10^5		2.40×10^3		9.00×10^4	
7	7.50×10^5	1.13×10^5	0.007	4.50×10^3	0.006	1.11×10^3	0.002
	9.20×10^5	4.30×10^4		6.30×10^2		1.26×10^3	
	1.09×10^6	7.80×10^4		3.08×10^3		1.18×10^5	
24	1.47×10^6	1.70×10^2	0.001	1.00×10^1	< 0.001	1.30×10^2	< 0.001
	1.44×10^6	2.40×10^2		2.90×10^2		4.00×10^1	
	1.40×10^6	1.92×10^5		3.01×10^3		8.50×10^1	

[a] CFU/ml; [b] Inoculum 1.01×10^6 CFU/ml; [c] Probability (comparison of treatment and control)

Table 6. Reduction[a] of *Mycobacterium fortuitum*[b] after exposure to silver (100 μg/L), copper (400 μg/L), and silver/copper (100/400 μg/L) at 4°C.

Time (h)	Control	Ag	P^c	Cu	P^c	Ag/Cu	P^c
1	3.06×10^5	7.60×10^4	0.001	2.88×10^5	0.025	1.44×10^5	0.001
	3.12×10^5	5.50×10^4		2.74×10^5		1.30×10^5	
	3.14×10^5	8.10×10^4		2.60×10^5		1.12×10^5	
2	3.60×10^5	4.90×10^4	0.008	2.84×10^5	0.468	7.60×10^4	0.002
	2.28×10^5	3.30×10^4		2.76×10^5		9.40×10^4	
	2.56×10^5	6.30×10^4		3.48×10^5		1.21×10^5	
3	3.34×10^5	4.40×10^4	0.002	2.54×10^5	0.182	7.60×10^4	0.009
	2.44×10^5	4.90×10^4		2.54×10^5		8.60×10^4	
	2.66×10^5	1.60×10^4		2.40×10^5		7.80×10^4	
5	3.66×10^5	3.10×10^4	0.003	3.46×10^5	0.285	1.60×10^4	0.003
	2.12×10^5	3.40×10^4		3.36×10^5		1.70×10^4	
	3.94×10^5	2.20×10^4		3.20×10^5		3.80×10^4	
7	3.08×10^5	1.60×10^4	0.002	2.48×10^5	0.407	9.02×10^3	0.002
	2.65×10^5	4.00×10^3		2.52×10^5		9.20×10^3	
	3.34×10^5	6.32×10^3		4.66×10^5		8.06×10^3	
24	2.56×10^5	4.00×10^3	< 0.001	1.31×10^5	0.013	2.18×10^3	< 0.001
	2.36×10^5	2.56×10^3		1.56×10^5		2.92×10^3	
	2.36×10^5	4.10×10^3		1.95×10^5		3.90×10^2	

[a] CFU/ml; [b] Inoculum 2.24×10^5 CFU/ml; [c] Probability (comparison of treatment and control)

Table 7. Reduction[a] of *Listeria monocytogenes*[b] after exposure to silver (100 µg/L), copper (400 µg/L), and silver/copper (100/400 µg/L) at 24°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	2.70×10^7	1.15×10^7	< 0.001	2.66×10^7	0.448	5.30×10^6	< 0.001
	3.08×10^7	8.60×10^6		3.20×10^7		7.10×10^6	
	2.88×10^7	1.06×10^7		2.88×10^7		4.20×10^6	
2	3.62×10^7	1.13×10^4	0.002	4.24×10^7	0.295	1.44×10^5	0.002
	4.40×10^7	1.08×10^4		3.62×10^7		1.80×10^5	
	3.98×10^7	1.13×10^4		3.60×10^7		1.91×10^5	
3	3.16×10^7	<10[d]	< 0.001	1.58×10^7	< 0.001	4.00×10^2	< 0.001
	2.92×10^7	<10[d]		1.71×10^7		1.50×10^2	
	2.90×10^7	<10[d]		1.55×10^7		3.70×10^2	
5	2.88×10^7	<10[d]	0.002	1.35×10^5	0.002	<10[d]	0.002
	3.58×10^7	<10[d]		1.22×10^5		<10[d]	
	3.32×10^7	<10[d]		1.10×10^5		<10[d]	
7	3.92×10^7	<10[d]	0.001	3.70×10^4	0.001	<10[d]	0.001
	3.54×10^7	<10[d]		5.50×10^4		<10[d]	
	3.58×10^7	<10[d]		6.20×10^4		<10[d]	

[a] CFU/ml
[b] Inoculum 3.27×10^7 CFU/ml
[c] Probability (comparison of treatment and control)
[d] Detection limit 10 CFU/ml

Table 8. Reduction[a] of *Listeria monocytogenes*[b] after exposure to silver (100 μg/L), copper (400 μg/L), and silver/copper (100/400 μg/L) at 4°C.

Time (h)	Control	Ag	*P*[c]	Cu	*P*[c]	Ag/Cu	*P*[c]
1	1.20×10^7	1.41×10^7	0.323	1.13×10^7	0.255	4.60×10^6	0.002
	1.21×10^7	1.07×10^7		1.11×10^7		6.80×10^6	
	1.22×10^7	8.60×10^6		1.26×10^7		2.20×10^6	
2	1.21×10^7	8.50×10^6	0.038	1.26×10^7	0.431	4.90×10^6	< 0.001
	1.20×10^7	5.90×10^6		1.12×10^7		6.00×10^6	
	1.19×10^7	9.80×10^6		1.19×10^7		4.10×10^6	
3	1.20×10^7	5.70×10^6	0.009	1.32×10^7	0.260	6.20×10^6	< 0.001
	1.21×10^7	4.20×10^6		1.06×10^7		4.80×10^6	
	1.18×10^7	7.10×10^6		1.40×10^7		4.20×10^6	
5	1.17×10^7	7.10×10^5	< 0.001	9.60×10^6	0.001	1.68×10^6	< 0.001
	1.20×10^7	2.71×10^5		8.50×10^6		1.01×10^6	
	1.19×10^7	7.47×10^5		8.40×10^6		9.25×10^5	
7	1.21×10^7	9.60×10^4	< 0.001	8.00×10^6	0.017	5.40×10^5	< 0.001
	1.22×10^7	1.21×10^5		9.90×10^6		5.82×10^5	
	1.16×10^7	7.20×10^4		8.40×10^6		4.80×10^5	

[a] CFU/ml
[b] Inoculum 1.24×10^7 CFU/ml
[c] Probability (comparison of treatment and control)

Table 9**.** Reduction[a] of *Escherichia coli*[b] after exposure to silver alone and in combination with organic matter[c] at 24°C.

Time (h)	Control	Ag 100 µg/L	Total organic carbon + Ag (100 µg/L)	
			3 mg/L	10 mg/L
1	0.01	2.51	2.51	2.39
2	0.03	4.40	4.40	4.40
3	0.05	5.55	5.55	5.55
5	0.07	>5.65[d]	>5.65[d]	>5.65[d]
7	0.09	>5.65[d]	>5.65[d]	>5.65[d]

[a] Average Log_{10} reduction of triplicate tests
[b] Inoculum 4.50×10^6 CFU/ml
[c] Humic acid
[d] Detection limit 10 CFU/ml

Table 10. Reduction[a] of *Escherichia coli*[b] after exposure to chlorine alone and in combination with organic matter[c] at 24°C.

Time (h)	Control	Cl 0.2 mg/L	Total organic carbon + Cl 0.2 mg/L	
			3 mg/L	10 mg/L
1	0.00	>5.70[d]	0.13	0.14
2	0.01	>5.70[d]	0.15	0.16
3	0.03	>5.70[d]	0.15	0.17
5	0.05	>5.70[d]	0.17	0.17
7	0.07	>5.70[d]	0.19	0.18

[a] Average Log_{10} reduction of triplicate tests
[b] Inoculum 5.00×10^6 CFU/ml
[c] Humic acid
[d] Detection limit 10 CFU/m

APPENDIX B:

EFFICACY OF PRODUCE SANITIZERS AGAINST ESCHERICHIA COLI, LISTERIA MONOCYTOGENES AND MS-2 ON LETTUCE

Enue E. Sicairos-Ruelas, Jonathan D. Sexton, Charles P. Gerba, and Kelly R. Bright*

The University of Arizona, Dept of Soil, Water & Environmental Science, Building 38, Room 429, Tucson, Arizona 85721

Running Title: Effectiveness of produce sanitizers
Key Words: lettuce, disinfection, chlorine, virus

Author for correspondence:
* Dr. Kelly R. Bright
The University of Arizona
Dept. of Soil, Water & Environmental Science
Building 38, Room 429
Tucson, Arizona 85721
Tel: (520) 626-8094
Fax:(520) 621-6163
Email: bright@email.arizona.edu

Abstract

The number of foodborne outbreaks associated with fresh produce has increased in the past decade. Microbial contamination of produce can occur at any of several points from the field through the time of consumption. Therefore, a proper sanitizer treatment is needed at the point of consumption to reduce the risk of foodborne disease. The objective of this study was to evaluate the antimicrobial activity of three produce sanitizers: Microdyn™ (colloidal silver), Gadicin® (10% stabilized chlorine dioxide) and Fit® (citric acid, grapefruit oil extract). Treatment with 150 ppm chlorine and a control (dechlorinated tap water only) were also included. The foodborne pathogens *Listeria monocytogenes*, *Escherichia coli*, and MS-2 coliphage (as a model for enteric viruses) were inoculated onto lettuce. The inoculated lettuce was exposed to the sanitizer treatment at concentrations recommended by the manufacturer. There was no significant difference between the Gadicin® and Microdyn™ treatments and the control. After 10 minutes of exposure to Fit®, *E. coli* was reduced by 2.36-$\log_{10}$, *L. monocytogenes* by 3.09-$\log_{10}$ and MS-2 by 2.28-log10. After 60 min, an additional 0.8-1.0-$\log_{10}$ reduction was observed. After 10 min of exposure, chlorine reduced *E. coli* by 1.82-$\log_{10}$, *L. monocytogenes* by 2.55-$\log_{10}$ and MS-2 by 1.44-$\log_{10}$. After 60 minutes of exposure to chlorine, a 3.61-$\log_{10}$ reduction in *L. monocytogenes* occured, whereas there was only a 2.25-$\log_{10}$ reduction in MS-2. Fit® and chlorine were the most effective. The use of produce sanitizers at a household level could help in reducing the probability of foodborne disease.

Introduction

Fruits and vegetables have been associated with an increasing number of foodborne outbreaks in recent years (Guan *et al*. 2005; Yaun *et al*. 2004). Fresh vegetables accounted for more than 45% of the total vegetable consumption between 1980 and 2001. During 2001, the most commonly consumed fresh vegetables were lettuce, tomatoes, carrots, potatoes, and onions (Sturchler 2006). Fruits and vegetables are the third leading vehicle in foodborne outbreaks, accounting for more than 10,000 cases of foodborne illness in the U.S. from 1990 to 2001 (CSPI 2001). Recently, an outbreak of *E. coli* O157:H7 affected more than 200 people, of which 30% developed a life-threatening complication, hemolytic uremic syndrome (HUS), and three people died. The outbreak was linked to the consumption of bagged spinach (Anonymous 2006). *Listeria monocytogenes* has been isolated from prepackaged salads, and epidemiological evidence has linked human listeriosis to the consumption of cabbage, lettuce, celery and tomatoes (Berrang *et al*. 1989). *L. monocytogenes* is a major public health concern due to its high mortality rate (~20%), and its ability to grow at refrigeration temperatures (Gandhi and Chikindas 2007; Mead *et al.* 1999).

The majority of pathogens implicated in produce-related outbreaks are transmitted via the fecal-oral route (Johnston *et al.* 2006). Microbial contamination of fruits and vegetables can occur during plant growth, harvesting, transport, processing, distribution and marketing, or in the home (Beuchat 1998). After harvesting, raw fruits and vegetables are treated with an aqueous chemical formulation to remove dirt, pesticides,

and microorganisms. Most fruits and vegetables are placed in flume tanks containing 150-200 ppm of free chlorine for a short period of time prior to packing (Yuk *et al.* 2006); however, this treatment results in less than 2-$\log_{10}$ colony forming units per gram (CFU/g) reduction of the microbial load (Koseki *et al.* 2004; Rodgers *et al.* 2004). In addition, circumstances such as contaminated irrigation water, runoff from animal feedlots, or the presence of feral animals further increase the number of pathogens on the produce. Washing fruits and vegetables with tap water cannot be relied upon to remove microorganisms completely at the household level (Harris *et al.* 2001; Beuchat 1998). Therefore, there is a critical need to develop more effective produce sanitizers that help to reduce the risks of contracting a foodborne illness.

The present study was undertaken to investigate the efficacy of commercially available alternative produce sanitizers in the removal of *Escherichia coli, Listeria monocytogenes* and MS-2 coliphage inoculated on lettuce. MS-2 was included as surrogate for enteric viruses.

Materials and methods

Maintenance and preparation of bacterial isolates

Escherichia coli (ATCC 25922) and *Listeria monocytogenes* (ATCC 19115) used in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA). The bacteria were maintained on Tryptic Soy Agar (TSA; Difco, Sparks, MD). Erlenmeyer flasks containing 100 ml of Tryptic Soy Broth (TSB; Difco, Sparks, MD)

were inoculated and incubated on an orbital shaker (250 rpm) at 37°C overnight prior to testing. After incubation, the bacteria were pelleted via centrifugation (15,300 x *g*, for 10 min in a Beckman J2-21 centrifuge; Beckman Coulter, Inc., Fullerton, CA). The pelleted cells were washed by resuspension in 100 ml of 0.01M phosphate buffer (3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4; pH 7.5), followed by centrifugation as before. This step was repeated an additional time. The final pellet was resuspended in 20 ml of phosphate buffer. The inoculum suspensions were prepared by adding small volumes of the bacterial suspension to 10 ml of phosphate buffer, resulting in an optical turbidity (BIOLOG turbidimeter, Hayward, CA) equivalent to a McFarland number 5 optical density standard [1.5 x 10^9 colony forming units per milliliter (CFU/ml)]. This suspension was then diluted further in dechlorinated tap water to achieve the desired final inoculum concentration (approximately 1.5 x 10^8 CFU/ml).

Maintenance and preparation of MS-2 bacteriophage

F-specific coliphage MS-2 (ATCC 15597-B1) was obtained from the American Type Culture Collection (ATCC; Manassas, VA). MS-2 was propagated by the double-top agar layer plaque technique (Adams 1959). An 18-hour culture of *Escherichia coli* (ATCC 15597) grown in tryptic soy broth (TSB; Difco, Detroit, MI) was transferred to fresh TSB and grown for 3-5 h at 37°C with continuous shaking. Stock MS-2 was serially diluted in Tris-buffered saline (0.14 M NaCl, 5.03 mM KCl, 30 mM Trizma base, 0.4 mM Na_2HPO_4 in ultrapure water at pH 7.2) to an approximate concentration of 10^5 plaque-forming units per milliliter (PFU/ml). A 1 ml suspension of the *E. coli* host cells and 0.1

ml of the phage dilution were mixed in 3 ml of molten overlay agar and poured over presolidified tryptic soy agar (TSA, 1.5% agar; Difco, Detroit, MI) petri dishes. After 24 hours of incubation at 37°C, 6 ml of sterile 0.01 M phosphate buffer was added to petri dishes with confluent plaques and allowed to sit for 1 hour. The phage was harvested from the plates and centrifuged at 15,300 x *g* for 10 min in a Beckman J2-21 centrifuge (Beckman Coulter, Inc., Fullerton, CA) to remove bacterial debris. The liquid fraction was passed through a 0.22 μm pore size acrodisc syringe filter pretreated with 0.3% beef extract at pH 7 (Pall, Ann Arbor, MI). The filtrate was used as the MS-2 stock suspension for the latter experiments. Phage titer was determined in the same way as phage propagation. The phage was stored at 4°C until needed. This suspension was then diluted further in dechlorinated tap water to achieve the desired final inoculum concentration (approximately 1.5 x 10^8 PFU/ml).

Lettuce

Iceberg lettuce was purchased from a local supermarket. The outer three or four leaves were removed and discarded. In each experiment, intact leaves were cut into pieces (5 x 3 cm) with a sterile surgical knife.

Inoculation procedure

Approximately 10 g of cut lettuce leaves (5 x 3 cm) were submerged in 250 ml of inoculum suspension (~10^8 CFU/ml) at room temperature (23 ± 2 °C) for 3 min with

continuous stirring. The lettuce was then drained using a commercial salad spinner (OXO, New York, NY) and left to dry for 2.5 hours under a safety cabinet.

Treatment with sanitizers

This study evaluated the antimicrobial activity of three commercially available alternative produce sanitizers: Microdyn™ (stabilized colloidal silver 0.35%, Mercancias Salubres S.A. de C.V.; Mexico, D.F.), Gadicin® (10% stabilized chlorine dioxide; Gadec S.A. de C.V.; Naucalpan, Estado de Mexico) and Fit® (citric acid, grapefruit oil extract, sodium lauryl sulfate, propylene glycol, sodium carbonate, magnesium carbonate; HealthPro Brands Inc., Cincinnati, OH). Treatment with dechlorinated tap water only was included as a control. The pH of each sanitizer solution was determined. All experiments were conducted in triplicate at room temperature (23 ± 2°C). Treatment of inoculated lettuce leaves was performed by immersing 10 g samples in 500 ml of sanitizer treatment at concentrations recommended by the manufacturer. At predetermined time intervals, the lettuce was drained using a commercial salad spinner (OXO, New York, NY), combined with 50 ml of 0.1% peptone water (Difco, Detroit, MI) in a polyethylene bag, and pummeled for 2 min at normal speed in a stomacher (Model 3500, Seward, London, UK). Samples were collected (3 ml each) and neutralized with Dey Engley neutralizing broth (D/E; Difco, Sparks, MD) containing 50 µl of 10% (wt/v) $Na_2S_2O_3$ (EMD chemicals, Gibbstown, NJ) at a ratio of 1:1. The samples were assayed the same day. The

concentration of silver in the Microdyn™ suspension was determined by coupled plasma mass spectrometry (Model Elan DCR-II, Perkin-Elmer, Shelton, CT).

Preparation of chlorinated water

A chlorine solution with a cocentration 150 mg/L was prepared using commercial bleach (6.0% sodium hypochlorite). A Hach test kit (Model DR/2000, Hach Company, Loveland Co) was used to quantitate free chlorine.

Microbial assays

Bacterial samples were serially diluted in 0.1% peptone water (Difco, Detroit, MI) and the surviving bacteria were enumerated in duplicate using the spread plate method on the following selective media with incubation at 37°C for 24 h: *E. coli,* mEndo (Difco, Sparks, MD); *L. monocytogenes,* Listeria Selective Agar (Oxford, Columbia, MD).

MS-2 was assayed by dilution of samples in 0.1% peptone water (Difco, Detroit, MI) and the surviving virus enumerated in duplicate using the double-top agar layer plaque technique previously described.

Determination of bioburden

The presence of microorganisms other than *E. coli, L. monocytogenes,* and MS-2 coliphage on lettuce that formed colonies or plaques on the selective media was also determined.

Data analyses

Data were reported as logarithmic reduction $-\log_{10} N_t/N_0$ where N_t is the concentration of microorganisms at time *t* and N_0 is the concentration of microorganisms at time zero. A Student's t-test was used for analysis of variance to determine significant differences between disinfection treatments. Differences were considered significant if *P* value was ≤ 0.05.

Results

No bacteria were isolated on mEndo and listeria selective agar from the uncontaminated lettuce. No MS-2 coliphage was found on the lettuce either. Microdyn™ sanitizer solution contained 1.40 mg/L of silver. The pH of the sanitizers were as follows: control pH 8.65, Fit® pH 2.2, Microdyn™ pH 8.36, Gadicin® pH 8.40, and chlorine pH 8.40. No significant difference was observed between the concentrations of chlorine at the beginning and at the end of each time interval.

Tables 1 through 6 show the results of the various sanitizer treatments on the test microorganisms. There was no significant difference ($P \leq 0.05$) between the reductions

observed with Microdyn™ or Gadicin® and the control (dechlorinated tap water) at all time intervals with all the microorganisms tested. Similarly, the microorganisms were not significally inactivated when Gadicin® was evaluated in an aqueous model system (dechlorinated tap water) under the same experimental conditions (Tables 2, 4 and 5). Gadicin® was not evaluated against *L. monocytogenes* inoculated on lettuce since this sanitizer did not show any effect on the organism in aqueous solution.

After 10 minutes of exposure to Fit®, *E. coli* was reduced by 2.36-$\log_{10}$, *L. monocytogenes* by 3.09-$\log_{10}$ and MS-2 by 2.28-log10. After 60 min, an additional 0.8 to 1.0-$\log_{10}$ was achieved against all of the tested organisms.

After 10 min, chlorine reduced *E. coli* by 1.82-$\log_{10}$, *L. monocytogenes* by 2.55-$\log_{10}$ and MS-2 by 1.44-$\log_{10}$. After 60 minutes exposure to chlorine, a 3.61-$\log_{10}$ reduction in *L. monocytogenes* occurred, whereas there was only a 2.25-$\log_{10}$ reduction in MS-2. Fit® and chlorine therefore yielded the best results.

Discussion

There are many factors influencing the efficacy of a produce sanitizer such as the presence of organic matter (e.g. vegetable tissue, fruit juices, soil particles), the produce surface (rough, smooth, hydrophilic, hydrophobic, injured tissue), and the type of disinfectant. Particularly with leafy vegetables, sanitizers are not as effective as they are with produce with smooth surfaces (e.g. tomatoes). Koseki *et al.* (2004) reported a 1.0-$\log_{10}$ reduction in *E. coli* O157:H7 inoculated on lettuce after treatment with 200 ppm of

free chlorine (Koseki *et al.* 2004). Similarly, Pirovani *et al.* (2004) observed a 1.8-$\log_{10}$ reduction in the total microbial count on lettuce after treatment with 150 ppm of chlorine (Pirovani *et al.* 2004). In contrast, a 4.0-$\log_{10}$ reduction was observed with *S. typhimurium* inoculated on the surface of a tomato after treatment with 83 ppm of free chlorine (Sicairos-Ruelas unpublished data 2007).

Bacteria in produce adhere to surface irregularities such as roughness, crevices, and pits, thus reducing the ability of washing or sanitizing treatments to remove or inactivate attached cells (Ukuku and Sapers 2006). The penetration of the microorganisms into the lettuce cut edges was enhanced by the method of inoculation used in this study (dip inoculation), conferring extra protection from the sanitizer. The inaccessibility of *E. coli* O157:H7 on lettuce has been demonstrated on lettuce using confocal laser microscopy. Agar plate culturing and microscopic observation have indicated that *E. coli* O157:H7 preferentially attaches to cut edges, as opposed to the intact leaf surface. Inoculated lettuce subjected to a chlorine treatment (200 ppm), resulted in only a 1.0-$\log_{10}$ reduction of the bacterium on the cut edges. Elongated cells were observed in stomata and within the tissues of the lettuce, indicating they were protected from contact with chlorine (Seo and Frank 1999; Takeuchi and Frank 2000). Even if the lettuce was washed with sanitizers and subject to physical manipulation similar to the stomacher method, microorganisms located within the interior of the lettuce would not be inactivated.

Researchers have shown that regardless of the size of the inoculated population of microorganisms on lettuce, pathogens still penetrated the interior of the lettuce tissue, and

treatment with 200 ppm chlorine did not completely inactivate them at low concentrations (10^0 to 10^2 CFU/g) (Beuchat 1999; Koseki *et al.* 2004). In addition, the hydrophobic waxy cuticle of the lettuce reduced the contact of the sanitizer with the organism. Bacteria arrangement on the surface of the produce could also confer some degree of protection. A stack arrangement was observed after strawberries and lettuce were treated with ozone, sodium hypochlorite and chlorine dioxide (Rodgers *et al.* 2004).

Organic matter can reduce the sanitizer efficacy by directly interacting with the chemical, or by indirectly binding to the microorganisms. Virto *et al.* (2005) found that the addition of organic matter to chlorinated water stabilized the cytoplasmic membrane of Gram-negative and Gram-positive bacteria against the permeabilization of external ions (Virto *et al.* 2005).

Microdyn™ wash solution contained 1.40 mg/L of silver as determined by atomic absorption. Silver is known for its antimicrobial properties at low concentrations in the range of μg/L and effectively inactivates pathogens in water (Landeen *et al.* 1989; Yahya *et al.* 1990); however, despite its high concentration in the wash solution, silver did not significaly inactivate the microorganisms tested in the present study.

Chlorine is generally considered to be a highly destructive, non-selective oxidant that reacts avidly with all biomolecules. HOCl can react with membranes, changing their permeability, inhibiting transport, fragmenting proteins, and reacting with nucleotides. It inactivates enzymes, with iron-sulfur clusters appearing as privileged targets (McKenna and Davies 1988).

Fit® has a very low pH (2.2) in comparison with the other sanitizers (approximately pH 8). The low pH probably contributes to the inactivation of the study organisms by interacting with the high number of protons (H^+) on the bacteria cell surface, outer membrane or cell wall, inner membrane or cytoplasmic membrane, and periplasmic space. This can adversely affect the ionic bonds of the macromolecules and thus can interfere with their three-dimensional structure and some of their functions. These changes can affect bacterial nutrient transport and energy generation, leading to bacterial inactivation (Brul and Coote 1999; Brul *et al.* 2002). Viruses undergo conformational changes at low pH, affecting the molecules used to attach to the host cell receptor. The low pH present in the Fit® solution could result in a rearrangement of the surface molecules on the MS-2, affecting its attachment to the lettuce. Once in the solution, possibly due to the acidity, the virus was inactivated. Fit® contains sodium lauryl sulfate which is an anionic surfactant (Feis *et al.* 2007) that enhances the contact of the sanitizer with the microorganisms, resulting in detachment of the organisms from the surface.

Overall, Fit® and chlorine yielded the greatest reductions of the microorganisms tested on lettuce. The use of these produce sanitizers at the household level could help in reducing the incidence of foodborne disease.

References

Adams, M. H. (1959) Bacteriophages. New York: Interscience Publishers, Inc.

Anonymous. (2006) Leafy, green, healthful--but contaminated. The *E. coli* outbreak from bagged spinach is a reminder that fruit and vegetables can harbor harmful bacteria. *Harv Health Lett* **32**, 4.

Berrang M. E, Brackett R.E and Beuchat L. R. (1989) Growth of *Listeria monocytogenes* on fresh vegetables stored under controlled atmosphere. *J Food Prot* **52**, 702-705.

Beuchat, L.R. (1999) Survival of enterohemorrhagic *Escherichia coli* O157:H7 in bovine feces applied to lettuce and the effectiveness of chlorinated water as a disinfectant. *J Food Prot* **62**, 845-849.

Beuchat L. R. (1998) Surface decontamination of fruits and vegetables eaten raw: a review. Food Safety Issues. World Health Organization, Geneva.WHO/FSF/FOS/ 98.2.

Brul, S. and Coote, P. (1999) Preservative agents in foods. Mode of action and microbial resistance mechanisms. *Int J Food Microbiol* **50**, 1-17.

Brul, S., Coote, P., Oomes, S., Mensonides, F., Hellingwerf, K. and Klis, F. (2002) Physiological actions of preservative agents: prospective of use of modern microbiological techniques in assessing microbial behaviour in food preservation. *Int J Food Microbiol* **79**, 55-64.

Gandhi, M. and Chikindas, M.L. (2007) *Listeria*: A foodborne pathogen that knows how to survive. *Int J Food Microbiol* **113**, 1-15.

Center for Science in the Public Interest. (2001) Closing the Gaps in our Federal food-Safety Net. Outbreak Alert. Washington, D.C.

Feis, A., Tofani, L., De Sanctis, G., Coletta, M. and Smulevich, G. (2007) Multiphasic kinetics of myoglobin/SDS complex formation. *Biophys J*. [Epub ahead of print]

Food Drug and Administration. (2007) Investigation of an *Escherichia coli* O157:H7 Outbreak associated with Dole pre-packaged spinach, Sacramento CA. http://www.marlerclark.com/2006_Spinach_Report_Final_01.pdf. Retrieved April 18 2007.

Guan, T.T., Blank, G. and Holley, R.A. (2005) Survival of pathogenic bacteria in pesticide solutions and on treated tomato plants. *J Food Prot* **68**, 296-304.

Harris, L.J., Beuchat, L.R., Kajs, T.M., Ward, T.E. and Taylor, C.H. (2001) Efficacy and reproducibility of a produce wash in killing *Salmonella* on the surface of tomatoes assessed with a proposed standard method for produce sanitizers. *J Food Prot* **64**, 1477-1482.

Johnston, M.L., Moe L.C., Moll D. and Jaykus L. (2006) The epidemiology of produce-associated outbreaks of foodborne disease. In: James J, ed. Microbial hazards identification in fresh fruits and vegetables. New Jersey: John Wiley and Sons, Inc., pp. 83-95.

Koseki, S., Yoshida, K., Isobe, S. and Itoh, K. (2004) Efficacy of acidic electrolyzed water for microbial decontamination of cucumbers and strawberries. *J Food Prot* **67**, 1247-1251.

Landeen, L.K., Yahya, M.T. and Gerba, C.P. (1989) Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila. Appl Environ Microbiol* **55**, 3045-3050.

McKenna, S.M. and Davies, K.J. (1988) The inhibition of bacterial growth by hypochlorous acid. Possible role in the bactericidal activity of phagocytes. *Biochem J* **254**, 685-692.

Mead, P.S., Slutsker, L., Dietz, V., McCaig, L.F., Bresee, J.S., Shapiro, C., Griffin, P.M. and Tauxe, R.V. (1999) Food-related illness and death in the United States. *Emerg Infect Dis* **5**, 607-625.

Pirovani, M., Piagentini, A., Guemes, D. and Silvana, A. (2004) Reduction of chlorine concentration and microbial load during washing-disinfection of shredded lettuce. *Int J Food Sci Tech* **39**, 341-347.

Rodgers, S.L., Cash, J.N., Siddiq, M. and Ryser, E.T. (2004) A comparison of different chemical sanitizers for inactivating *Escherichia coli* O157:H7 and *Listeria monocytogenes* in solution and on apples, lettuce, strawberries, and cantaloupe. *J Food Prot* **67**, 721-731.

Seo, K.H. and Frank, J.F. (1999) Attachment of *Escherichia coli* O157:H7 to lettuce leaf surface and bacterial viability in response to chlorine treatment as demonstrated by using confocal scanning laser microscopy. *J Food Prot* **62**, 3-9.

Sturchler D.A. (2006) Plant -derived food. In: Exposure a guide to sources of infection. Washington, D.C.: ASM press, pp. 177-178.

Takeuchi, K. and Frank, J.F. (2000) Penetration of *Escherichia coli* O157:H7 into lettuce tissues as affected by inoculum size and temperature and the effect of chlorine treatment on cell viability. *J Food Prot* **63**, 434-440.

Ukuku D.O. and Sapers G.M. (2006) Microbiological safety issues of fresh melons. In: Sapers GM, Gorny JR, Yousef AE eds. Microbiology of fruits and vegetables. Boca Raton, Florida: CRC Press, pp. 29-30.

Virto, R., Manas, P., Alvarez, I., Condon, S. and Raso, J. (2005) Membrane damage and microbial inactivation by chlorine in the absence and presence of a chlorine-demanding substrate. *Appl Environ Microbiol* **71**, 5022-5028.

Yahya, M.T., Landeen, L.K., Messina, M.C., Kutz, S.M., Schulze, R. and Gerba, C.P. (1990) Disinfection of bacteria in water systems by using electrolytically generated copper:silver and reduced levels of free chlorine. *Can J Microbiol* **36**, 109-116.

Yaun, B.R., Sumner, S.S., Eifert, J.D. and Marcy, J.E. (2004) Inhibition of pathogens on fresh produce by ultraviolet energy. *Int J Food Microbiol* **90**, 1-8.

Yuk, H.G., Bartz, J. A. and Schneider, K. R. (2006) The effectiveness of sanitizer treatments inactivation of *Salmonella spp*. from bell pepper, cucumber, and strawberry. *J Food Sci* **71**, 95-99.

Table 1. Reduction[a] of *Escherichia coli*[b] inoculated on lettuce following exposure to various sanitizers

Time (min)	Control	Fit®	*P*[c]	Mycrodyn™	*P*[c]	Gadicin ®	*P*[c]	Chlorine[d]	*P*[c]
	0.42	2.79		0.44		0.41		1.77	
10	0.45	2.22	0.006	0.50	0.090	0.40	0.218	2.12	0.006
	0.35	2.08		0.46		0.58		1.58	
	0.75	3.01		0.80		0.79		3.16	
30	0.79	3.29	0.001	0.83	0.062	0.81	0.074	2.51	0.006
	0.76	3.15		0.78		0.78		2.50	
	0.84	3.71		1.48		0.90		2.47	
60	1.39	3.08	< 0.001	1.47	0.060	1.55	0.378	2.57	0.002
	1.12	3.40		1.37		1.15		3.12	

[a] Log_{10} reduction

[b] Inoculum 1.24 x 10^6 CFU/g

[c] *P*. Probability (comparision treatment and control)

[d] 150 mg/L

Table 2. Reduction[a] of *Escherichia coli*[b] in dechlorinated tap water following exposure to various sanitizers

Time (min)	Control	Fit®	*P*[c]	Mycrodyn™	*P*[c]	Gadicin ®	*P*[c]	Chlorine[d]	*P*[c]
	0.05	>6.38[e]		0.03		0.06		>6.38[e]	
10	0.03	>6.38[e]	< 0.001	0.10	0.162	0.06	0.072	>6.38[e]	< 0.001
	0.02	>6.38[e]		0.05		0.04		>6.38[e]	
	0.03	>6.38[e]		0.08		0.04		>6.38[e]	
30	0.03	>6.38[e]	< 0.001	0.11	0.059	0.05	0.187	>6.38[e]	< 0.001
	0.04	>6.38[e]		0.05		0.03		>6.38[e]	
	0.03	>6.38[e]		0.41		0.04		>6.38[e]	
60	0.04	>6.38[e]	< 0.001	0.36	< 0.001	0.04	0.115	>6.38[e]	< 0.001
	0.04	>6.38[e]		0.38		0.05		>6.38[e]	

[a] Log_{10} reduction

[b] Inoculum 2.40 x 10^7 CFU/ml

[c] *P*. Probability (comparision treatment and control)

[d] 150 mg/L

[e] Detection limit 10 CFU/ml

Table 3. Reduction[a] of *Listeria monocytogenes*[b] inoculated on lettuce following exposure to various sanitizers

Time (min)	Control	Fit®	*P*[c]	Mycrodyn™	*P*[c]	Chlorine[d]	*P*[c]
	1.88	3.32		1.16		2.22	
10	1.30	3.18	0.001	1.53	0.209	2.66	0.007
	1.50	2.78		1.44		2.77	
	2.42	2.78		1.18		2.44	
30	1.71	3.44	0.026	1.55	0.038	2.62	0.053
	2.18	2.69		1.60		3.22	
	2.31	4.15		2.59		3.69	
60	2.40	3.53	0.002	2.21	0.423	3.14	0.008
	2.00	3.93		1.74		4.00	

[a] Log_{10} reduction

[b] Inoculum 6.38 x 10^6 CFU/g

[c] *P*. Probability (comparision treatment and control)

[d] 150 mg/L

Table 4. Reduction[a] of *Listeria monocytogenes*[b] in dechlorinated tap water following exposure to various sanitizerss

Time (min)	Control	Fit®	*P*[c]	Mycrodyn™	*P*[c]	Chlorine[d]	*P*[c]
10	0.03	>6.48[e]	< 0.001	0.39	0.032	>6.48[e]	< 0.001
	0.07	>6.48[e]		0.18		>6.48[e]	
	0.05	>6.48[e]		0.28		>6.48[e]	
30	0.08	>6.48[e]	< 0.001	0.46	< 0.001	>6.48[e]	< 0.001
	0.03	>6.48[e]		0.46		>6.48[e]	
	0.06	>6.48[e]		0.48		>6.48[e]	
60	0.10	>6.48[e]	< 0.001	0.47	0.001	>6.48[e]	< 0.001
	0.08	>6.48[e]		0.54		>6.48[e]	
	0.09	>6.48[e]		0.51		>6.48[e]	

[a] Log_{10} reduction

[b] Inoculum 3.00 x 10^7 CFU/ml

[c] *P*. Probability (comparision treatment and control)

[d] 150 mg/L

[e] Detection limit 10 CFU/ml

Table 5. Reduction[a] of MS-2 coliphage [b] inoculated on lettuce following exposure to various sanitizers

Time (min)	Control	Fit®	*P* [c]	Mycrodyn™	*P* [c]	Gadicin ®	*P* [c]	Chlorine[d]	*P* [c]
	0.76	2.42		0.80		0.73		1.67	
10	0.80	2.32	< 0.001	0.77	0.436	0.75	0.398	1.22	0.010
	0.93	2.10		0.96		0.94		1.55	
	0.78	2.79		0.79		0.80		2.31	
30	0.80	2.50	0.007	0.92	0.480	0.95	0.483	2.30	0.014
	0.91	2.12		0.77		0.75		1.67	
	0.77	3.08		0.82		0.79		2.59	
60	0.84	3.67	0.003	0.98	0.195	0.91	0.332	2.18	0.009
	0.96	3.43		1.00		0.98		1.99	

[a] Log_{10} reduction

[b] Inoculum 1.58 x 10^6 CFU/g

[c] *P.* Probability (comparision treatment and control)

[d] 150 mg/L

Table 6. Reduction[a] of MS-2 coliphage [b] in dechlorinated tap water following exposure to various sanitizers

Time (min)	Control	Fit®	*P* [c]	Mycrodyn™	*P* [c]	Gadicin ®	*P* [c]	Chlorine[d]	*P* [c]
	0.12	3.04		0.13		0.09		4.86	
10	0.08	3.18	< 0.001	0.10	0.371	0.07	0.434	4.59	< 0.001
	0.09	3.11		0.08		0.12		4.72	
	0.10	4.80		0.09		0.11		5.10	
30	0.08	3.59	0.004	0.10	0.386	0.12	0.339	5.25	< 0.001
	0.11	4.31		0.11		0.08		5.22	
	0.07	4.97		0.12		0.09		5.21	
60	0.15	3.99	0.002	0.14	0.458	0.11	0.407	5.34	< 0.001
	0.13	4.86		0.08		0.13		5.41	

[a] Log_{10} reduction

[b] Inoculum 9.97 x 10^6 CFU/ml

[c] *P*. Probability (comparision treatment and control)

[d] 150 mg/L

APPENDIX C:

A NATURAL PEPTIDE-BASED ANTIMICROBIAL WITH BROAD-SPECTRUM ACTIVITY

Enue E. Sicairos-Ruelas, Charles P. Gerba, and Kelly R. Bright*

The University of Arizona, Dept of Soil, Water & Environmental Science, Building 38, Room 429, Tucson, Arizona 85721

Running Title: Natural Peptide-Based Antimicrobial
Key Words: Antimicrobial, peptides, plant extract, broad-spectrum

Author for correspondence:
* Dr. Kelly R. Bright
The University of Arizona
Dept. of Soil, Water & Environmental Science
Building 38, Room 429
Tucson, Arizona 85721
Tel: (520) 626-8094
Fax:(520) 621-6163
Email: bright@email.arizona.edu

Abstract

A quantitative suspension test was used to assess the efficacy of a new natural peptide-based antimicrobial, Absolute Fx, against representative Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) and Gram-negative (*Escherichia coli, Salmonella typhimurium, Pseudomonas aeruginosa*) bacterial species. Greater than a 5-$\log_{10}$ reduction was observed for *E. coli*, *S. typhimurium*, *P. aeruginosa* and *L. monocytogenes* after a 30 second exposure to a 1:400 dilution of Absolute Fx. Similar results were observed for a 1:800 dilution with the exception of *P. aeruginosa* (3.90-$\log_{10}$ reduction). A 3.23-$\log_{10}$ and a 2.71-$\log_{10}$ reduction were observed for *S. aureus* after 30 seconds of exposure to 1:400 and 1:800 dilutions, respectively. After 5 minutes, greater than a 5-$\log_{10}$ reduction was observed for both *P. aeruginosa* and *S. aureus.* Absolute Fx was also tested against human coronavirus strain 229E (enveloped) and feline calicivirus strain F-9 (non-enveloped). Greater than a 4-$\log_{10}$ loss of human coronavirus 229E was observed within 30 seconds of exposure to a 1:200 and a 1:400 dilution. A one to 2-$\log_{10}$ reduction was observed for feline calicivirus within 10 minutes to 1 hour of exposure. Absolute Fx shows promise as a natural antimicrobial against a broad spectrum of microorganisms including both bacteria and viruses. Further studies are needed to determine the potential use of this product in specific applications.

Introduction

A disinfectant is an agent that significantly reduces the numbers of specific species of infectious or otherwise undesirable microorganisms, but not necessarily bacterial spores, on inanimate surfaces [2]. A sanitizer is an agent that reduces the number of microorganisms to a level considered safe. This usually corresponds to a reduction of at least 99.9% (3-$\log_{10}$) of the test bacteria within 5 minutes under specific conditions on inanimate and non-food contact surfaces [5]. The primary difference between a sanitizer and a disinfectant is that, at a specified dilution, the disinfectant must have a higher kill capability for pathogenic bacteria. A large number of compounds are available for use as disinfectants; however, some are highly toxic, corrosive and produce harmful by-products [6, 7, 13].

Natural antimicrobial products are a possible alternative. Numerous plant extracts and their components (e.g. cinnamaldehyde, thymol, carvacrol, eugenol) have significant antibacterial properties [4]. Absolute Fx is a proprietary peptide-based antimicrobial containing three plant extracts with the combined properties of thionins, amino acid-enriched peptides and a new class of cationic peptides (The Center for Lifestyle Disease, Surprise, AZ). There are also some enzymatic components that mimic the antimicrobial activities of diterpenes. The U.S. Environmental Protection Agency (EPA) classifies it as a non-toxic category IV compound (tests performed by DuPont Haskell^{SM} Laboratory for Health and Environmental Sciences, Newark, DE). Cationic peptides are widely

distributed in animals and plants, but their mechanism of action has not been established. It has been proposed that cationic peptides interact with bacterial membranes, increasing their permeability, or that part of the peptide penetrates into the cell, exerting action on target molecules [9].

The objective of this study was to determine the antimicrobial efficacy of this new peptide-based antimicrobial, Absolute Fx (chemical formulation is shown on table. 3), against representative Gram-negative (*Escherichia coli*, *Salmonella typhimurium*, *Pseudomonas aeruginosa*) and Gram-positive (*Staphylococcus aureus, Listeria monocytogenes*) bacterial pathogens and non-enveloped (feline calicivirus strain F-9) and enveloped (human coronavirus strain 229E) viruses in aqueous solutions.

Materials and methods

Maintenance and preparation of bacterial isolates

All test bacteria in this study were obtained from the American Type Culture Collection (ATCC; Manassas, VA). Strains included *E. coli* ATCC 25922, *L. monocytogenes* ATCC 19115, *S. typhimurium* ATCC 23564, *P. aeruginosa* ATCC 27313 and *S. aureus* ATCC 25923. The bacteria were maintained on Tryptic Soy Agar (TSA; Difco, Sparks, MD). Erlenmeyer flasks containing 100 ml of Tryptic Soy Broth (TSB; Difco, Sparks, MD) were inoculated and incubated on an orbital shaker (250 rpm) at 37°C overnight prior testing.

After incubation, the bacteria were pelleted via centrifugation (15,300 x *g*, for 10 min in a Beckman J2-21 centrifuge; Beckman Coulter, Inc., Fullerton, CA). The pelleted cells were washed by resuspension in 100 ml of sterile physiological saline (0.85% NaCl; Difco, Sparks, MD) followed by centrifugation as before. This step was repeated an additional time. The final pellet was resuspended in 20 ml of phosphate buffer 0.01M (pH 7.5; 3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4). The test suspensions were prepared by adding small volumes of the bacterial suspension to 10 ml of sterile saline (0.85% NaCl; Difco, Sparks, MD), resulting in an optical turbidity (BIOLOG turbidimeter, Hayward, CA) equivalent to a McFarland number 0.5 optical density standard (1.5 x 10^8 bacterial/ml). This solution was then diluted further in saline to achieve the desired final test concentration (approximately 1.0 x 10^7 bacteria/ml).

Maintenance and preparation of viruses

Human coronavirus strain 229E (ATCC VR-740) and feline calicivirus strain F-9 (ATCC VR-782) were obtained from the American Type Culture Collection (ATCC, Manassas, VA) and were maintained on either MRC-5 (human coronavirus; fetal human lung fibroblasts, ATCC CCL-171) or CRFK (feline calicivirus; Crandell Rees feline kidney, ATCC CCL-94) cell line monolayers with Eagle's minimal essential medium (MEM; modified with Earle's salts, Irvine Scientific, Santa Ana,) containing 2% fetal bovine serum (FBS; Hyclone, Logan, UT) at an incubation temperature of 35°C with 5% CO_2.

The viruses were purified by centrifugation (756 × *g*) to remove cell debris followed by polyethylene glycol (9% w/v PEG, 0.5 M NaCl) precipitation. Viral

titrations were performed using a plaque-forming technique (feline calicivirus) described by Bidawid et al. [3] or the Reed-Muench method [11] to determine the tissue culture infectious dose that affected 50% of the cultures ($TCID_{50}$).

Bacterial experimental prodecure

A 1:40 and a 1:80 stock solution of the Absolute Fx were made from a full strength solution (60.7% active) provided by The Center for Lifestyle Disease (Surprise, AZ). All experiments were conducted in triplicate at room temperature (23 ± 2°C).

The inactivation of bacterial strains was carried out in sterile tubes filled with 9 ml of sterile saline (0.85% NaCl; Difco, Sparks, MD). A final concentration of 1:400 or 1:800 of the Absolute Fx was achieved by adding 1 ml of the stock solution to this 9 ml bacterial test suspension. The pH of the 1:400 and the 1:800 reaction solutions were 3.83 and 4.10, respectively. Physiological saline (0.85% NaCl; Difco, Sparks, MD) with a pH of 3.0 was inoculated with the target organism and included as a control to determine if any reductions observed could be ascribed to the acidic pH alone. Bacteria in Letheen neutralizing broth (Difco, Sparks, MD) alone were included as a control to verify that the neutralizer had no antimicrobial action. In addition, the bacteria were added to Absolute Fx solutions that had previously been neutralized with Letheen neutralizing broth. The surviving bacteria were quantified to confirm that the amount of neutralizer used in the experiments was able to completely neutralize the 1:200, 1:400 and 1:800 dilutions of the Absolute Fx.

A 1 ml sample was removed after 30 seconds, 5 minutes and 10 minutes and placed in 9 ml of Letheen neutralizing broth to neutralize the Absolute Fx. Ten-fold serial dilutions were made and the surviving bacteria were enumerated in duplicate using the spread plate method on the following selective media with incubation at 37°C for 24 hours: *E. coli,* mEndo (Difco, Sparks, MD, USA); *S. aureus,* Mannitol Salt Agar (MSA; Difco, Sparks, MD), *L. monocytogenes,* RAPID'L.Mono agar (Bio-Rad, Hercules, CA) *S. typhimurium*, XLD (Difco, Sparks, MD)*, P. aeruginosa,* Tryptic Soy Agar (TSA; Difco, Sparks, MD).

Virus experimental procedure

The virus experiments were performed following the same general protocol as the bacterial experiments; however, a final Absolute Fx dilution of 1:200 was utilized in place of the 1:800 dilution. The test concentration of each virus was approximately $1.0x10^6$ plaque-forming units per ml (PFU) or $TCID_{50}$ per ml. A 1 ml sample was removed after 30 seconds of exposure to the Absolute Fx in the human coronavirus experiment and after 10 minutes and one hour in the feline calicivirus experiment and placed in 9 ml of Letheen neutralizing broth. Ten-fold serial dilutions were made in either Eagle's MEM (feline calicivirus) or phosphate buffer 0.01 M (human coronavirus) (pH 7.5; 3.8 mM Na_2HPO_4, 6.5 mM KH_2PO_4).

The remaining feline calicivirus were enumerated utilizing a plaque-forming assay [3] in 6-well tissue culture plates with CRFK monolayers and agar overlays

containing Eagle's minimal essential medium (MEM; modified with Earle's salts, Irvine Scientific, Santa Ana,) with 2% fetal bovine serum (FBS; Hyclone, Logan, UT). After incubation for 5 days at 35 °C with 5% CO_2, agar overlays were removed, the remaining monolayers were stained with crystal violet and plaque-forming units (PFU) were counted.

Human coronavirus was enumerated utilizing the Reed-Muench method [11] to determine the tissue culture infectious dose that affected 50% of the cultures ($TCID_{50}$) in 24-well tissue culture plates with MRC-5 monolayers with Eagle's MEM containing 2% FBS after incubation for 5 days at 35 °C with 5% CO_2. This method is based on the observation of monolayer cytopathic effects (CPE).

Results

The effect of Absolute Fx on the test bacteria is shown in Table 1. Greater than a 5-$\log_{10}$ reduction was observed for all of the Gram-negative species and the Gram-positive *L. monocytogenes* after a 30 second exposure to both Absolute Fx concentrations, with the exception of *P. aeruginosa* (3.90-$\log_{10}$ reduction). In contrast, *S. aureus* (Gram-positive) was reduced by 3.23-$\log_{10}$ and 2.71-$\log_{10}$ in 30 seconds exposure to 1:400 and the 1:800 dilutions. After 5 minutes, greater than a 5.0-$\log_{10}$ reduction was observed for *P. aeruginosa* and *S. aureus* at both dilutions.

The effect of Absolute Fx on feline calicivirus F-9 and human coronavirus 229E are shown in Table 2. Coronavirus 229E was reduced by > 4-$\log_{10}$ at both dilutions.

Feline calicivirus was more resistant, with only a 1 to 2-$\log_{10}$ reduction within 10 to 60 minutes of exposure. No reductions occured for any of the bacteria or virus in the low pH saline or the Letheen control solutions over the course of the experiments. The neutralizing effect of Letheen on all dilutions of Absolute Fx was also confirmed.

Discussion

There is considerable variation in the response of different microorganisms to antimicrobials. The underlying reasons are poorly understood, but the chemical composition of the outer cell layers is likely to be a key factor [10, 12]. The structure, chemical composition, and thickness of the bacterial cell wall differ greatly between Gram-positive and Gram-negative bacteria. Porin proteins in the Gram-negative outer membrane form channels to allow the entry of essential substances as well as many antimicrobial drugs [8]. Non-enveloped viruses are more stable in the environment than enveloped viruses [1]. Thus, enveloped viruses are often more sensitive to environmental insults and to many antimicrobials than are non-enveloped viruses [14].

Absolute Fx was found to be highly effective against both Gram-negative and Gram-positive bacteria with slight variability in resistance between species. Although it did effect a reduction in the number of the non-enveloped feline calicivirus, it was more effective against the lipid containing human coronavirus. Absolute Fx appears to exhibit antimicrobial activity against a wide variety of both bacteria and viruses.

References

1. Barker J, Stevens D, Bloomfield SF (2001) Spread and prevention of some common viral infections in community facilities and domestic homes. J Appl Microbiol 91: 7-21.

2. Block SS (2001) Disinfection, sterilization and preservation. 5th ed. Lippincott Williams & Wilkins, Philadelphia, PA, USA, p. 19.

3. Bidawid S, Malik N, Adegunrin O, Sattar SS, Farber JM (2003) A feline kidney cell line-based plaque assay for feline calicivirus, a surrogate for Norwalk virus. J Virol Methods 107: 163–167.

4. Didry N, Dubreuil LB, Pinkas MA (1994) Activity of thymol, carvacrol, cinnamaldehyde and eugenol on oral bacteria. Pharm Acta Helv 69: 25-28.

5. Environmental Protection Agency (1976) Sanitizer Test for Inanimate Surfaces DIS/TSS-10 Efficacy data requirements Supplemental Efficacy Sanitizer Test (for inanimate, non-food contact surfaces). Proposed method prepared by Registration Division, Office of Pesticide Programs, EPA, Washington, DC, USA.

6. Guzzella L, Monarca S, Zani C, Feretti D, Zerbini I, Buschini A, Poli P, Rossi C, Richardson SD (2004) In vitro potential genotoxic effects of surface drinking water treated with chlorine and alternative disinfectants. Mutat Res/Genet Toxicol Environ Mutagen 564: 179-193.

7. Kahdre MA, Yousef MA, Kim JG (2001) Microbial aspects of ozone applications in food: review. Concise Rev Food Sci 6: 1242-1252.

8. Levinson W, Jawetz E (2000) Structure of Bacterial Cells. In: Foltin J, Lebowitz H, Holton B. (eds) Medical Microbiology and Immunology. 6th ed. McGraw Hill, New York, NY, USA, pp. 4-8.

9. Marshall SH, Arenas G (2003) Antimicrobial peptides: a natural alternative to chemical antibiotics and a potential for applied biotechnology. J Biotechnol 6: 271-284.

10. McDonnell G, Russell AD (1999) Antiseptics and Disinfectants: Activity, Action, and Resistance. Clin Microbiol Rev 12: 147-179.

11. Payment P, Trudel M (1993) Isolation and Identification of Viruses. In: Payment P, Trudel M (eds) Methods and Techniques in Virology. Marcel Dekker, Inc., New York, NY, USA, pp. 32-33.

12. Russell AD (2003) Similarities and differences in the responses of microorganisms to biocides. J Antimicrob Chemother 52: 750–763.

13. Veschetti E, Cittadini B, Citti G, Ottaviani M (2005) Inorganic by-products in waters disinfected with chlorine dioxide. Microchem J 79: 165-170.

14. Watanabe Y, Miyata H, Sato H (1989) Inactivation of laboratory animal RNA-viruses by physicochemical treatment. Jikken Dobutsu 38: 305-311.

Table 1. Reduction[a] of bacteria after exposure to Absolute Fx.

Time (sec)	Gram-positive				Gram-negative					
	S. aureus		***L. monocytogenes***		***E. coli***		***S. typhimurium***		***P. aeruginosa***	
	1:400[b]	1:800	1:400	1:800	1:400	1:800	1:400	1:800	1:400	1:800
30	3.23	2.71	> 6.25	> 6.25	> 5.87	> 5.87	5.03	5.47	5.16	3.90
300	> 5.69	5.39	> 6.25	> 6.25	> 5.87	> 5.87	5.87	5.87	5.89	5.58
600	> 5.69	> 5.69	> 6.25	> 6.25	> 5.87	> 5.87	> 5.87	> 5.87	6.05	5.65

a - Average of Log_{10} reduction of triplicate experiments. The initial bacterial concentration was $2.4x10^7$ cfu/ml for *S. aureus*, $8.9x10^7$ cfu/ml for *L. monocytogenes*, $3.7x10^7$ cfu/ml for *E. coli*, $3.7x10^7$ cfu/ml for *S. typhimurium*, and $8.3x10^7$ cfu/ml for *P. aeruginosa*.

b - Dilution of Absolute Fx.

Table 2. Reduction[a] of Human Coronavirus 229E and Feline Calicivirus F-9 after exposure to Absolute Fx.

	Human Coronavirus				**Feline Calicivirus**			
	1:200[b]		**1:400**		**1:200**		**1:400**	
Exposure time (min)	# virus[c]	Log_{10} reduction	# virus[c]	Log_{10} reduction	# virus[d]	Log_{10} reduction	# virus[d]	Log_{10} reduction
½	$1.9x10^2$	4.05	3.2x10	4.83	ND[f]	ND	ND	ND
10	< 3.7[e]	> 5.76	< 3.7	> 5.76	$2.7x10^4$	1.41	$2.7x10^4$	1.41
60	< 3.7	> 5.76	< 3.7	> 5.76	$2.5x10^4$	1.44	$3.6x10^3$	2.28

a - Average of Log_{10} reduction triplicate experiments.
b - Dilution of Absolute Fx.
c - $TCID_{50}$ counts/ml recovered. Initial virus titer was $2.14x10^6$ viruses/ml ($TCID_{50}$).
d - Plaque-forming units (PFU)/ml recovered. Initial virus titer was $6.85x10^5$ PFU/ml.
e - Detection limit.
f - Not determined

Table 3. Chemical composition of Absolute Fx

Absolute Fx Liquid	Full Strength sample 33285	
Analyte	Result	Unit
O-benzyl-p-chlorophenol	0.184	% wt / wt
O-phenylphenol	0.123	% wt / wt
Tetradecyl-dimethylbenzyl ammonium chloride	0.052	% wt / wt
Dodecyl-dimethylbenzyl ammonium chloride	0.390	% wt / wt
Hexadecyl-dimethylbenzyl ammonium chloride	0.016	% wt / wt
Didecyl-dimethyl ammonium chloride	0.085	% wt / wt
Octyl-decyl-dimethyl ammonium chloride	0.094	% wt / wt
Dioctyl-dimethyl ammonium chloride	0.077	% wt / wt
2, 4, 4'- trichloro-2'-hydroxydiphenyl ether	0.644	% wt / wt
Ascorbic acid	2.452	% wt / wt
Glycerin	41.160	% wt / wt
Water	37.490	% wt / wt
Ethyl alcohol	16.390	% wt / wt
Isopropyl alcohol	1.106	% wt / wt
Mass balance	99.912	% wt / wt

APPENDIX D:

SILVER AS A DISINFECTANT

Nadia Silvestry-Rodriguez[1], **Enue E. Sicairos-Ruelas**[2], Charles P. Gerba[2], Kelly R. Bright[2]*.

[1] Department of Agricultural and Biosystems Engineering, The University of Arizona, Building 38, Room 403, Tucson, Arizona 85721

[2] Department of Soil, Water and Environmental Science, The University of Arizona, Building 38, Room 429, Tucson, Arizona 85721

Running Title: Silver as disinfectant
Key Words: silver, disinfection, water, bacteria

Author for correspondence:
* Dr. Kelly R. Bright
The University of Arizona
Dept. of Soil, Water & Environmental Science
Building 38, Room 429
Tucson, Arizona 85721
Tel: (520) 626-8094
Fax:(520) 621-6163
Email: bright@email.arizona.edu

List of contents

Introduction and History

The antimicrobial effects of silver (Ag) have been recognized for thousands of years. In ancient times, it was used in water containers (Grier 1983) and to prevent putrefaction of liquids and foods. In ancient times in Mexico, water and milk were kept in silver containers (Davis and Etris 1997). Silver was also mentioned in the Roman pharmacopoeia of 69 B.C. (Davis and Etris 1997).

In 1884, silver nitrate drops were introduced as a prophylactic treatment for the eyes of newborns. This became a common practice in many countries throughout the world (Wahlberg 1982) to prevent infections caused by *Neisseria gonorrhoeae* transmitted from infected mothers during childbirth (Klueh et al. 2000; Slawson et al. 1992). In 1928, the "Katadyn-Process", based on the use of silver in water at low concentrations, was introduced (Krause 1928).

Silver ions have the highest level of antimicrobial activity of all the heavy metals. Gram-negative bacteria appear to be more sensitive than Gram-positive species (Feng et al. 2000; Kawahara et al. 2000; Klueh et al. 2000). Kawahara et al. (2000) posited that some silver binds to the negatively charged peptidoglycan of the bacterial cell wall. Since Gram-positive species have a thicker peptidoglycan layer than do Gram-positive species, perhaps more of the silver is prevented from entering the cell.

Generally speaking, the observed bactericidal efficacy of silver and its associated ions, is through the strong binding with disulfide (S-S) and sulfhydryl (-SH) groups found in the proteins of microbial cell walls. Through this binding event, normal metabolic processes are disrupted, leading to cell death. Silver (Ag), copper

(Cu) and zinc (Zn) have thus found their way into a number of applications.

Applications And Uses

Drinking water

Chlorine has been used as the principal disinfectant for drinking water since the early 1900's. In the 1970's, it was discovered that chlorination caused the formation of numerous chlorinated compounds in water including trihalomethanes and other disinfection by-products (DPB) that are known to be hazardous to human health (Moudgal et al. 2000; Von Gunten et al. 2001). There is therefore a need to assess alternative disinfectants (Yahya et al. 1992).

Silver electrochemistry experiments suggest that silver may have potential as a chlorine alternative in drinking water disinfection in applications in which chlorine may be considered too hazardous (Pedahzur et al. 2000). Silver has been used as an effective water disinfectant for many decades (Kim et al. 2004), primarily in Europe (Russell and Hugo 1994). It has also been used to treat recycled water aboard the MIR space station and aboard NASA space shuttles (Butkus 2004; Gupta et al. 1998).

Both the Environmental Protection Agency (EPA) and the World Health Organization (WHO) regard silver as safe for human consumption. Only argyria (irreversible skin discoloration) occurs with the ingestion of gram quantities of silver over several years or by the administration of high concentrations to ill individuals. There have been no reports of argyria or other toxic effects caused by silver in healthy persons (World Health Organization 1996). Based on epidemiological and

pharmacokinetic data, a lifetime limit of 10 grams of silver can be considered a NOAEL (No Observable Adverse Effect Level) for humans (World Health Organization 1996). In the United States, no primary standards exist for silver as a component in drinking water. The EPA recommends a secondary non-enforceable standard of 0.1 mg/L (100 ppb)(Environmental Protection Agency 2002). The World Health Organization (1996) has stated this amount of silver in water disinfection could easily be tolerated since the total absorbed dose would only be half of the NOAEL after 70 years.

Silver has been used as an integral part of EPA and National Sanitation Foundation (NSF) approved point-of-use (POU) water filters to prevent bacterial growth. Home water purification units (e.g. faucet-mounted devices and water pitchers) in the U.S. contain silverized activated carbon filters along with ion exchange resins (Gupta et al. 1998). Today, some 50 million consumers obtain drinking water from POU devices that utilize silver (Water Quality Association 2001). These products leach silver at low levels (1 to 50 ppb) with no known observable adverse health effects. Such filters have been shown to prevent the growth of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* in water supplies (Russell and Hugo 1994); however, several studies have raised questions about their efficacy (Bell 1991). Reasoner et al. (1987) established that bacterial colonization of such devices occurs within a matter of days and may result in a large number of bacteria in the product water.

Cooling Towers / Large Building Water Distribution Systems

Cooling towers provide cooling water for air compressors and industrial processes that generate heat (Broadbent 1993). They provide an ideal environment and a suitable balance of nutrients for microbial multiplication (Martinez et al. 2004). Chlorine is a popular method for controlling such bacterial growth, but there are difficulties in maintaining disinfection efficacy, particularly at a high temperature or pH (Kim et al. 2004). Chlorination can also cause corrosion of cooling tower facilities (Kim et al. 2004).

Ag/Cu ionization has been used in cooling towers to control bacterial growth (Lin et al. 2002). In a study by Martinez et al. (2004), an appreciably reduced chlorine concentration of 0.3 parts per million (ppm or mg/L) was combined with 200 ppb Ag and 1.2 ppm Cu. This had an appreciable impact on levels of coliform bacteria, iron-related bacteria, sulfate-reducing bacteria and slime-forming bacteria in a cooling tower (Martinez et al. 2004).

Large hot water distribution systems in hospitals and hotels have also often been attributed as a source of contaminating bacteria (Kim et al. 2002). Contaminated systems are usually treated by either superheating the water with flushing of the distal sites (heat-flush), hyperchlorination, or by installing Ag/Cu ionization units (Stout and Yu 1997). Greater bacterial reductions have been observed with Ag/Cu ionization than with the heat-flush method (Stout et al. 1998). Ag/Cu ionization is known to provide long-term control (Liu et al. 1994; Mietzner et al. 1997) and may be used in older buildings in which the pipes could be damaged by hyperchlorination (Stout and Yu 1997). Such systems are easy to install and maintain, are relatively inexpensive and do not produce toxic by-products (Liu et al. 1994).

One microorganism that has been commonly isolated from cooling towers is

Legionella pneumophila, the causative agent of Legionnaires' disease (Fliermans et al. 1981; Landeen et al. 1989). Many outbreaks have been linked to cooling towers (Bentham and Broadbent 1993; Brown et al. 1999; CDC 1994) and evaporative condensers (Breiman et al. 1990). *L. pneumophila* is also commonly isolated from the periphery of hot water systems in large buildings such as hospitals, hotels and apartment buildings where temperatures tend to be lower (Zacheus and Martikainen 1994). Ag/Cu systems have been in common use in hospitals to control *Legionella* for more than a decade (Stout and Yu 2003). Mietzner et al. (1997) reported that one such ionization system maintained effective control of *L. pneumophila* for at least 22 months. *Legionella* may develop a tolerance to silver after a period of years, requiring higher concentrations to achieve the same effect (Rohr et al. 1999).

Recreational Waters (Swimming Pools/Spas/Hot Tubs)

Bacteria, protozoa, and viruses may occur naturally in recreational waters or be introduced into swimming pools by bathers or through faulty connections between the filtration and sewer systems (Beer et al. 1999). Species carried by bathers include the intestinal *Streptococcus faecalis* and *Escherichia coli,* as well as skin, ear, nose and throat organisms such as *Staphylococcus aureus, Staphylococcus epidermidis, Streptococcus salivarius, Pseudomonas aeruginosa* and *Mycobacterium marinum* (Singer 1990). Mild to serious illnesses caused by ingestion of or contact with contaminated water (Beer et al. 1999; Craun 1988) can be the result of improperly maintained pools, spas and hot tubs (Kebabjian 1995).

In recent years, there has been a rapid increase in the number of public, semi-

public and private pools built in Europe and America. Adequate disinfection of such waters is becoming an increasingly important health issue (Singer 1990). Traditionally, chlorine based products are used for disinfection of swimming pools (Borgmann 2003). Chlorine produces harmful by-products (DBP) caused by the halogenation of organic compounds (urine, mucus, skin particles, hair, etc.) released into the water by swimmers (Kim et al. 2002). Thus, there is also a need for alternative disinfectants for recreational waters (Yahya et al. 1992).

Silver (Ag_2SO_4) at a low concentration (10 ppb) has been shown to kill greater than 99.9% of heterotrophic bacteria in swimming pools within 30 min (Albright et al. 1972). Silver has been used commercially in pools, but it is too slow to be used as a primary disinfectant. Regulatory agencies in some countries have recommended its use only in combination with another disinfectant (Anonymous 2006). Electrolytic generation of Ag and Cu ions allows ppb concentrations to be maintained in a convenient and reproducible manner.

Food and Dietary Supplements

Silver has been used to treat vinegar, fruit juices, and effervescent drinks and wine (Foegeding and Busta 1991). It is also available in Mexico as colloidal silver in gelatin (‘Microdyn’) for use as a consumer fruit and vegetable wash and in the U.S. as an alternative health supplement or in silver citrate complexes as food additives (Silver 2003).

Medical Applications

Silver has been used in numerous medical applications (Hotta et al. 1998; Yoshida et al. 1999). In dentistry, silver nitrate is effective against a number of oral bacteria including Gram-negative periodontal pathogens and Gram-positive Streptococci that cause periodontitis (Spacciapoli et al. 2001). Dental amalgams contain approximately 35% Ag(0) and 50% Hg(0). It is unclear whether sufficient Ag(0) is released and oxidized to Ag(I) to produce an antimicrobial effect; however, the release of Hg(II) selects for metal-resistant bacteria (Silver 2003). New amalgams have therefore been introduced that contain silver alone (Silver 2003).

Silver salts have traditionally been administered to the eyes of newborn infants to prevent neonatal eye infections (Isenberg 1990). Silver ions are the most commonly used topical antimicrobial agents used in burn wound care in the western world (Poon and Burd 2004). Both silver nitrate and silver sulphadiazine have also been used as topical antiseptics for cutaneous wounds (Fox and Modak 1974; Gupta et al. 1998; Li et al. 1997; Rosenkranz and Carr 1972). A topical cream containing 1.0% silver sulphadiazine and 0.2% chlorhexidine digluconate has been marketed as Silvazine^{TM} in the U.S. (Silver 2003).

Silver sulphadiazine has recently been incorporated directly into bandages used on burns and large open wounds (Furr et al. 1994; Innes et al. 2001; Silver 2003). Unlike silver nitrate, silver sulphadiazine does not react with sulfhydryl groups or proteins. Thus, its action is not diminished in the wound (Liau et al. 1997; Modak et al. 1988). Nevertheless, the silver is still the antimicrobial portion of the molecule. Two commercial silver coated dressings (Acticoat^{TM} and Silverdin) prevented muscular invasion by *P. aeruginosa* in experimental burns in rats (Ulkur et al. 2005).

P. aeruginosa and *S. aureus* populations were similarly affected by Silverlon, an FDA-approved wound dressing (Heggers et al. 2005).

Silver has also been used to coat vascular, urinary, and peritoneal catheters (Cicalini et al. 2004; Gentry and Cope 2005), prosthetic heart valve sewing rings (Auer et al. 2001; Ionescu et al. 2003), vascular grafts, sutures, and fracture fixation devices (Blaker et al. 2005; Darouiche 1999). Plastic indwelling catheters coated with silver compounds retard the formation of microbial biofilms (Silver 2003). Manal et al. (1996) determined that the adherence of four strains of *E. coli* was decreased by 50% to 99% in comparison to silicone and latex catheters. In two separate clinical studies, from 10-12% of patients with silver treated catheters developed bacteriuria (>100 microorganisms/ml) versus 34-37% of patients with standard Foley catheters after three days. The onset of bacteriuria was thus delayed in comparison to latex catheters (Liedberg et al. 1990; Lunderberg 1986). Gentry and Cope (2005) also found a 33.5% reduction in catheter-associated urinary tract infections following the introduction of silver coated catheters.

The complex of silver with antibiotics on the surfaces of polytetrafluoroethylene vascular grafts has been examined in a number of studies. Silver increased the elution and prolonged the duration of ciprofloxacin release in one such study (Darouiche 1999).

Antimicrobial Surfaces/Materials

Silver may be added to polymers (Brady et al. 2003) to confer antimicrobial activity. The result is consumer products such as washing machines, refrigerators and ice

machines that have incorporated silver (http://www.agion-tech.com/CorporateOverview.pdf, retrieved May 30, 2006; http://www.samsung.com/silvercare/index.htm, retrieved May 30, 2006). Silver has been added to plastics to produce items such as public telephones and public toilets (in Japan), toys and infant pacifiers (Silver 2003). Johnson Matthey Chemicals (UK) utilizes an inorganic composite with immobilized slow release silver as a preservative in their cosmetics (Silver 2003). Synthetic fabrics with silver are popular in areas such as in sports wear, sleeping bags, bed sheets and dishcloths (Silver 2003; Takai et al. 2002). These are believed to reduce the level of bacterial contamination and thus odors (Silver 2003).

Silver may also be added to inorganic ceramics (e.g. zirconium phosphate, zeolite)(Cowan et al. 2003; Galeano et al. 2003; Kim et al. 1998; Kim et al. 2004) that are able to trap metal ions and may then be added to other materials (e.g. paints, plastics, waxes, polyesters) to confer antimicrobial properties (Quintavalla and Vicini 2002; Takai et al. 2002). Zeolite ceramic (sodium aluminosilicate) has a porous three-dimensional crystalline structure in which ions are able to reside. It has a strong affinity for silver ions and can electrostatically bind up to 40% silver (wt/wt)(Kawahara et al. 2000; Uchida 1995). Zeolites act as ion exchangers, releasing silver into the environment in exchange for other cations (Hotta et al. 1998; Kawahara et al. 2000). The amount of silver released is dependent upon the concentration of cations in the environment (Kawahara et al. 2000). The bactericidal activity of Ag-zeolite appears to be due to both the effect of silver ions (Matsumura et al. 2003) and to the generation of reactive oxygen species (under aerated conditions) such as superoxide anions, hydroxyl radicals, hydrogen peroxide, and singlet oxygen (Inoue

et al. 2002).

Studies on stainless steel surfaces coated with zeolites containing 2.5% Ag and 14% Zn ions demonstrated significant reductions in *L. pneumophila* (Rusin et al. 2003), *S. aureus* (Bright et al. 2002), *Campylobacter jejuni*, *Salmonella typhimurium*, *Listeria monocytogenes* and *Escherichia coli* O157:H7 (Bright, K.R. and Gerba, C.P., unpublished data). Vegetative cells of *Bacillus subtilis*, *B. anthracis*, and *B. cereus* were also inactivated by at least three orders of magnitude within 24 hours by a Ag/Zn-zeolite whereas *Bacillus* spores were completely resistant under the same conditions (Galeano et al. 2003).

Antimicrobial Efficacy

The antimicrobial effect of silver has been demonstrated in numerous and varied applications against many different types of microorganisms including bacteria, viruses and protozoa. An overview of the available experimental data on silver disinfection is presented in Table 1.

Antimicrobial Mechanisms

Proposed mechanisms of the antibacterial and antiviral actions of silver are summarized in Table 2.

Antibacterial Action

The antibacterial effects of silver are not completely understood. Numerous mechanisms have been proposed. Several are generally accepted:

1. Extracellular binding or precipitation of silver to bacterial cell walls and membranes (Bellantone et al. 2002; Efrima and Bronk 1998; Goddard and Bull 1989; Slawson et al. 1992). Bacterial cell walls contain negatively charged peptidoglycans that will most likely electrostatically bind some Ag^{+} on their own (Thurman and Gerba 1989).
2. Energy-dependent or independent accumulation of silver inside cells (Slawson et al. 1992). Possible active uptake of silver by a transport system for an essential metal with a similar charge or ionic size (Slawson et al. 1992; Solioz and Odermatt 1995).
3. Binding of silver to cellular proteins, including enzymes (Slawson et al. 1992). Silver is known to stain proteins (Slawson et al. 1992). It binds to sulfhydryl (-SH) groups on enzymes, leading to their inactivation (Feng et al. 2000; Liau et al. 1997; Slawson et al. 1992; Thurman and Gerba 1989) and eventually to the inactivation of the bacteria (Liau et al. 1997). Monovalent silver ions bind to these functional groups, resulting in a stable –S-Ag group that inhibits hydrogen transfer, the source of energy transfer (Davis and Etris 1997). Silver also complexes with sulfhydryl groups in the cell membrane that are components of enzymes that participate in transmembrane energy generation and electrolyte transport (Klueh et al. 2000). This may cause the formation of R-S-S-R bonds that block respiration and electron transfer (Davis and Etris 1997; Heining 1993).
4. Binding of silver to deoxyribonucleic acid (DNA)(Thurman and Gerba 1989).

Silver displaces the hydrogen bonds between adjacent nitrogens of purine and pyrimidine bases (Klueh et al. 2000; Richards 1981). This may stabilize the DNA helix and prevent replication of the DNA and subsequent cell division (Modak and Fox 1973; Richards 1981; Thurman and Gerba 1989).

5. Binding of silver to electron donor groups (Thurman and Gerba 1989) containing nitrogen, oxygen, and sulfur such as amines, hydroxyls, phosphates, and thiols in cells (Grier 1983; Modak and Fox 1973).

Several observations support these proposed mechanisms. Compounds with thiol groups such as sodium thiosulfate, sodium thioglycollate and lysozyme are able to neutralize silver activity. Silver binds to the thiol groups on these compounds and is no longer able to bind to proteins (Liau et al. 1997; Richards 1981). In a study by Bellantone et al. (2002), silver was depleted from an aqueous solution over time in the presence of bacteria. This was assumed to be due to silver binding to the cell wall, or accumulation inside of cells. In a separate study, silver iodide inside a polymer was able to bind sulfhydryl groups on proteins on bacterial outer membranes. It was then transported intracellularly where it accumulated until it reached a toxicity threshold, leading to bacterial death (Brady et al. 2003). Silver accumulation has been observed in non-growing *E. coli* cells due to both binding at the surface and to intracellular uptake (Ghandour et al. 1988).

Feng et al. (2000) visualized the fate and action of silver in *E. coli* and *S. aureus* by transmission electron microscopy. In both species, the cytoplasmic membrane shrank and detached from the cell wall. An electron-light region appeared in the central region that contained large amounts of phosphorous, as determined by

x-ray microanalysis. It therefore likely contained highly condensed DNA molecules. Numerous electron-dense granules surrounded both the cell wall and deposited inside cells, surrounding, but not found within, the electron-light central region. The electron-dense granules contained significant amounts of both silver and sulfur, suggesting a combination of silver and proteins. It was proposed that the cells might produce proteins that aggregate around this nuclear region to protect DNA molecules (Feng et al. 2000). A similar mechanism has been found for heat shock proteins (Nover et al. 1983). Condensed DNA is unable to replicate. No cell growth or multiplication was observed during continuous cultivation with fresh liquid nutrient medium over the course of the experiment. Proteins were inactivated after the silver treatment and the cell wall was severely damaged in some cells. The effects were milder in *S. aureus* than in *E. coli*. The thicker cell wall of the Gram-positive *S. aureus* protects it to some degree from penetration of silver ions into the cytoplasm (Feng et al. 2000).

Several other potential antibacterial mechanisms have been proposed for silver in recent years. Silver collapses the proton motive force on the cell membrane (Dibrov et al. 2002; Williams et al. 1989). Dibrov et al. (2002) found that there was a total collapse of the respiration-generated transmembrane pH gradient in vesicles and also of the membrane electric potential (in the absence of added Na^+). Low concentrations of silver ions induced massive leakage of protons (H^+) through the membrane of *Vibrio cholerae*. This resulted in the complete deenergization of the cells and most likely cell death. This effect might have been the result of modified membrane proteins or a modified phospholipid bilayer (Dibrov et al. 2002). Toxicity may also cause leakage of cellular metabolites and intracellular ions such as potassium

(Slawson et al. 1992).

Silver blocks the respiratory chain of bacteria in the cytochrome oxidase and NADH-succinate-dehydrogenase region (Klueh et al. 2000). One of the primary targets of Ag^+ ions is the Na^+-translocating NADH: ubiquinone oxidoreductase (NQR). Submicromolar Ag^+ ions inhibit energy-dependent Na+ transport in membrane vesicles. This is one of the proposed mechanisms of inactivation at low Ag^+ concentrations (Dibrov et al. 2002).

Silver also inhibits the oxidation of glucose, glycerol, fumarate, succinate, D- and L-lactate and endogenous substrates of *E. coli* cells by the inhibition of the *b* cytochromes and cytochrome *d* at the site of substrate entry into the respiratory chain and also flavoproteins in the NADH and succinate dehydrogenase regions (Bragg and Rainnie 1973). Schreurs and Rosenberg (1982) described a mechanism specifically for silver nitrate in which it inhibits the uptake of inorganic phosphate and causes efflux of accumulated phosphate. This also induces leakage of mannitol, succinate, glutamine and proline, causing metabolite leakage (Slawson et al. 1990).

Adsorption of atomic oxygen on the surface of silver provides a reservoir of oxygen. As a result of the catalytic action of silver, oxygen is converted to active oxygen (such as hydroxyl radicals). Silver can thus catalyze the complete destructive oxidation of bacteria (Davis and Etris 1997; Yoshida et al. 1999).

Antiviral Action

To date, there have been no detailed studies describing the interaction between silver and viruses. Viruses that contain sulfhydryl termini may bind silver. This might affect

their replication cycle (Davis and Etris 1997). One theory is that there is a site-specific Fenton mechanism, where the metal binds to a biological molecule and is reduced by superoxide radicals or other reductants and then reoxidized by hydrogen peroxide. Continous redox reactions in a cyclic manner result in damage, as radical formation occurs near the target site of the molecule (Samuni et al. 1984; Thurman and Gerba 1989; Yahya et al. 1992).

Tzagoloff and Pratt (1964) proposed that silver modifies the adsorption of viruses to cells. Thurman and Gerba (1989) suggested that the inactivation mechanism should be one that does not require a metabolic process, for example, the immobilization of the virus to a surface, the blocking or destruction of host-cell receptors, or the inactivation of the nucleic acid within the viral capsid.

Anti-protozoal Action

The mechanisms by which silver acts against protozoa are not presently understood; nevertheless, many of the mechanisms that have been reported for bacteria most likely play some role against protozoa as well. For instance, silver will most certainly be able to bind to proteins on the cell membrane and, if transported inside the cell, to DNA as well. Binding to DNA could prevent replication and binding to proteins could inhibit their function. If some of these proteins are transmembrane proteins, this might also inhibit transport and nutrient uptake.

It has been reported that silver and copper inactivate *Tetrahymena pyriformis* more easily than they do amoebas (Rohr et al. 2000). *Hartmannella* is inactivated by a concentration of 100 ppm Ag and 1000 ppm copper (Rohr et al. 2000). There are also

reports of the inactivation of *Naegleria fowleri* by the use of silver, copper and free chlorine when used in combination (Cassells et al. 1995).

Silver Resistance

Rusin and Gerba (2001) defined resistance as the ability of a bacterial population to grow in working concentrations of an active disinfectant. Tolerance was defined as the ability of an organism to survive short-term exposure to a disinfectant or to survive for a longer period of time than more sensitive bacterial strains. Many papers have been published describing silver resistance that would be considered as mere tolerance following these criteria. This makes a thorough discussion of silver resistance somewhat problematic. For the purpose of this review, the term "resistance" includes both true silver resistance as well as silver tolerance since the terms are not always discernable based on published descriptions of empirical data.

Some bacteria appear to have natural resistance to silver (Wood 1984). Silver-resistant bacteria are usually found in areas where bacteria are regularly exposed to silver such as in hospital burn wards, hospital water distribution systems and in contaminated soil near silver mines (Silver 2003). Two proposed mechanisms of this resistance are that silver ions are excluded from the cell or mobilized outside of the cell (Slawson et al. 1992). These processes are typically performed by membrane proteins that are energy-dependent and function as either ATPases or chemiosmotic cation/proton antiporters (Silver 2003). Bioaccumulation or sequestration of silver, although it does exist, is not common and its relationship to silver resistance is unclear (Silver 2003). Silver-resistant strains of *E. coli* do not accumulate intracellular

silver deposits whereas sensitive strains contain dense deposits (Starodub and Trevors 1990). The Gram-positive organism *Enterococcus hirae* (formerly *Streptococcus faecalis*) possesses a homeostatic mechanism to manage intracellular copper concentration via an ion pump. The *E. hirae* CopB ATPase in membrane vesicles was found to expel both Cu^+ and Ag^+ from the cytoplasm, causing an accumulation of Cu^+ and Ag^+ inside native inside-out membrane vesicles (Solioz and Odermatt 1995).

In Gram-negative bacteria, plasmid-mediated silver resistance is believed to be the most common and typically involves energy-dependent efflux of silver from the cell. Plasmid-mediated silver resistance in *Salmonella* involves a total of nine genes and is unusual in that it includes three separate types of resistance mechanisms: a periplasmic metal-binding protein (SilE) that binds silver at the cell surface, a chemiosmotic efflux pump and an ATPase efflux pump (SilCBA and SilP)(Silver 2003). This resistance system is somewhat homologous to the plasmid-mediated *pco* copper resistance system in *E. coli* (Silver 2003).

The *agr* gene cluster (containing genes formerly named *ybdE, ylcABCD* and *ybcZ*) encodes a silver resistance system in *E. coli* that is homologous to the central six genes (*silA* through *silS*) of the *sil* resistance system (Silver 2003).

Synergism With Other Disinfectants

Synergy between silver ions and other antimicrobials such as potassium permanganate, potassium peroxymonosulfate (Bright, K.R. and Gerba, C.P., unpublished data), hydrogen peroxide (Armon et al. 2000; Rafter et al. 1999), biguanides (Bright, K.R. and Gerba, C.P., unpublished data), chlorine (Yahya et al.

1992), chlorite and chlorate (Rafter et al. 1999) and UV light (Butkus et al. 2004) has been observed by a number of investigators against a variety of microbiological species including bacteria, viruses and oocysts (see Table 3). Interestingly, metal ions in many instances enhance the effectiveness of the system well beyond that predicted by the individual components; that is, a synergistic effect is observed. It has been postulated that the oxidizer disrupts the cell wall and effects the rapid penetration of the metallic ions into the cell where irreversible precipitation of the DNA occurs (Armon et al. 2000; Straub et al. 1995; Yahya et al. 1992). Other mechanistic interpretations are, of course, possible. For instance, at higher levels of chlorine, silver is precipitated as $AgCl_2^-$ that actually increases the sensitivity of silver-sensitive bacteria (Silver 2003).

Inactivation of *L. pneumophila* by combined copper and silver has been shown to be relatively slow when compared with that of free chlorine; nonetheless, when they were included in addition to low levels of free chlorine, the inactivation rates of bacterial indicator organisms were greater than those for free chlorine alone (Landeen et al. 1989; Yahya et al. 1990). Beer et al. (1999) found that electrolytically generated copper and silver ions used in swimming pool water along with lower levels of chlorine provided control of total coliform and heterotrophic bacteria equivalent to the control provided by high levels of chlorine. Yahya et al. (1990) demonstrated that adding 400 ppb of copper and 40 ppb of silver to water systems containing contaminants similar to those in swimming pools allowed the concentration of free chlorine to be reduced at least three-fold (from 0.1 to 0.3 ppm). Enhanced inactivation rates for *E. coli, S. aureus, L. pneumophila*, *S. faecalis* (Landeen et al. 1989; Yahya et al. 1990) and *P. aeruginosa* (Landeen et al. 1989) were also obtained when water was

treated with 400 ppb copper, 40 ppb silver and 0.2 ppm free chlorine. These studies suggest a synergistic effect upon microorganisms subjected to copper or silver ions in the presence of low levels of chlorine.

Silver has also been shown to have synergistic activity with other metal ions such as copper and zinc. In one study (Lin et al. 1998), both copper and silver ions were found to be effective in inactivating *L. pneumophila* and the combined effect was greater than the sum of the individual effects when each was administered alone.

In two studies, silver-resistant strains of *Acinetobacter baumannii* were found to accumulate high amounts of silver, most of which was surface bound. This resistance was reduced by the purging of a plasmid (Deshpande and Chopade 1994; Shakibaie et al. 1999). In one experiment, the plasmid was successfully transferred to *E. coli* by conjugation; however, the subsequent increased silver resistance conferred to *E. coli* was due to the efflux of silver ions from the cell rather then accumulation (Deshpande and Chopade 1994).

If the oxidizing effect of other disinfectants damages outer cellular structure, it may permit silver ions to rapidly penetrate into the cell. This may bypass the role of silver accumulation on the cell surface as a resistance mechanism.

Conclusions

Both the EPA and the WHO regard silver as safe for human consumption. It does not pose a risk to human health (World Health Organization 1996) and, unlike numerous other commonly utilized disinfectants, is not considered a hazardous substance (Ibarluzea et al. 1998; Kim et al. 2002; World Health Organization 1996). Silver

inactivates a wide variety of microorganisms such as bacteria, viruses, and protozoa, alone or in combination with other disinfectants (Cassells et al. 1995; Davis and Etris 1997; Inoue et al. 2002), although this is not instantaneous.

To date, the development of resistance to silver does not appear to be a concern in real world applications. Silver has successfully been utilized for centuries (Davis and Etris 1997; Grier 1983) and is still effective against a wide variety of microorganisms (Hotta et al. 1998; Kim et al. 2004; Rohr et al. 2000; Yahya et al. 1992). Resistance does exist in certain microorganisms (Silver 2003); however, this usually occurs in environments with high silver concentrations such as near silver mines (Silver 2003). Silver tolerance is more likely to develop under more typical circumstances and silver usages. For example, organisms found in hospital wards and hospital water distribution systems are probably only tolerant since silver has been shown to be effective in hospital water distribution systems for several years (Blanc et al. 2005; Liu et al. 1994; Rohr et al. 1999; Stout and Yu 2003).

Further research needs to be undertaken in order for silver to be accepted as a disinfectant in certain applications by regulatory agencies. This research should provide sufficient information to corroborate real world observations about the efficacy of silver as a disinfectant and any potential problems related to its use such as the development of microbial resistance.

Summary

Silver has been used as an antimicrobial for thousands of years. Over the past several decades, it has been introduced into numerous new venues such as in the treatment of

water, in dietary supplements, in medical applications, and to produce antimicrobial coatings and products. Silver is often used as an alternative disinfectant in applications in which the use of traditional disinfectants such as chlorine may result in the formation of toxic by-products or cause corrosion of surfaces. Silver has also been demonstrated to produce a synergistic effect in combination with several other disinfectants. Many mechanisms of the antibacterial effect of silver have been described, but its antiviral and anti-protozoal mechanisms are not well understood. Both microbial tolerance and resistance to silver have been reported; however, the effect of silver has been observed against a wide variety of microorganisms over a period of years. Further research is needed to determine the antimicrobial efficacy of silver in these new applications and the effects of its long-term usage

References

Albright LJ, Wentworth W, Wilson EM (1972) Technique for measuring metallic salt effects upon the indigenous heterotrophic microflora of a natural water. Water Res 6: 1589-1596.

Anonymous (2006) Australian pesticides and veterinary medicines authority. http://www.apvm.gov.au/qa/poolspa_Q&A.shtml. Retrieved Feb. 1, 2006.

Antelman MS (1992) Anti-pathogenic multivalent silver molecular semiconductors. Precious Metals 16: 141-149.

Armon R, Laot N, Lev O, Shuval H, Fattal B (2000) Controlling biofilm formation by hydrogen peroxide and silver combined disinfectant. Water Sci Technol 42: 187-192.

Auer J, Berent R, Ng CK, Punzengruber C, Mayr H, Lassnig E, Schwarz C, Puschmann R, Hartl P, Eber B (2001) Early investigation of silver-coated Silzone heart valves prosthesis in 126 patients. J Heart Valve Dis 10: 717-723.

Beer CW, Guilmartin LE, McLoughlin TF, White TJ (1999) *S*wimming pool disinfection. J Environ Health 61: 9-13.

Bell FA (1991) Review of Effects of Silver Impregnated Carbon Filters on Microbial Water Quality. J Am Water Works Assoc 83: 74-76.

Bellantone M, Williams HD, Hench LL (2002) Broad-spectrum bactericidal activity of Ag_2O-doped bioactive glass. Antimicrob Agents Chemother 46: 1940-1945.

Bentham RH, Broadbent CR (1993) A model for autumn outbreaks of Legionnaires' disease associated with cooling-towers, linked to system operation and size. Epidemiol Infect 111: 287-295.

Blaker JJ, Boccaccini AR, Nazhat SN (2005) Thermal Characterizations of silver-containing bioactive glass-coated Sutures. J Biomater Appl 20: 81-98.

Blanc DS, Carrara P, Zanetti G, Francioli P (2005) Water disinfection with ozone, copper and silver ions, and temperature increase to control *Legionella:* seven years of experience in a university teaching hospital. J Hosp Infect 60: 69-72.

Borgmann SR (2003) Comparative assessment of different biocides in swimming pool water. Int Biodeterior Biodegradation 51: 291-297.

Brady MJ, Lisay CM, Yurkovetskiy AV, Sawan SP (2003) Persistent silver disinfectant for the environmental control of pathogenic bacteria. Am J Infect Control 31: 208-214.

Bragg PD, Rainnie DJ (1973) The effect of silver ions on the respiratory chain of *Echerichia coli*. Can J Microbiol 20: 883-889.

Breiman RF, Cozen W, Fields BS, Mastro TD, Carr SJ, Spika JS, Mascola L (1990) Role of air sampling in investigation of an outbreak of Legionnaires' disease associated with exposure to aerosols from an evaporative condensor. J Infect Dis 161: 1257-1261.

Bright KR, Gerba CP, Rusin PA (2002) Rapid reduction of *Staphylococcus aureus* populations on stainless steel surfaces by zeolite ceramic coatings containing silver and zinc ions. J Hosp Infect 52: 307-309.

Broadbent CR (1993) *Legionella* in cooling towers: practical research, design, treatment, and control guidelines. In: Barbaree JM, Breiman RF, Dufour AP (eds) *Legionella:* current status and emerging perspectives. American Society for Microbiology, Washington, D.C., pp. 217-222.

Brown CM, Nuorti PJ, Breiman RF, Hathcock L, Fields BS, Lipman HB, Llewellyn GC, Hofmann J, Cetron M (1999) A community outbreak of Legionnaires' disease linked to hospital cooling towers; an epidemiological method to calculate dose of exposure. Int J Epidemiol 28: 353-359.

Butkus MA, Labare MP, Starke JA, Moon K, Talbot M (2004) Use of aqueous silver to enhance inactivation of coliphage MS-2 by UV disinfection. Appl Environ Microbiol 70: 2848-2853.

Cassells JM, Yahya MT, Gerba CP, Rose JB (1995) Efficacy of a combined system of copper and silver and free chlorine for inactivation of *Naegleria fowleri* Amoebas in water. Water Sci & Tech 31: 119-122.

Centers for Disease Control and Prevention. (1994) Legionnaires' disease associated with cooling-towers. MMWR 43: 491-493, 499.

Cicalini S, Palmieri F, Petrosillo N (2004) Clinical review: new technologies for prevention of intravascular catheter-related infections. Crit Care 8: 157-62.

Cowan MM, Abshire KZ, Houk SL, Evans SM (2003) Antimicrobial efficacy of a silver-zeolite matrix coating on stainless steel. J Ind Microbiol Biotechnol 30: 102-106.

Craun GF (1988) Surface water supplies and health. J Am Water Works Assoc 80: 40-52.

Darouiche RO (1999) Anti-infective efficacy of silver-coated medical prostheses. Clin Infect Dis 29: 1371-1377.

Davis RI, Etris SF (1997) Development and functions of silver in water-purification and disease-control. Catalysis Today 36: 107-114.

Deshpande LM, Chopade BA (1994) Plasmid mediated silver resistance in *Acinetobacter baumannii*. Biometals 7: 49-56.

Dibrov P, Dzioba J, Gosink KK, Hase CC (2002) Chemiosmotic mechanism of antimicrobial activity of Ag^{+} in *Vibrio cholerae*. Antimicrob Agents Chemother 46: 2668-2670.

Efrima S, Bronk BV (1998) Silver colloids impregnating or coating bacteria. J Phys Chem B 102: 5947-5950.

Environmental Protection Agency (2002) National Secondary Drinking Water regulations. http://www.epa.gov/safewater/mcl.html; Retrieved July 7, 2006.

Feng QL, Wu J, Chen GQ, Cui FZ, Kim TN, Kim JO (2000) A mechanistic study of the antibacterial effect of silver ions on *Escherichia coli* and *Staphylococcus aureus*. J Biomed Mater Res 52: 662-668.

Fliermans CB, Cherry WB, Orrison LH, Smith SJ, Tison DL, Pope DH (1981) Ecological distribution of *Legionella pneumophila*. Appl Environ Microbiol 41: 9-16.

Foegeding PM, Busta FF (1991) Chemical food preservatives. In: Block SS (ed) Disinfection, sterilization, and preservation, 4th Ed. Lea & Febiger, Philadelphia, p 842.

Fox CL, Modak SM (1974) Mechanisms of silver sulfadiazine action on burn wound infections. Antimicrobiol Agents Chemother 5: 582-588.

Furr JR, Russell AD, Turner TD, Andrews A (1994) Antibacterial activity of Actisorb Plus, Actisorb and silver nitrate. J Hosp Infect 27: 201-208.

Galeano B, Korff E, Nicholson WL (2003) Inactivation of vegetative cells, but not spores, of *Bacillus anthracis*, *B. cereus*, and *B. subtilis* on stainless steel surfaces coated with an antimicrobial silver- and zinc-containing zeolite formulation. Appl Environ Microbiol 69: 4329-4331.

Gentry H, Cope S (2005) Using silver to reduce catheter-associated urinary tract infections. Nurs Stand 19: 51-54.

George N, Faoagali J, Muller M (1997) Silvazine (silver sulfadiazine and chlorhexidine) activity against 200 clinical isolates. Burns 23: 493-495.

Ghandour W, Hubbard JA, Diestrung J, Hughes MN, Poole PK (1988) The uptake of silver ions by *Escherichia coli* K12: Toxic effects and interaction with copper ions. Appl Microbiol Biotechnol 28: 559-565.

Goddard PA, Bull TA (1989) Accumulation of silver by growing and non-growing populations of *Citrobacter intermedius* B6. Appl Microbiol Biotechnol 31: 314-319.

Grier N (1983) Silver and its compounds. In: Block SS (ed) Disinfection, sterilization, and preservation, 3rd Ed. Lea & Febiger, Philadelphia, pp 375-389.

A, Maynes M, Silver S (1998) Effects of halides on plasmid-mediated silver resistance in *Escherichia coli*. Appl Environ Microbiol 64: 5042-5045.

Han J, Duan S, Yang Q, Gao C, Zhang B, He H, Dong X (2005) Efficient and quick inactivation of SARS coronavirus and others microbes exposed to the surfaces of some metals catalysts. Biomed Environ Sci 18: 176-180.

Heggers J, Goodheart RE, Washington J, McCoy L, Carino E, Dang T, Edgar P, Maness C, Chinkes D (2005) Therapeutic efficacy of three silver dressings in an infected animal model. J Burn Care Rehabil 26: 53-56.

Heining CF, Jr. (1993) Catalyst-assisted oxidative sanitation. Ozone Sci & Eng 12: 533.

Hotta M, Nakamima H, Yamamoto K, Aono M (1998) Antibacterial temporary filling materials: the effect of adding various ratios of Ag-Zn-zeolite. J Oral Rehabil 25: 485-489.

Ibarluzea J, Moreno B, Zigorraga C, Castilla T, Martinez M, Santamaria J (1998) Determinants of the microbiological water quality of indoor swimming-pools in relation to disinfection. Water Res 32: 865-871.

Innes ME, Umraw N, Fish JS, Gomez M, Cartotto RC (2001) The use of silver coated dressings on donor site wounds: a prospective, controlled matched pair study. Burns 27: 621-627.

Inoue Y, Hoshino M, Takahashi H, Noguchi T, Murata T, Kanzaki Y, Hamashima H, Sasatsu M (2002) Bactericidal activity of Ag-zeolite mediated by reactive oxygen species under aerated conditions. J Inorg Biochem 92: 37-42.

Ionescu A, Payne N, Fraser AG, Giddings J, Grunkemeier GL, Butchart EG (2003) Incidence of embolism and paravalvar leak after St Jude Silzone valve implantation: experience from the Cardiff Embolic Risk Factor Study. Heart 89: 1055-1061.

Isenberg SJ (1990) The dilemma of neonatal ophthalmic prophylaxis. West J Med 153: 190-191.

Kadar M, Janossy L, Nagy G, Takatsy ZS, Koller M, Simon M, Pohl Ö (1993) Antiviral effect of a new disinfectant containing a silver complex and hydrogen peroxide as active agents. Wiener Mitteilungen: Wasser-Abwasser–Gewaesser 112: 62-64.

Kebabjian RS (1995) Disinfection of public pools and management of fecal accidents. J Environ Health 58: 8-12.

Kawahara K, Tsuruda K, Morishita M, Uchida M (2000) Antibacterial effect of silver-zeolite on oral bacteria under anaerobic conditions. Dent Mater 16: 452-455.

Kim H, Shim J, Lee S (2002) Formation of disinfection by-products in chlorinated swimming pool water. Chemosphere 46: 123-130.

Kim J, Cho M, Oh B, Choi S, Yoon J (2004) Control of bacterial growth in water using synthesized inorganic disinfectant. Chemosphere 55: 775-780.

Kim TN, Feng QL, Kim JO, Wu J, Wang H, Chen GC, Cui FZ (1998) Antimicrobial effects of metal ions (Ag^{+},Cu^{2+}, Zn^{2+}) in hydroxyapatite. J Mater Sci Mater Med 9: 129-134.

Klueh U, Wagner V, Kelly S, Johnson A, Bryers JD (2000) Efficacy of silver-coated fabric to prevent bacterial colonization and subsequent device-based biofilm formation. J Biomed Mater Res 53: 621-631.

Krause GA (1928) Neue Wege zur Wasserterilisierung. Bermann, München.

Landeen LK, Yahya MT, Gerba CP (1989) Efficacy of copper and silver ions and reduced levels of free chlorine in inactivation of *Legionella pneumophila*. Appl Environ Microbiol 55: 3045-3050.

Li XZ, Nikaido H, Williams KE (1997) Silver-resistant mutants of *Escherichia coli* display active efflux of Ag+ and are deficient in porins. J Bacteriol 179: 6127-6132.

Liau SY, Read DC, Pugh WJ, Furr JR, Russell AD (1997) Interaction of silver-nitrate with readily identifiable groups – relationship to the antibacterial action of silver ions. Lett Appl Microbiol 25: 279-283.

Liedberg H, Lundeberg T, Ekman P (1990) Refinements in the coating of urethral catheters reduces the incidence of catheter-associated bacteriuria. An experimental and clinical study. Eur Urol 17: 236-240.

Lin YS, Vidic RD, Stout JE, Yu VL (2002) Negative effect of high pH on biocidal efficacy of copper and silver ions in controlling *Legionella pneumophila*. Appl Environ Microbiol 68: 2711-2715.

Lin YS, Stout JE, Yu VL, Vidic RD (1998) Disinfection of water distribution systems for *Legionella*. Semin Respir Infect 13: 147-159.

Liu Z, Stout JE, Tedesco L, Boldin M, Hwang C, Diven WF, Yu VL (1994) Controlled evaluation of copper-silver ionization in eradicating *Legionella pneumophila* from a hospital water distribution system. J Infect Dis 169: 919-922.

Lundeberg T (1986) Prevention of catheter-associated urinary-tract infections by use of silver-impregnated catheters. Lancet 2: 1031.

Manal MG, Mayo MS, May LL, Simmons RB, Ahearn DG (1996) In Vitro Evaluation of the Efficacy of a Silver-Coated Catheter. Curr Microbiol 33: 1-5.

Martinez SS, Alvarez AG, Esteban M (2004) Electrolytically generated silver and copper ions to treat cooling water: an environmentally friendly novel alternative. Int J Hydrogen Energy 29: 921-932.

Matsumura Y, Yoshikata K, Kunisaki S, Tsuchido T (2003) Mode of bactericidal action of silver zeolite and its comparison with that of silver nitrate. Appl Environ Microbiol 69: 4278-4281.

Mietzner S, Schwille RC, Farley A, Wald ER, Ge JH, States SJ, Libert T, Wadowsky RM, Miuetzner S (1997) Efficacy of thermal treatment and copper-silver ionization for controlling *Legionella pneumophila* in high-volume hot water plumbing systems in hospitals. Am J Infect Control 25: 452-457.

Modak SM, Fox CL Jr. (1973) Binding of silver sulfadiazine to the cellular components of *Pseudomonas aeruginosa*. Biochem Pharmacol 22: 2391-2404.

Modak SM, Sampath L, Fox CL Jr. (1988) Combined topical use of silver sulfadiazine and antibiotics as a possible solution to bacterial resistance in burn wounds. J Burn Care Rehabil 9: 359-363.

Moudgal CJ, Lipscomb JC, Bruce RM (2000) Potential health effects of drinking water disinfection by-products using quantitative structure toxicity relationship. Toxicology 147: 109-31.

Nover L, Scharf KD, Neuman D (1983) Formation of cytoplasmic heat shock protein granules in tomato cell cultures and leaves. Mol Cell Biol 3: 1648-1655.

Pedahzur R, Katzenelson D, Barnea N, Lev O, Shuval H, Ulitzur S (2000) The efficacy of long-lasting residual drinking water disinfectants based on hydrogen peroxide and silver. Water Sci & Tech 42: 293-298.

Poon VK, Burd A (2004) In vitro cytotoxity of silver: implication for clinical wound care. Burns 30: 140-147.

Quintavalla S, Vicini L (2002) Antimicrobial food packaging in meat industry. Meat Sci 62: 373-380.

Rafter J, Grenier J, Denkewicz R (1999) US Patent #5,858,246. January 12, 1999; US Patent #5,935,609. August 10, 1999.

Reasoner DJ, Blannon JC, Geldreich EE (1987) Microbial characteristics of third faucet point-of -use devices. J Am Water Works Assoc 79: 60-66.

Richards RM (1981) Antimicrobial action of silver nitrate. Microbios 31: 83-91.

Rohr U, Senger M, Selenka F, Turley R, Wilhelm M (1999) Four years of experience with silver-copper ionization for control of *Legionella* in a German university hospital hot water plumbing system. Clin Infect Dis 29: 1507–1511.

Rohr U, Weber S, Selenka F, Wilhelm M (2000) Impact of silver and copper on the survival of amoebae and ciliated protozoa in vitro. Int J Hyg Environ Health 203: 87-89.

Rosenkranz HS, Carr HS (1972) Silver sulfadiazine: effect on the growth and metabolism of bacteria. Antimicrob Agents Chemother 2: 367-372.

Rusin P, Bright K, Gerba C (2003) Rapid reduction of *Legionella pneumophila* on stainless steel with zeolite coatings containing silver and zinc ions. Lett Appl Microbiol 36: 69-72.

Rusin P, Gerba C (2001) Association of chlorination and UV irradiation to increasing antibiotic resistance in bacteria. Rev Environ Contamination Toxicology 171: 1-52.

Russell AD, Hugo WB (1994) Antimicrobial activity and action of silver. Prog Med Chem 31: 351-370.

Shakibaie MR, Kapadnis BP, Dhakephalker P, Chopade BA (1999) Removal of silver from photographic wastewater effluent using *Acinetobacter baumannii* BL54. Can J Microbiol 45: 995-1000.

Samuni A, Aronovitch J, Chevio M, Czapski G (1984) In: Rottilio G, Bannister JV (eds) Life Chemistry Reports, Suplement, 2nd Ed. Hardwood Academic Publishers, New York, pp 39-47.

Schreurs WJ, Rosenberg H (1982) Effect of silver ions on transport and retention of phosphate by *Escherichia coli*. J Bacteriol 152: 7-13.

Silver S (2003) Bacterial silver resistance: Molecular biology and uses and misuses of silver compounds. FEMS Microbiol Rev 27: 341-353.

Singer M (1990) The role of antimicrobial agents in swimming pools. Int Biodeterior 26: 159-168.

Slawson RM, Lee H, Trevors JT (1990) Bacterial interactions with silver. Biol Metals 3: 151-154.

Slawson RM, Van Dyke MI, Lee H, Trevors JT (1992) Germanium and silver resistance, accumulation, and toxicity in microorganisms. Plasmid 27: 72-79.

Solioz M, Odermatt A (1995) Copper and silver transport by CopB-ATPase in membrane vesicles of *Enterococcus hirae*. J Biol Chem 270: 9217-9221.

Spacciapoli P, Buxton D, Rothstein D, Friden P (2001) Antimicrobial activity of silver nitrate against periodontal pathogens. J Periodontal Res 36: 108-113.

Starodub ME, Trevors JT (1990) Silver accumulation and resistance in *Escherichia coli* R1. J Inorg Biochem 39: 317-325.

Stout JE, Lin YS, Goetz AM, Muder RR (1998) Controlling *Legionella* in hospital water systems: experience with the superheat-and-flush method and copper-silver ionization. Infect Control Hosp Epidemiol 19: 911-914.

Stout JE, Yu VL (1997) Legionellosis. N Engl J Med 337: 682-687.

Stout JE, Yu VL (2003) Experiences of the first 16 hospitals using copper-silver ionization for *Legionella* control: implications for the evaluation of other disinfection modalities. Infect Control Hosp Epidemiol 24: 563-568.

Straub TM, Gerba CP, Zhou X, Price R, Yahya MT (1995) Synergistic inactivaction of *Escherichia coli* and MS-2 coliphage by chloramine and cupric chloride. Water Res 29: 811-818.

Takai KT, Ohtsuka T, Senda Y, Nakao M, Yamamoto K, Matsuoka-Junji J, Hirai Y (2002) Antibacterial properties of antimicrobial-finished textile products. Microbiol Immunol 46: 75-81.

Thurman RB, Gerba CP (1989) The molecular mechanisms of copper and silver ion disinfection of bacteria and viruses. CRC Crit Rev Environ Contr 18: 295-315.

Tzagoloff H, Pratt D (1964) The initial steps in infection with coliphage M13. Virology 24: 372-380.

Uchida M (1995) Antimicrobial zeolite and its application. Chem Ind 46: 48-54.

Ulkur E, Oncul O, Karagoz H, Celikoz B, Cavuslu S (2005) Comparison of silver-coated dressing (Acticoat), chlorhexidine acetate 0.5% (Bactigrass), and silver sulfadiazine 1% (Silverdin) for topical antibacterial effect in *Pseudomonas aeruginosa*-contaminated, full-skin thickness burn wounds in rats. J Burn Care Rehabil 26: 430-433.

Von Gunten U, Driedger A, Gallard H, Salhi E (2001) By-products formation during drinking water disinfection: a tool to assess disinfection efficiency? Water Res 35: 2095-2099.

Wahlberg V (1982) Reconsideration of Crede Prophylaxis. Acta Paediatr Scand, Supp 295.

Water Quality Association (2001) Use/Purchase of Home Water Treatment Systems. National Consumer Water Quality Survey. Naperville, IL.

Williams RL, Grashoff GJ, Williams DF (1989) The biocompatibility of silver. Crit Rev Biocompat 5: 221-243.

Wood JM (1984) Microbiological strategies in resistance to metal ion toxicity. In: Sigel H (ed) Metal ions in biological systems, vol. 18. Marcel Dekker, New York, pp 333-351.

Yahya MT, Landeen LK, Messina MC, Kutz SM, Schulze R, Gerba CP (1990) Disinfection of bacteria in water systems by using electrolytically generated copper:silver and reduced levels of free chlorine. Can J Microbiol 36: 109-116.

Yahya MT, Straub TM, Gerba CP (1992) Inactivation of coliphage MS-2 and poliovirus by copper, silver and chlorine. Can J Microbiol 38: 430-435.

Yoshida K, Tanagawa M, Matsumoto S, Yamada T, Atsuta M (1999) Antibacterial activity of resin composites with silver-containing materials. Eur J Oral Sci 107: 290-296.

Zacheus OM, Martikainen PJ (1994) Occurrence of legionellae in hot water distribution systems of Finnish apartment buildings. Can J Microbiol 40: 993-999.

Table 1. Microorganisms for which silver has been shown to be effective.

Organism	Treatment	Reference
Hartmannella vermiformis *Tetrahymena pyriformis*	100 ppb Ag + 1000 ppb Cu or 500 ppb Ag + 5000 ppb Cu	Rohr et al. 2000
Naegleria fowleri	400 ppb Cu + 40 ppb Ag or 800 ppb Cu + 80 ppb Ag, and combined with 1.0 ppm free chlorine	Cassells et al. 1995
Mouse malaria	Silver sulphadiazine	Davis and Etris 1997
SARS-Coronavirus	Ag/Al_2O_3 wafers	Han et al. 2005
Coronavirus 229E (human) Feline coronavirus Feline calicivirus	Ag/Cu zeolite	Bright KR, Gerba CP, unpublished data
HIV-1 (AIDS)	1.0, 5.0, 10.0 and 20.0 ppm of Ag_4O_4	Antelman 1992
HIV-1103	Silver thiosulfate complex encapsulated in silica gel microspheres	Davis and Etris 1997
Poliovirus (type 1 Mahoney)	400 ppb Cu + 40 ppb Ag or in combination with free chlorine at 0.2 and 0.3 ppm	Yahya et al. 1992
Poliovirus (type 1 Mahoney)	Sanosil Super 25 (contains	Kadar et al. 1993

Organism	Treatment	Reference
Papovavirus SV-40, A 426 Adenovirus (prototype 6) Vaccinia (Elstree strain) Herpes simplex type 1	silver and hydrogen peroxide) at a concentration of 0.025 % and 0.1% 0.05% Sanosil Super 25	
Herpes vesicular stomatitis	Silver sulphadiazine	Davis and Etris 1997
Bacteriophage MS-2	400 ppb Cu + 40 ppb Ag or in combination with free chlorine at 0.2 and 0.3 ppm	Yahya et al. 1992; Thurman and Gerba 1989
Saccharomyces cerevisiae	Minimum inhibitory concentration of Ag_4O_4 is 1.25 ppm	Antelman 1992
Candida albicans	Minimal inhibitory concentration of Ag_4O_4 is 2.5-5.0 ppm	Antelman 1992
Escherichia coli Pseudomonas aeruginosa *Staphylococcus aureus*	AgBC (bioactive glass doped with Ag_2O) at concentrations 0.05 to 0.20 mg/ml	Bellantone et al. 2002
Escherichia coli *Staphylococcus aureus*	$AgNO_3$	Feng et al. 2000
Escherichia coli	Ceramic balls coated with Ag	Kim et al. 2004

Organism	Treatment	Reference
	and Cu at a concentration of 0.05 ppm Ag and 0.05 ppm Cu	
Pseudomonas aeruginosa *Micrococcus lutena* *Staphylococcus agalactiae*	Minimum inhibitory concentration of Ag_4O_4 is 1.25-2.5 ppm	Antelman 1992
Escherichia coli *Enterobacter cloacae* *Staphylococcus pyogenes*	Minimum inhibitory concentration of Ag_4O_4 is 2.5 ppm	Antelman 1992
Bacillus subtilis *Staphylococcus aureus* *Staphylococcus faecium*	Minimum inhibitory concentration of Ag_4O_4 is 5.0 ppm	Antelman 1992
Staphylococcus epidermidis	Minimum inhibitory concentration of Ag_4O_4 is 0.625 ppm	Antelman 1992
Escherichia coli	Ag-Zeolite	Inoue et al. 2002
Pseudomonas aeruginosa *S. aureus* (MRSA) *S. aureus* (non-MRSA) *Listeria monocytogenes* *Escherichia coli*	Ag/Zn zeolite	Takai et al. 2002; Cowan et al. 2003;

Organism	Treatment	Reference
Staphylococcus aureus *Pseudomonas aeruginosa* *Bacillus anthracis* *Bacillus subtilis* *Bacillus cereus*		Galeano et al. 2003
Staphylococcus aureus *Legionella pneumophila* *Escherichia coli O157:H7* *Campylobacter jejuni* *Salmonella typhimurium* *Listeria monocytogenes*	2.5% Ag / 14% Zn (wt/wt) zeolite	Bright et al. 2002; Rusin et al. 2003; Bright KR, Gerba CP, unpublished data
Streptococcus mutans *Streptococcus mitis* *Streptococcus salivarius* *Streptococcus sanguis*	Ag-Zn Zeolite and SiO_2 at ratio concentrations of 5/55, 10/50, 20/40, and 30/30 wt%	Hotta et al. 1998
S. aureus (non-MRSA) *S. aureus* (MRSA)	0.1 ml of Silvazine™ (1% silver sulphadiazine + 2% chlorhexidine digluconate)	George et al. 1997
Vibrio cholerae	1.0 and 2.0 ppm of Ag_4O_4;	Antelman 1992;

Organism	Treatment	Reference
	low concentration of Ag^{+}	Dibrov et al. 2002
Neisseria gonorrhoeae *Treponema pallida* *Trichomonas*	Treated with silver sulphadiazine	Davis and Etris 1997
Legionella pneumophila	Treated with Ag + Cu	Davis and Etris 1997

Table 2. Summary of mechanisms of inactivation of bacteria and viruses using silver

Scientific Observation	Reference	Type of Microbe
Release of silver into the system	Slawson et. al 1990; Inoue et al. 2002	Bacteria
Oxidative destruction catalyzed by silver	Modak and Fox 1973; Richards 1981	Bacteria
Affinity for sulfhydryl groups	Davis and Etris 1997; Feng et al. 2000	Bacteria and Viruses
Targeting of Na^+-translocating NADH: ubiquinone oxidoreductase (NQR) at low concentration of Ag^+	Dibrov et al. 2002	Bacteria
Targeting of membrane proteins	Dibrov et al. 2002	Bacteria
Inhibits oxidative metabolism required by the cells	Davis and Etris 1997; Heining 1993	Bacteria
Inhibits uptake of nutrients	Slawson et al. 1990	Bacteria
Causes metabolite leakage	Slawson et al. 1990	Bacteria
Binds to DNA	Modak and Fox 1973; Richards 1981; Thurman and Gerba 1989	Bacteria and Viruses
Site-specific Fenton mechanism	Thurman and Gerba 1989;	Viruses

Scientific Observation	Reference	Type of Microbe
	Samuni et al. 1984; Yahya et al. 1992	
Immobilization of the virus to a surface	Thurman and Gerba 1989	Viruses
Blocks or destroys host-cell receptors	Thurman and Gerba 1989	Viruses
Inactivation of the nucleic acid within the viral capsid	Thurman and Gerba 1989	Viruses

Table 3. Synergism with Other Disinfectants.

Scientific Observation	Reference
Copper and silver metals are capable of inactivating poliovirus and coliphages. This effect is greatly enhanced in the presence of oxidizers.	Yahya et al. 1992
Silver significantly enhances the effectiveness of UV light against MS-2 virus.	Butkus et al. 2004
Synergistic effect between silver, copper and free chlorine in the inactivation of *Naegleria fowleri*.	Cassells et al. 1995
Silver shown to be synergistic with chlorite, chlorate and oxidizers (peroxymonosulfate and hydrogen peroxide).	Rafter et al. 1999
Silver is effective in preventing biofilm formation in water. This effect is enhanced in the presence of hydrogen peroxide.	Armon et al. 2000
	Bright KR, Gerba CP,

Scientific Observation	**Reference**
Silver exhibits synergistic effect with potassium monoperoxysulfate against *Acinetobacter baumannii* and *Bacillus globigii* spores.	unpublished data
Silver and copper ions shown to have synergistic effect against *Salmonella typhimurium* and *Escherichia coli*.	Bright KR, Gerba CP, unpublished data