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EFFICACY OF CHEMICAL SANITIZERS TO INACTIVATE *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT104, AND *LISTERIA MONOCYTOGENES* ON ALFALFA SEEDS AND SPROUTS

presented by

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has been accepted towards fulfillment
of the requirements for the

Masters degree in Food Science

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8/26/04
Date

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A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

MASTER OF SCIENCE

Department of Food Science and Human Nutrition

2004

ABSTRACT

EFFICACY OF CHEMICAL SANITIZERS TO INACTIVATE *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT 104 AND *LISTERIA MONOCYTOGENES* ON ALFALFA SEEDS AND SPROUTS

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Alfalfa seeds and sprouts were inoculated with a 3-strain cocktail of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104 or *Listeria monocytogenes* by immersion so as to contain ~ 6 to 8 log CFU/g and subjected to various sanitizer treatments to reduce the pathogen load ≥ 5 logs, while maintaining acceptable seed germination and sprout quality. Exposing seeds and sprouts for ≤ 10 minutes to Clorox™ (sodium hypochlorite, 200 to 20,000 ppm), Tsunami™ (peroxyacetic acid / hydrogen peroxide, 80 and 800 ppm) or Vegi-Clean™ (anionic surfactant, 1%, 2%, 5%) was generally unable to decrease pathogen populations 5 logs. No appreciable differences in pathogen reduction were observed using either sonication (20 kHz) or copper ions (1 ppm) alone or in combination with the previous sanitizers. An FDA-approved fatty acid based-sanitizer containing 3,750 ppm peroxyacid, 15,000 ppm caprylic and capric acid (E 658), 15,000 ppm lactic acid and 7,500 ppm glycerol monolaurate reduced *E. coli*, *Salmonella* and *Listeria* > 5.45, > 5.62 and > 6.92 logs, respectively, on seeds, after 3 minutes, with no injury and no significant loss in seed germination rate or sprout yield. The combination of lactic acid (chelator of metal ions from bacterial cell membrane) and E 658, which was responsible for the observed reduction, may provide a viable alternative to the recommended 20,000 ppm chlorine.

To the two dearest microbes of my life: my sons, Laurent-David and Alain-Nicolas César

ACKNOWLEDGMENTS

I would like to thank God, with all my soul, for my faith that provides me with comfort, strength, wisdom, confidence and the deep certainty that no situation is too complicated that He and I cannot get through together. I am thankful for all the opportunities that were offered to me and all the miracles that happen so often in my life.

I would like to extend my heartfelt gratitude to Dr Jerry N. Cash and his wife, Stella and to Dr. Eleanor Bossi for their determinant impact at critical times of my life at Michigan State University. They were the compass that kept the boat of my life afloat and on track.

I am genuinely thankful to Dr. Elliot T. Ryser for his careful and patient guidance throughout my research, his support and his dedication to create a comfortable and enjoyable work atmosphere for his lab group. The deep relationship that we built together during these years is so meaningful to me.

I would like to thank Dr. Randolph Beaudry for being part of my graduate Committee and for all his suggestions and recommendations. His ability to listen and understand are greatly appreciated.

I also like to thank my previous Committee members, Dr. Perry K.W. Ng, Dr.Wanda Chenoweth and Dr. Robert Ofoli.

I had one of the most interesting learning experience with Dr. Maija Zile. Her guidance style contributes to reinforce my self-confidence in my ability to explore new frontiers.

I am thankful to all the professors who have contributed to my academic experience at Michigan State University. A particular thought goes for Dr. Gale Strasburg, Dr. Maurice Bennink, Dr. Janice Harte, Dr. Zeynep Ustunol, Dr. John Linz and Dr. James Pestka.

I would like to extend my sincere appreciation to the Haitian Ministry of Agriculture, Natural Resources and Rural Development, the College of Agriculture and Veterinary Medecine (FAMV) of the State University of Haiti, the Fulbright-Laspau Scholarship program and the Inter-American Foundation Fellowship program of the United States Government, the National Association of Mango Exporters of Haiti (ANEM), the Office of International Students and Scholar (OISS) at Michigan State University for sponsoring and funding my graduate studies, contributing to my research projects or providing the required papers and authorizations

I am deeply thankful to my family members, my mother, Marie-Lisette David, my sons, Laurent-David and Alain-Nicolas, my sisters, Danielle, Brigitte and Murielle for their love, encouragement, caring and support. Thank you Maman for always being there for me and believing in my abilities, my goals and my dreams. Thank you Laurent and Alain for your maturity and your love. You are the blessing of my life.

I am grateful to all the friends that gave me so precious token of friendship and encouragement during this step of my life. It is impossible to mention all the names here but my heart will always remember. Among them, my childhood friends, Régine and Jean-Richard Beauboeuf, Marcelle Chevallier, Fabienne and Alix Fleury, Claudine Gourgues Jeannot my family's friend, Claude and Adrien Nicolas, Norma-Jean Ek, Lully Thélémaque, Monique Paul Garrity who, despite her responsibilities as Resident

Representative of the World Bank at the United Nations took the time to call me each and every day during more than two months at a critical moment of my Master program, my friends and colleagues of the Center for Agricultural Research and Documentation(CRDA), particularly Colette Blanchet, Myrlene Chrysostome and Emmanuel Prophete. A special thought for my mentors, my friend and CRDA boss, Danielle Avin and my BS advisor, Dr. Frantz Flambert.

I will dearly remember my labmates, the "Ryser's Research Monkeys", Arzu, Rajesh, Finny, Lindsey, Keith, Alyssa, Yan, Iuliano and Geetha for all the memories that we shared during this step of our academic life.

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1.- INTRODUCTION

With the growing trend toward a healthier diet, market demand and consumption of fresh or minimally processed fruits and vegetables continue to increase. Moreover, new production and packaging technologies allow for year-long availability of numerous of fruits and vegetables. Presence of salad-bars in restaurants, displays of fresh produce in receptions and availability of fresh cut ready-to-eat salads in supermarkets have become increasingly common.

A wide range of technologies have been developed to increase the shelf-life of fresh fruits and vegetables. However, none of these technologies are without some negative side effects. Changes in consumption patterns, delays in storage and consumption of fruits and vegetables and numerous manipulations required by new technologies have led to increased risks of cross-contamination with added shelf-life raising additional concerns.

The emergence of new pathogens is an undeniable fact. Pathogens that were previously less virulent have increased in virulence and are now considered public health concerns to certain high risk segments of the population. Some of these organisms have acquired antibiotic resistance while others have been able to grow in environments and conditions (low temperature, low pH) where they were presumed to be unable to survive. Thus, the number of produce-associated food-borne disease outbreaks where fruits and vegetables have been clearly identified as vectors of bacterial infections and the number of cases of illness due to food pathogens have significantly increased in recent years. (Tauxe 1997; Xu, 1999). The bacterial pathogens of greatest concern include *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104 and *Listeria*

monocytogenes, all three of which are targeted in this study. These microorganisms have been linked to, at least, 20 outbreaks and 22 Class I recalls involving sprouts (CDC 1997; FDA, 2004) and have thus gathered significant public and government attention. In one such outbreak involving *Salmonella* Muenchen in Wisconsin, over 12,500 pounds of alfalfa seeds and 2,700 pounds of sprouts were recalled. In a similar outbreak involving *E. coli* O157:H7, 30,000 pounds of sprouts were recalled by a Michigan grower.

Improvements in health care have significantly increased the number of immuno-compromised, elderly and chronically ill patients. Infants and pregnant women are very susceptible to acute bacterial infections. Fruits and vegetables are known to be a good source of vitamins and minerals and because of their high digestibility and good nutritional value, they are recommended as part of the daily diet. Therefore, more efficient strategies are clearly needed to enhance the safety of fresh fruits and vegetables.

Raw alfalfa sprouts, formerly considered to be a "safe","healthy" and nutritious product, have been repeatedly incriminated in food-borne outbreaks involving *E. coli* O157:H7 and *Salmonella* sp. The first consumer warning about sprouts was issued by the CDC in 1997 (Powell et al., 2002). CDC and FDA recommended that individuals at high risk for systemic infections (i.e., the elderly, young children and immunocompromised persons) not eat raw sprouts. In July of 1999, the FDA advised all consumers to be aware of the risks associated with raw sprouts and informed the public that, at that time, the best way to control this safety risk was to avoid eating raw sprouts (Powell et al., 2002). For people who continued to eat sprouts, FDA recommended cooking sprouts before consumption, to reduce the risk of illness (Mohle-Boetani et al., 2002; Powell et al., 2002).

Since then, considerable research efforts have focused on increasing the microbial safety of raw alfalfa sprouts by identifying consumer-acceptable strategies that can reduce the microbial load on alfalfa seeds or alfalfa sprouts by 5 logs or greater. Any proposed seed treatment must also maintain a commercially acceptable seed germination rate. Although, some progress has been made in regard to bean sprouts, no effective treatment has been developed to guarantee safe consumption of organoleptically acceptable alfalfa sprouts. Therefore, the need for further investigations still remains.

Sonication is a physical technique involving the use of ultrasound. Stresses and strains produced during cavitation result in mechanical disruption of bacterial cells (Shukla, 1992; Lillard, 1993). One hypothesis for the present study is that sonication may play a role in damaging the bacterial cell wall, thereby making such organism more susceptible to chemical sanitizers. Nevertheless, sonication is, at least, expected to contribute in declumping and dislodging of bacteria from the surface of alfalfa seeds and sprouts and more effectively expose the pathogens to the bactericidal action of various chemical sanitizers including Clorox™ (sodium hypochlorite), Tsunami™ (peroxyacetic acid), Vegi-Clean™ (anionic surfactant) and copper ions. This synergistic effect between sonication and the chemical sanitizers should lead to enhanced microbial reduction.

Copper has long been known for its antimicrobial properties (Yeager 1991). The second hypothesis in the present study is that copper ions alone will exert a certain level of toxicity toward bacterial pathogens on alfalfa seeds and sprouts. Moreover, a synergistic antimicrobial effect is expected from the combined use of copper ion with Clorox™, Tsunami™ or Vegi-Clean™. Copper ions are generated through an electrolytic process and dispersed into a circulating water stream. The formation of electrostatic

bonds between positively charged ions and negatively charged sites on the bacterial cell surface (Superior Water Solutions, Inc.) should enhance sanitizer contact and performance, thereby leading to greater bacterial reductions.

As part of this work, a novel FDA-approved fatty acid-based sanitizer is also being assessed for inactivation of pathogens on alfalfa seeds. This novel sanitizer concentrate is diluted in water to a reference concentration of 1x, so as to contain 250 ppm peroxyacid, 1000 ppm fatty acid [caprylic (octanoic,C_8) and capric (decanoic,C_{10}) acids], 1000 ppm lactic acid and 500 ppm glycerol monolaurate. The combination of these ingredients produces a synergistic effect, providing a much more potent biocide than what could be obtained using these components separately and offers the unique advantage of having antimicrobial activity at substantially lower concentrations. In addition, lactic acid and glycerol monolaurate also react with peroxyacids and free fatty acids to enhance antimicrobial activity (Guthery, 2002). All components in this fatty acid-based sanitizer have attained Generally Recognized As Safe (GRAS) status. These fatty acids and their esters, which are non-toxic, naturally occurring substances in foods (Kabara, 1984; Oh and Marshall, 1993), carry a considerable advantage over other types of chemical sanitizers developed to control microorganisms. The goal of this research is to determine the optimal concentration and length of exposure to this fatty acid-based sanitizer for decreasing *E. coli* O157:H7, *Salmonella* Typhimurium DT 104 and *Listeria monocytogenes* populations 5 logs on alfalfa seeds while maintaining an optimal germination rate and sprout yield.

2.- LITERATURE REVIEW

2.1.- ALFALFA SEEDS AND SPROUTS

2.1.1.- CHARACTERIZATION OF ALFALFA.

Alfalfa *(Medicago sativa,* Linn.*)* is a perennial legume (botanical family *Leguminosae*) mostly grown for forage production. Originally from southwestern Asia, it has spread throughout the world and can be found in very diverse ecological and agricultural areas. It has been grown for forage since the Roman era. (Ivanov, 1988; Scheaffer et al., 1993). Alfalfa is considered the most nutritious and palatable forage species because of its high protein content and balance of amino acids as well as vitamins and minerals (Orloff, 1995).

The alfalfa plant herbage originates from a large crown and usually reaches 1 to 4 feet or more in height, depending on the soil, climate or cultivation technique. The root system may grow as deep as 20 feet into the soil. Being a legume, this plant enriches the soil in nitrogen. The leaves are comprised of three leaflets. The flowers, most often purple, resemble pea blossoms and are dispersed through the alfalfa branches and stems. The seeds, which are small, usually kidney shaped, brown-olive-green in color measure approximately 2 mm x 1 mm. (Coburn, 1907; Sheaffer, 1993) and weigh 2-3 mg each.

2.1.2.- SPROUTING PROCESS

In commercial settings, alfalfa seeds are sanitized before being processed according to recommendations from the International Food Growers Association (ISGA website, 2004) and FDA requirements (FDA/CFSAN, 1999). The seeds are sprouted hydroponically, at ambient temperature, under dim light, in flat open trays or in relatively

closed rotating drums. Treated potable water is applied through sprinklers at regular intervals. After harvest, fresh sprouts are thoroughly washed, centrifuged and packed (Hooper, 2000; Fett, 2000). Typical seed to sprout yield ratios range from 1:5 to 1:9 (DeVitto, 1982). Fresh yield and nutritive value of alfalfa sprouts are affected by light, temperature, moisture conditions, cultivar and length of sprouting time (Bass et al.,1988). A variety of home-sprouting devices are also available.

2.1.3.- SIGNIFICANCE OF ALFALFA SPROUTS

A.- Nutritional value

Sprouts benefit from a strong public perception as being a very healthy product since alfalfa sprouts are most often viewed as an "unprocessed" or " natural" food (Hooper, 2000). Sprouts are considered a health food because they are low in fat and calories and high in fiber (Weissinger and Beuchat 2000), with the nutritional content of dry seeds increasing as a result of the sprouting process (Rajkowski and Thayer, 2000).

Alfalfa sprouts contain several nutrients of interest: minerals, proteins and vitamins such as ascorbic acid, thiamine, riboflavin and niacin (Bass et al., 1988). DeVitto (1982) reported the following nutritional composition for 100 g of alfalfa sprouts: 16 mg of ascorbic acid, 5 g of protein, 2 g of fiber, 28 mg of calcium and 1.6 mg of niacin. Nutritional content of alfalfa sprouts was investigated by Pennington (1989) who published the following data for 33 g (1 cup) of sprouts : 10 Kcal, 30.1 g of water, 0.2 g of fat, 0.1 g of polyunsaturated fatty acids, 1.3 g of protein, 1.3 g of carbohydrate, 0.7 g of dietary fiber, 5 Retinol Equivalent / 51 International Unit of vitamin A, 3 mg of ascorbic acid, 2 mg of sodium, 10 mg of calcium, 9 mg of magnesium, 0.30 mg of zinc,

26 mg of potassium, 23 mg of phosphorus, 0.32 mg of iron, 44 mg of threonine, 47 mg of isoleucine, 88 mg of leucine, 71 mg of lysine and 48 mg of valine.

Vitamin C (ascorbic acid) is considered a key nutrient in alfalfa sprouts. DeVitto (1982) found a mean initial ascorbic acid level of 15 mg/100 g of alfalfa sprouts followed by a statistically significant decreased to 10.7 mg/100 g of product after 9 days of storage. On a fresh-weight basis, alfalfa sprouts provide higher amounts of ascorbic acid and iron than other vegetables such as cabbage, lettuce and carrots (DeVitto, 1982; Bass et al., 1988). Nevertheless, their ascorbic acid content is far less than high vitamin C-fruits like citrus (DeVitto, 1982; Bass et al., 1988).

B.- Economic significance

The sprouting industry encompasses approximately 475 sprout growers in the United States and Canada, 850 in Japan, 200 in Europe, 35 in Australia and New-Zealand, 3000 in Korea and more than a million in China (Snider, 2000).

On a world-wide basis, sales of sprouts are generating revenues in the range of 1 billion dollars, with sprouts representing total sales of $250 millions / year in the United States and Canada from an annual production of 600 millions pounds of sprouts (Snider, 2000). In 1984, an estimated 32,000 Kg of alfalfa seeds were processed for commercial sprouting with an estimated farm value of $63 millions (Bass et al, 1988).

2.1.4.- HEALTH CONCERNS

A.- Nature of the problem with the sprouts

Alfalfa sprouts found in retail salad bars are minimally processed and most often eaten raw or briefly cooked (Weissinger and Beuchat, 2000). As is true for many other types of fresh produce, alfalfa sprouts typically contain high levels of bacteria, some of which may be pathogenic. The sprouting process itself involves extensive use of water. The sprouting environment, characterized by high moisture and warm temperatures, creates a favorable environment for growth and spread of bacteria (Taormina and Beuchat, 1999a; Fett, 2000). Therefore, contamination of seeds with low levels of pathogens can result in a final product that supports rapid growth of these organisms (Fett, 2000). A pathogen population of 2.5 $\log_{10}$ CFU/g on alfalfa seeds would be considered large in a commercial setting and, in reality, would be unlikely to occur. On alfalfa seeds, pathogen populations would more likely be at least 100-fold lower (Jaquette et al., 1996).

Seeds and sprouts have been recognized as an important cause of foodborne illness (FDA/CFSAN, 1999). They have been linked to, at least, 20 outbreaks of *E. coli, Salmonella* or *L. monocytogenes* infection and targeted by 22 Class I recalls (CDC 1997; FDA, 2004). The potential for sprouts to transmit pathogenic microbes has been linked, especially, to the fact that they are usually eaten uncooked (Piernas and Guiraud, 1997). The first consumer warning about sprouts was issued by the CDC in 1997 (Powell et al., 2002). CDC and FDA recommended that people at high risk for systemic infections (i.e., the elderly, young children and immunocompromised individuals) not eat raw sprouts, with the California Department of Health Services (1998) issuing an interim advisory on

raw alfalfa sprouts. In July of 1999, the US Department of Health and Human Services (1999) advised consumers about the risks associated with raw sprouts and informed them that, at that time, the best way to control this safety risk was to avoid raw sprouts. For persons who continue to eat sprouts, the FDA recommended cooking as a means to reduce the risk of illness (FDA, 2002; Mohle-Boetani et al., 2002; Powell et al., 2002). The California Department of Health Services and the California Department of Education also recommended that schools stop serving uncooked sprouts to young children (Mohle-Boetani et al., 2002).

B.-Source of contamination of Alfalfa sprouts

Alfalfa seeds may become contaminated from animal waste while growing in the field. Contaminated irrigation water, run-off water, sewage and improperly composted manure may also serve as sources of contamination (Como-Sabetti et al., 1997; Mahon et al. 1997; Taormina and Beuchat, 1999a ; Beuchat, 1999; Mohle-Boetani et al., 2002). Harvest, transportation, storage and distribution operations must be considered potential points of contact with pathogens (Beuchat, 1996; Park et al., 2000; Mohle-Boetani et al., 2002).

The procedures inherent to the sprouting process make contamination very easy. Sprouting involves the use of large quantities of water and creates an environment characterized by high moisture and warm temperatures which encourage the growth of bacteria (Mahon et al., 1997; Taormina and Beuchat, 1999a; Mohle-Boetani et al., 2002). While contaminated water, contaminated equipment on the farm or at the processing facility, poor handling and poor hygiene can serve as vectors for contamination

(FDA/NACMCF, 1999; Rajkowski and Thayer, 2000; Park et al. 2000; Mohle-Boetani et al., 2002), most sprout-related outbreaks have been traced to contaminated seeds (Fett, 2000; Mohle-Boetani et al., 2002). Considering this issue, Mohle-Boetani et al. (2002) suggested the designation of sprout seed production lots for human consumption at seed planting, in order to encourage producers to focus on reducing potential seed contamination during production and harvest.

Pathogens can grow on sprouts without modifying their appearance (Taormina, 1999). Jaquette (1996) reported that *Salmonella* Stanley populations of 10^2 to 10^3 CFU/g, on seeds, began increasing after 6 hours of soaking, increasing 3 and 4 logs during the first 24 and 48 hours of germination, respectively. The bacterial population eventually stabilized at 6.75 to 7.08 logs, 54 hours after sprouting with populations decreasing only slightly during 10 days of refrigerated storage at 5 °C.

Therefore, it can be easily understood why seeds contaminated with very low levels of pathogens can be implicated in outbreaks. This observation may explain why, in a 1994 *Salmonella* outbreak, in Sweden and Finland, the organism was found in sprouts but not in seeds (Taormina and Beuchat, 1999a). In cases where spot contamination occurs in one lot of seeds, sprouting water can act as a vector for contaminating the entire batch (Taormina and Beuchat, 1999a).

C.- State of present research

No bactericidal treatment has been developed to effectively guarantee safe consumption of organoleptically acceptable bean sprouts (Rajkowski and Thayer, 2000, Mohle-Boetani et al., 2002). After a multistate *Salmonella* Muenchen outbreak in

September 1999, even the US Food and Drug Administration (FDA) reported that the recommended 20,000 ppm Ca (OCl_2) soak for 15 minutes did not completely eliminate the safety risk as this outbreak occured despite the incriminated sprouts having been grown from seeds that were previously sanitized with the recommended treatment (Proctor, 2001). While several other options have led to a better understanding of the problem, alternative decontamination methods are still needed.

a.- Chemical sanitizers

Various disinfectants have been used on seeds (Tables 2.1 and 2.2) and on sprouts, most notably, sodium hypochlorite (section 2.3.1), peroxyacetic acid (section 2.3.2) and acid-anionic surfactants (section 2.3.3). However, no chemical or water rinse treatments have thus far been able to completely decontaminate fresh fruits and vegetables leaving a satisfying edible raw product (FDA/NACMCF, 1999; Rajkowski and Thayer, 2000). Moreover, cracks and crevices in alfalfa seeds may trap pathogenic bacteria, making them less accessible to lethal concentrations of disinfectants (Taormina and Beuchat, 1999a; Mohle-Boetani et al., 2002). Therefore potential hazards will likely remain after treatment, particularly for the high risk populations.

Hydrogen peroxide (H_2O_2)

The antimicrobial activity of hydrogen peroxide is due to its oxidizing capacity (Piernas and Guiraud, 1997). These authors showed that post-harvest treatment of biologically cultivated brown rice with a 1% solution of hydrogen peroxide for 10 minutes decreased the aerobic plate count by 2 logs. No significant improvement

TABLE 2.1.- INACTIVATION OF *E. COLI* O157:H7 ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	EXPOSURE TIME (Min)
$Ca(OCl)_2$ (20,000 ppm chlorine)	~ 2 to 3	3 and 10
Hydrogen peroxide (1%)	~ 3	3 and 10
Trisodium phosphate (4%)	~ 2	0.5 and 2
Vortexx™ (40 and 80 ppm)	~ 2	3 and 10
Tsunami™ (80 ppm)	> 1.70	3 and 10
Vegi-Clean™ (1 and 2%)	~ 1.50 to 2.1	3 and 10

From Taormina and Beuchat (1999a)

TABLE 2.2.- INACTIVATION OF *SALMONELLA* ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	EXPOSURE TIME (Min)
$Ca(OCl)_2$ (20,000 ppm)	1.95	10
Tsunami™ (530 and 1,060 ppm)	1.12 and 1.50, respectively	10
Trisodium phosphate (2 and 5%)	0.90 and 1.99, respectively	10
Acid. NaClO (500 & 1,200 ppm)	1.26 and 1.43, respectively	10
Vortexx™ (530 and 1,060 ppm)	1.29 and 1.62, respectively	10
Lactic acid (2%)	1.19	10
Lactic (5%)	2.98	10
Citric acid (5%)	2.98	10
Acetic acid (5%)	1.74	10
Hydrogen peroxide (8%)	3.22	10
Calcium hydroxide (1%)	2.84	10
Calcinated calcium (1%)	2.88	10

From Weissinger and Beuchat (2000)

resulted either from increasing the soaking time or from raising the concentration of the bactericidal agent. Hydrogen peroxide at 1% did not affect germination of treated rice seeds or growth of seedlings.

Taormina and Beuchat (1999a) used 1% hydrogen peroxide to decrease *E. coli* O157:H7 from 3.21 to < 0.3 $\log_{10}$ CFU/g in 10 minutes on alfalfa seeds. These seeds were artificially contaminated by dipping 1 kg dry seeds in an 5-strain *E. coli* inoculum cocktail for 1 minute and then drying the seeds for 48 hours. However, even 8% H_2O_2 did not eliminate the pathogen, which was detected by enrichment of alfalfa seeds after treatment

When alfalfa seeds inoculated with *Salmonella* were exposed to 10% hydrogen peroxide for 30 seconds, Beuchat (1997) reported that populations decreased from 3.57 $\log_{10}$ CFU/g to <1 CFU/g; however, the pathogen was not eliminated. Weissinger and Beuchat (2000) found that 0.2%, 2% and 8% hydrogen peroxide respectively reduced *Salmonella* populations 0.22, 0.67 and 3.22 logs on experimentally contaminated alfalfa seeds, following a 10 minute exposure. These concentrations did not affect the germination rate which was 93.3% for the water control compared to 96.1 to 96.5% for the treated seeds (Weissinger and Beuchat, 2000).

Trisodium phosphate (Na_3PO_4)

Taormina and Beuchat (1999a) did not recover *E. coli* O157:H7 by direct plating when alfalfa seeds artificially contaminated to contain 2.20 $\log_{10}$ CFU/g were treated with ≥ 4% trisodium phosphate. However, *E. coli* O157:H7 was detected in all samples, after enrichment.

Weissinger and Beuchat (2000) reduced the number of *Salmonella* by 0.90 and 1.99 $\log_{10}$ CFU/g (compared to water control) by respectively exposing *Salmonella*-inoculated alfalfa seeds to 2% and 5% Na_3PO_4 for 10 minutes. The seed germination rate was not significantly affected by these treatments.

Calcium hydroxide $Ca(OH)_2$

Weissinger and Beuchat (2000) artificially contaminated alfalfa seeds with *Salmonella* by mixing 1 kg of dry seeds in a 6-strain cocktail inoculum, followed by drying 24 hours. *Salmonella* population on the previously inoculated seeds decreased by 0.31, 2.06 and 2.84 logs following a 10 minute exposure to 0.1%, 0.5% and 1% calcium hydroxide, respectively. The seed germination rate remained unaffected.

Calcium hypochlorite $Ca(OCl)_2$

Taormina and Beuchat (1999a) reported that *E. coli* O157:H7 populations decreased from 2.68 and 2.80 logs to < 0.3 $\log_{10}$ CFU/g, respectively, after 3 and 10 minute applications of $Ca(OCl)_2$ (20,000 ppm of active chlorine) in 0.05 M potassium phosphate buffer (pH 6.8) to previously inoculated alfalfa seeds, with no significant loss of germination rate (70.3 and 70.7%, respectively compared to their corresponding water control 78.3 and 77.0%). FDA currently recommends a 20,000 ppm calcium hypochlorite soak before sprouting to reduce the risk for sprout-related illnesses (FDA/NACMCF, 1999). However, this high dose was not completely effective in preventing outbreaks (Proctor, 2001) and doses > 20,000 ppm Ca (OCl_2) can impair seed germination (Mohle-Boetani et al. 2002).

Weissinger and Beuchat (2000) reported a 1.95 log reduction in *Salmonella* on alfalfa seeds after a 10 minute exposure to 20,000 ppm of free chlorine as $Ca(OCl_2)$ in 0.05 M potassium phosphate buffer (pH 6.8), with a significantly lower germination rate of 91.6% compared to 94.8% for the control. Beuchat et al. (2001) observed a germination loss of approximately 10% when 20,000 ppm chlorine were applied to inoculated alfalfa seed for 30 minutes, which led to a 2.3 log reduction in *Salmonella*.

Calcium oxide (CaO)

Bari et al. (1999) found that the addition of 0.4% calcium oxide to a radish seed sprouting medium containing 3.0 to 3.2 $\log_{10}$ CFU of *E. coli* O157:H7/ml completely inhibited the growth or inactivated the pathogen.

Weissinger and Beuchat (2000) reported a 2.88 log reduction in *Salmonella* after alfalfa seeds were exposed to 1% calcinated calcium for 10 minutes with the seed germination rate unaffected by this treatment.

Acidified sodium hypochlorite

Piernas and Guiraud (1997) applied acidified sodium hypochlorite to biologically cultivated brown rice seeds destined for sprouting. Exposure to solution of acidified sodium hypochlorite (pH 4 to 7) had little effect on the mesophilic aerobic bacterial load until 1,000 ppm when aerobic plate counts decreased by 2 to 3 logs after 20 minutes. Decontamination efficacy was not improved by extending contact time, nor by increasing the solution concentration 10,000 ppm. Decreasing the pH of sodium hypochlorite to 4 to 7 did not increase the microbial reduction. Weissinger and Beuchat (2000) tried acidified

sodium hypochlorite on seeds contaminated with low level of *Salmonella* spp. Reductions of 1.26 and 1.43 logs were observed after 10 minute exposure to concentrations of 500 and 1,200 ppm, respectively.

Ethanol

Ethanol at high concentrations will denature bacterial proteins and seed enzymes involved in germination (Piernas and Guiraud, 1997). These authors assessed the efficacy of 70% ethanol for disinfecting biologically cultivated brown rice seeds. A 10 minute exposure to 70% ethanol decreased mesophilic aerobic populations 2 to 4 logs in 10 minutes, after which populations stabilized. Germination was, however, greatly affected, as only 11.5% of disinfected seeds germinated after 24 h, producing abnormal seedlings. Reducing the ethanol concentration to 10% suppressed the undesirable effects on germination. However, decontamination at that concentration was no more efficacious than washing with water.

Active oxygen solution (Vortexx, Ecolab, Mendota Heights, Minn.)

Vortexx (40 and 80 ppm) was applied by Taormina and Beuchat (1999a) to inactivate *E. coli* O157:H7 on alfalfa seeds. After either 3 or 10 minutes of exposure, *E. coli* O157:H7 populations of 0.30 log remained on the seeds, compared to ~ 2 logs for the corresponding water controls.

Vortexx (270, 530 and 1,060 ppm) was used by Weissinger and Beuchat (2000) to reduce populations of *Salmonella* on alfalfa seeds. After a 10 minute exposure, microbial

reductions of 0.78, 1.29 and 1.62 logs, respectively, compared to the water control, were observed without any significant reduction in germination rate.

Organic acids

The antimicrobial effect of organic acids is partly attributed to low pH. Weissinger and Beuchat (2000) used 2% and 5% acetic, lactic or citric acid to decontaminate alfalfa seeds experimentally inoculated with *Salmonella.* The lethal effects of the 5% organic acid treatments were substantial: 1.74, 2.98 and 2.98 log reduction, respectively, after 10 minutes of exposure. Nevertheless, seed germination rates were also significantly reduced: 46.7%, 56.8%, 81.4%, respectively, compared to 92.3% for the water control. Sprouts produced from these seeds were slightly etiolated and were inferior to the control sprouts.

Allyl isothiocyanate

Allyl isothiocyanate (AIT) results from hydrolysis of glucosinolates by myrosinase in cruciferous plants, including mustard and horseradish (Park et al., 2000). Although the antimicrobial activity of AIT varies widely (Delaquis and Mazza, 1995; Park et al., 2000), the volatile compound has been shown to inhibit the growth of *E. coli* (Kyung and Fleming, 1997; Park et al., 2000), including serotype O157:H7 (Delaquis and Scholberg, 1997; Park et al., 2000).

Park et al. (2000) hypothesized that a volatile compound such as allyl isothiocyanate, which is potentially lethal to microorganisms, could more easily reach *E. coli* O157:H7 cells in areas otherwise protected from contact with aqueous solutions.

Incubation of Trypic Soy Agar (TSA) disks inoculated with *E. coli* O157:H7 in a 950 ml jar containing 8 µl of AIT, at 37 °C, for 48 hours, resulted in reductions > 7 logs, with these reductions lower when similar experiments were repeated at 20 °C. Nevertheless, 8 µl AIT was not completely lethal to *E. coli* O157:H7 cells inoculated on the agar disk, as subsequent incubation at 37 °C for 48 h in an atmosphere free of AIT resulted in growth of the pathogen. Colonies developed slowly, however, indicating that exposure of cells to AIT may have caused sublethal injury (Park et al., 2000).

These authors applied 50 µl of AIT on 2 g of dry (6.8% moisture) alfalfa seeds with an initial *E. coli* O157:H7 population of 2.9 logs CFU/g and on 2 g of wet (22.5% moisture) alfalfa seeds with an initial pathogen population of 2.7 logs CFU/g at 25, 37 and 47 °C for 24 h. The pathogen was not recovered by direct plating or by enrichment from wet seeds held at 37 and 47 °C for 24 h. However, *E. coli* O157:H7 was recovered from wet seeds held at 25 °C, after enrichment, indicating that the effect of AIT is temperature dependent. AIT is clearly more effective in killing *E. coli* O157:H7 on wet seeds than on dry seeds as enrichment of the treated dry seeds revealed the presence of the pathogen, and as exposure to 100 µl of AIT at 47 °C for 24 h did not eliminate the pathogen from dry seeds.(Park et al., 2000).

The drawback of this method is that AIT drastically reduced the germination rate which fell from 90% to 3% after wet seeds were exposed to 50 µl AIT at 25 °C for 24 h. (Park et al., 2000). As the mechanism of action is believed to involve respiratory inhibition in bacterial cells, AIT may also adversely affect the respiratory mechanism of alfalfa seeds (Park et al., 2000).

Fit ™, (Procter and Gamble, Cincinnati, OH)

Fit™ is a GRAS alkaline produce-sanitizing solution composed of water, oleic acid, glycerol, ethanol, potassium hydroxide, sodium bicarbonate, citric acid and distilled grapefruit oil. Beuchat et al. (2001) compared the antimicrobial performance of Fit™ to 20,000 ppm chlorine solution as $Ca(OCl)_2$ in 0.05 M potassium phosphate buffer (pH 7.0) These authors found that treatment with 2% chlorine or Fit™ applied for 15 or 30 minutes reduced *Salmonella* populations by 2.3 to 2.9 logs. Treating seeds with 2% chlorine for 15 or 30 minutes resulted in equal or a significantly greater reduction in viable *E. coli* O157:H7 (1.6 and 2.0 logs, respectively) compared to Fit™ (1.5 to 1.7 logs, respectively). The actual difference in numbers of *E. coli* recovered from seeds treated with 2% chlorine or Fit™ for 30 minutes, although significant, was only 0.3 $\log_{10}$ CFU/g. However, exposing seeds to 2% chlorine for 15 or 30 minutes or to Fit™ for 30 minutes significantly reduced the seed germination rate from 95.7% to 85.7 - 87.7%.

Chlorine dioxide

Applying 500 ppm of acidified ClO_2 under the form of USS-1400 (Universal Sanitizers and Supplies, Knoxville, TN), for 3 and 10 minutes reduced *E. coli* O157:H7 on alfalfa seeds from approximately 2.65 logs to <0.30 logs, with the pathogen detected after enrichment. After the 3 minute exposure, the germination rate declined from 73.7% to 55% (Taormina and Beuchat, 1999a,b). Although chlorine dioxide will effectively destroy microorganisms present in solutions and those attached to equipment surfaces, this sanitizer is less efficacious for fruits and vegetables (Reina et al., 1995). Mari et al. (1999) mentioned that some limitations affect the efficacy of chlorine. They are mostly

related to a rapid drop in chlorine activity in the presence of organic substances that modify the pH of the solution.

b.- Heat treatment

Alfalfa seeds have been exposed to high temperatures, with heat also having been combined with various chemical treatments to assess possible synergistic effects.

Jaquette et al. (1996) studied the effect of a mild heat treatment on the microbial load of alfalfa seeds inoculated with *Salmonella* Stanley. No reduction in populations of *S.* Stanley was seen on seeds soaked in water at 21 °C for 5 or 10 minutes. Treating seeds in water at 54 °C for 5 and 10 minutes reduced *S.* Stanley populations from 263 to 9 CFU/g and from 261 to 6 CFU/g, respectively. All other treatment temperatures (57, 60, 63, 66 and 71 °C) led to populations of <1CFU/g after 5 minutes.

Jaquette et al. (1996) also assessed the effect of temperature on alfalfa seed viability. Compared with dipping seeds in water at 21 °C (control), immersing seeds in water at 54, 57, or 60 °C for 5 minutes did not substantially reduce the germination rate after 48 h of storage at 30 °C. However, treatments at 54, 57 and 60 °C for 10 minutes reduced seed viability from 96% (control) to 88, 84 and 42%, respectively. Treating seeds at 63 and 66 °C for 5 minutes reduced the germination rate to 83 and 82%, respectively, while treatment at the same temperature for 10 minutes reduced viability to 21 and 6 %, respectively. While heating appears to effectively inactivate *S.* Stanley on alfalfa seeds, the range of temperatures that can be used is narrow, i.e., between 57 and 60 °C for no longer than 5 minutes because lower temperatures may not kill *S.* Stanley and perhaps other Salmonellae with higher temperatures or a longer exposure time (10

minutes) decreasing germination. Therefore, this hot water treatment is not practical in commercial settings.

Piernas and Guiraud (1997) experimented with heat as an antimicrobial treatment for rice seeds. These authors reported that temperatures of 70 or 90 °C inactivated the seeds after 30 seconds. Immersing the seeds at 60 °C for 5 minutes led to a 3 log decrease in total aerobes without affecting germination.

Piernas and Guiraud (1997) observed substantially lower aerobic plate counts on biologically cultivated brown rice seeds destined for sprouting after soaking in a 1, 000 ppm sodium hypochlorite solution at 60 °C for 5 minutes.

c.- Gamma irradiation

Rajkowski and Thayer (2000) reported D-values of 0.54 and 0.46 kGy when radish sprouts were inoculated with a *Salmonella* cocktail and irradiated. D-values were 0.34 and 0.30 kGy when alfalfa sprouts were inoculated with *E. coli* O157:H7. These sprouts were previously irradiated at 6 kGy / 19 °C to eliminate background flora before inoculation. After enrichment, *Salmonella* was not detected on sprouts irradiated at ≥ 0.5 kGy. Nevertheless, only a 4 log reduction in total aerobes was observed using 3.0 kGy (Rajkowski and Thayer, 2000). Although radish sprouts used in these experiments kept their structure following sterilization by irradiation, inoculation and irradiation, the authors acknowledged that further research is necessary to determine the effect of ionizing radiation on the structure and keeping quality of other sprout varieties. They suggested the combination of low level radiation, an effective chemical wash and modified atmosphere packaging to control pathogens on fresh fruits and vegetables

The organoleptic quality of sprouts should be unaffected by gamma irradiation. This process may be economically feasible for sprout growers. However, a major drawback is that sprout growers have small operations scattered over large areas, and therefore, 2 to 3 days may be required for irradiation. Considering the relatively short shelf-life of sprouts, this solution may not be commercially viable for all growers. Moreover, the germination rate and production yield are essential concerns for sprout growers. Irradiation does not affect germination rate while sprout production yield is affected (Rajkowski and Thayer, 2001).

d.- Pre-soaking treatment

Experiments conducted either on seeds or sprouts actually show that the microbial reduction is sometimes less after longer rather than shorter exposure times. This observation is drawn from work by Taormina and Beuchat (1999a) and Weissinger and Beuchat (2000). The explanation offered is that, in the case of longer solution-seed contact times, the seeds imbibe more water and therefore release bacterial cells that have been more strongly attached to the seed surface or were previously trapped and hidden within crevices or between the testae and the cotyledons. This explanation can also be extended to sprouts.

In order to verify the previous hypothesis, Weissinger and Beuchat (2000) assessed the effect of pre-soaking alfalfa seeds in water for 30 minutes on the release of bacteria hidden in their crevices. The effect of pre-soaking on the efficacy of subsequent exposure to 2,000 ppm chlorine was negligible; however, it might slightly enhance the efficacy of lactic acid.

e.- Surfactants

Surfactants were used by Zhang and Farber (1996) to increase surface wetting. Surfactants have a capacity to penetrate and adhere to porous surfaces (Piernas and Guiraud, 1997).

Use of Orenco Peel 40 (Rio Linda Chemical Co., Inc., Sacramento, CA) and Tergitol (Sigma Chemical Co., St Louis, MO) as surfactants added to a chlorine treatment did not lead to a lower microbial load in comparison with the use of chlorine alone (Zhang and Farber, 1996).

Addition of 1 g/L Tween 80, as a surfactant, to a sodium hypochlorite solution by Piernas and Guiraud (1997) improved the disinfection of rice seeds, although this microbial reduction was not due to any bactericidal effect of Tween 80 at that concentration. No significant decrease in quantity of free available chlorine was observed after adding Tween 80 at concentrations up to 1g/L.

Although benzalkonium chloride, as a quaternary ammonium compound, is not permitted as a food sanitizer due to the possibility of generating toxic residues, Piernas and Guiraud (1997) assessed its use as a surfactant. Dipping biologically cultivated brown rice seeds in a solution containing 0.1 mg/L of benzalkonium chloride for 30 minutes decreased the natural microflora about 2 logs. Decontamination was more effective at 1 mg/L when aerobic plate counts decreased 2.5 to 3 logs after 10 minutes. Prolonged soaking had no effect with neither germination nor seedling growth affected.

f.- Addition of antimicrobials to irrigation water

Addition of antimicrobials to sprout irrigation water was investigated as a means to inhibit the growth of human pathogens introduced after seed decontamination due to inadequate water quality or insufficient worker hygiene (Fett, 2000).

Fett (2000) reported that it is very difficult to achieve a significant reduction in the natural microflora of growing sprouts by addition of antimicrobials to irrigation water. Such a treatment would likely be unable to kill human pathogens that are located in the internal tissues of sprouts (Fett 2000).

Work by Taormina and Beuchat (1999b) led to the same conclusions. These authors treated alfalfa seeds previously inoculated with *E. coli* O157:H7. The seeds were soaked in one of the following chemicals for 20 minutes : NaOCl (200 and 2,000 ppm active chlorine), $Ca(OCl)_2$ (200 and 2,000 ppm active chlorine), acidified $NaClO_2$ (100, 500 and 1,200 μg/ml), Na_3PO_4 (1%), Vegi-Clean™ (1%), Tsunami™ (40 and 80 ppm), Vortexx™ (40 and 80 ppm) or H_2O_2 (1%). After soaking, the seeds were drained for 2 minutes, immersed in sterile tap water for 1 hour, and then placed in plastic boxes for germination. During sprouting, 40 to 45 ml of each test chemical was sprayed evenly onto sprouts after 24 hours of germination and 24, 48 and 72 hours later. Chemical solutions sprayed onto sprouts were rinsed 5 minutes after application by spraying 20 to 25 ml of sterile tap water evenly. Samples of sprouts were removed from the sprouting boxes and analyzed for *E. coli* populations prior to and immediately after spray applications. No chemical treatment reduced numbers of *E. coli* O157:H7 on sprouts when compared with numbers recovered from water-treated sprouts. With the exception of $NaClO_2$ at 1,200 μg/ml, spray applications of these chemicals did not significantly

reduce populations or control the growth of *E. coli* O157:H7 on alfalfa sprouts during the sprouting process. Populations of *E. coli* O157:H7 peaked at *ca.* 6 to 7 logs, 48 hours after initiation of the sprouting process and remained stable despite further spraying with chemicals. No difference were seen between various treatments after 6 days of cold storage at 9 ± 2 °C.

g.- Effect of duration of seed storage

Storing dry alfalfa seeds at 8 °C for 9 weeks reduced populations of *Salmonella* Stanley from 2.53 to 1.81 logs. Inactivation was enhanced at 21 °C. Storing these dry seeds at 8 °C for 1 week and then at 21 °C for 8 weeks decreased the *Salmonella* population from 2.53 to 0.92 log (Jaquette et al., 1996).

h.- Examination of sprouts using scanning electron microscopy

Scanning electron microscopy allows for observing biofilm formation on all parts of alfalfa sprouts (Fett, 2000). Mature biofilms are structured communities of microbes adherent to a surface and embedded in a self-produced glycocalyx material composed primarily of exopolysaccharides. The process of biofilm formation is believed to start with adhesion of individual microbes to a surface, aggregation into microcolonies, intercellular communication and finally maturation into structured biofilms (Costerton et al., 1999; Fett, 2000). Biofilm bacteria are ≥ 500 times more resistant to antimicrobial compounds and interact physiologically with other microbes as a complex community (Costerton et al., 1995; Fett, 2000). Rod-shaped bacteria of various sizes were the predominant microbes observed on all sprout surfaces (cotyledons, hypocotyls and roots).

Cocci-shaped bacteria, as well as yeast, were rarely seen, while structures resembling filamentous fungi were not observed (Fett, 2000). After 2 days of growth, biofilms were already seen on laboratory grown sprouts. Rinsing under running tap water did not remove these biofilms from the surface of sprouts. By the 4th day of growth, 29 to 59% of the total mesophyllic bacteria were present in biofilms (Fett, 2000).

i.- Sensory evaluation

Taormina and Beuchat (1999b) evaluated the overall visual appearance of mature alfalfa sprouts that had been dipped in chemical solutions and rinsed with tap water or not rinsed. A hedonic scale ranging from 1 (inedible) to 5 (excellent quality) was used to rate the appearance of the sprouts during 10 days of storage at 9 ± 2 °C. Chemical treatments used used in this study included NaClO at 200, 1,000 and 2,000 ppm active chlorine; $Ca(OCl)_2$ at 200, 1,000 and 2,000 ppm active chlorine; acidified $NaOCl_2$ at 850 and 1,200 ppm; Na_3PO_4 at 40,000 and 120,000 ppm; Tsunami™ at 40 and 80 ppm; Vortexx™ at 40 and 80 ppm, and H_20_2 at 1 and 5%. Rinsing with tap water after applying the chemical sanitizer clearly helped to maintain overall appearance. After 1 day of storage, the rinsed sprouts usually (except Na_3PO_4 120,000 ppm) compared well to the water control. By the 6th day, the rinsed sprouts previously treated with NaClO, Tsunami and H_20_2 were still similar to the water control while some alterations were detected in the appearance of those treated with Vortexx™ and Na_3PO_4.

D.- Sprouts industry regulations

In order to counteract the "sprout problem", several guidelines and regulations has been published. On October 27, 1999, the FDA and the Center for Food Safety and Applied Nutrition (CFSAN) published a series of papers as "guidance for the industry" (FDA/CFSAN, 1999). A paper entitled "Reducing microbial food safety hazards for sprouted seeds" identifies the preventive controls that the FDA believes should be taken immediately to reduce the public health risks associated with raw sprouts and to ensure that sprouts are not adulterated under the food safety provisions of the Food, Drug and Cosmetic (FDC) Act. Failure to adopt effective preventive methods of control assumes that the product was prepared under unsanitary conditions which may render it injurious to health. Food produced under such conditions is adulterated under the act [21 U.S.C. 342 (a) (4)]. The FDA would consider enforcement actions against any party who does not have effective preventive controls in place, particularly, microbial testings. This paper provides guidelines and recommends good agricultural practices (GAPs) for seed production, conditioning, storage and transportation and good manufacturing practices (GMPs) for sprout production. It also advocates seed treatment with an approved antimicrobial (i.e., use of the recommended 20,000 ppm calcium hypochlorite soak), testing for pathogens in the spent irrigation water, and implementation of traceback systems (FDA/CFSAN, 1999).

2.2.- BACTERIAL PATHOGENS ASSOCIATED WITH ALFALFA SPROUTS

2.2.1.- *ESCHERICHIA COLI* O157:H7

A.- Health significance of *E. coli* O157:H7

Escherichia coli O157:H7 was first isolated in 1975 from the stool of a woman with bloody diarrhea. In 1982, *E. coli* O157:H7 was identified as a human pathogen when it was involved in two outbreaks of hemorrhagic colitis in Oregon and Michigan. Since these years, *E. coli* O157:H7 has gone from a little known enteric pathogen to a foodborne pathogen of international importance. Indeed, it is the predominant cause of enterohemorrhagic-associated diseases in the United States and in many other countries (Doyle et al., 1997; Ryser, 1998).

Issues of concern with *E. coli* O157:H7 include a very low infective dose (< 100 cells), unusual tolerance to acid and the development of antibiotic resistance. Recent evidence suggests that isolates of *E. coli* O157:H7 have developed a trend toward resistance to streptomycin, sulfisoxazole and tetracycline (Doyle et al., 1997; Ryser, 1998).

Infections with *E. coli* O157:H7 lead to symptoms ranging from a mild nonbloody diarrhea to hemorrhagic colitis (characterized by grossly bloody diarrhea accompanied by severe appendicitis-like abdominal pain), and hemolytic uremic syndrome (HUS) which can produce renal failure, thrombocytopenic purpura and eventually death. These symptoms are related to the adherence of the pathogen to the intestinal tract lining followed by production of one or more verotoxins (VTs) also called Shiga-like toxins (SLTs) (Doyle et al., 1997; Ryser, 1998).

B.- Evidence of *E. coli* O157:H7 and *E. coli* O157: NM in fresh produce

Contamination of fresh produce by *E. coli* has been well documented (Beuchat, 1996). Alfalfa sprouts are reported among foods often linked to *E. coli* outbreaks (Table 2.3) and, rather than ground beef, have been found by the CDC to be incriminated in the greatest number of *E. coli* O157:H7-related illnesses. Of 285 cases of *E. coli* O157:H7 infection reported by CDC as of 1998, 108 were caused by alfalfa sprouts whereas 20 were attributed to tainted ground beef and 52 to other sources (FDA, 1998). Cattle and chickens are well known reservoirs of *E. coli* O157: H7. Manure, in general, and manure from cattle and chickens, in particular, should be avoided in fertilization of fresh produce intended for raw consumption.

The world's largest *E. coli* O157:H7 oubreak occured in Japan, from May to August 1996, and involved about 10,000 people, including 6,000 primary school children, in Sakai City, Osaka Prefecture and factory workers in Kyoto. *E. coli* O157:H7 isolates from Sakai City and Kyoto had identical PFGE and RAPD patterns. Based on strong evidence, white radish (daikon) sprouts were incriminated as the source of infection although *E. coli* could not be isolated from either the seeds or the sprouts. As a consequence of this outbreak, 12 deaths were reported and at least one person died of HUS-associated encephalopathy (Gutierrez, 1997; Como-Sabetti et al., 1997; Itoh et al., 1998; Watanabe et al., 1999; Taormina and Beuchat, 1999a; Taormina 1999; Taormina et al., 1999; FDA/NACMCF, 1999).

The following year, *E. coli* O157:H7 was again implicated through radish sprouts in two different outbreaks in Yokohama and Gamagori City, Japan (Gutierrez, 1997; Itoh et al., 1998, Watanabe et al., 1999; Taormina and Beuchat, 1999a; Taormina, 1999) .

TABLE 2.3.- SUMMARY OF *E.COLI* O157:H7 AND *E.COLI* O157: NM OUTBREAKS ASSOCIATED WITH RAW SPROUT CONSUMPTION

TYPE OF SPROUTS	LOCATION AND DATE	NUMBER OF CASES	REMARKS	REFERENCES
White radish (daikon) sprouts	Japan, May to August 1996	10,000 cases 12 deaths	*E. coli* O157:H7 incriminated but not isolated from either the seeds or the sprouts	Gutierrez, 1997; Como-Sabetti et al., 1997; Itoh et al. 1998; Watanabe et al., 1999; Taormina and Beuchat, 1999a; Taormina, 1999; Taormina et al.,1999; FDA/NACMCF,1999
Radish sprouts	Japan (2 outbreaks) 1997	126 cases	-	Gutierrez, 1997; Hara-Kudo et al., 1997; Itoh et al., 1998; Watanabe et al., 1999; Taormina and Beuchat, 1999a; Taormina, 1999
Alfalfa sprouts	Michigan and Virginia, July 1997	~ 108 cases	Strains found indistinguishable by molecular subtyping	Como-Sabetti et al., 1997; Taormina and Beuchat, 1999a; FDA/NACMCF, 1999; Taormina et al., 1999
Alfalfa / clover sprouts	California and Arizona, June 1998	8 cases	Pathogen not recovered from seeds.	FDA/NACMCF, 1999 Taormina et al., 1999 Mohle-Boetani et al., 2001
Alfalfa sprouts	San Luis Obispo (California), August 2002	5 cases	-	Prado and Huff, 2002; Huff, 2002a,b,c; Stone, 2002; Wootson, 2002; FDA, 2002

Hara-Kudo et al. (1997) reported an *E. coli* O157:H7 population of 7.8 $\log_{10}$ CFU/g on sprouts obtained from the implicated seeds. This outbreak consisted of 126 cases, including one fatality (Gutierrez, 1997).

The first reported outbreak of *E. coli* O157:H7 infection associated with alfalfa sprouts occurred in Michigan and Virginia during June and July of 1997. This outbreak was traced to alfalfa sprouts produced from a seed lot common to both Michigan and Virginia producers and supplied by the same seed distributor. Forty to 60 persons were affected in Michigan, with an additional 48 cases in Virginia. The strains implicated in both states were identical by molecular subtyping (Como-Sabetti et al., 1997; Taormina and Beuchat 1999a; FDA/NACMCF, 1999; Taormina et al., 1999). Following these *E. coli* O157:H7 outbreaks, a class I recall (Table 2.4) was issued for an undetermined volume of alfalfa sprouts grown in Norfolk, VA that were distributed in North Carolina, Maryland, Virginia and the District of Columbia (FDA, 2004).

In June 1998, a cluster of infections by a non-motile *E. coli* O157 strain (lacking the flagellar antigen) producing Shiga toxins I and II occurred in Northern California and Arizona. These cases were associated with eating an alfalfa and clover sprout mixture produced by the same sprouter implicated in a simultaneous *Salmonella* Seftenberg outbreak in California and Nevada. *E. coli* O157:NM isolates from 8 patients had the same PFGE pattern but laboratory analysis of seeds, sprouted seeds and environmental samples did not yield *E. coli* O157:NM. This producer inconsistently used chlorine disinfection before sprouting (Taormina et al., 1999, FDA/NACMCF, 1999; Mohle-Boetani et al., 2001). A class I recall (Table 2.4) was issued to recover the sprouts jointly associated with the two *E. coli* O157:NM and *Salmonella* Seftenberg outbreaks.

TABLE 2.4.- RECALLS OF SPROUT PRODUCTS LINKED TO CONTAMINATION BY *E. COLI* O157:H7 AND *E. COLI* O157:NM.

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
4/1/1998	8/1-4-5/1997	Class I # F-191-8	Alfalfa sprouts	Undetermined	North Carolina Maryland, Virginia, District of Columbia	*E. coli* O157:H7
11/25/1998	7/10/1998	Class I # F-049/051-9	Alfalfa sprouts, alfalfa/clover sprouts, alfalfa/clover/radish sprouts, clover sprouts	Firm estimates none remains on the market.	California, Nevada	*E. coli* O157:NM jointly with *Salmonella*

Source: FDA (2004) Enforcement reports.

* Class I : a situation in which there is a reasonable probability that the use of, or exposure to, a violative product will cause serious adverse health consequences or death.
Class II : a situation in which the use of, or exposure to a violative product may cause temporary or medically reversible adverse health consequences or where the probability of serious adverse health consequences is remote.

An outbreak of *E. coli* O157:H7 associated with alfalfa sprouts occurred during the summer of 2002 in San Luis Obispo County, California (FDA, 2002, Huff, 2002c). The victims, four women and one teenage girl, experienced mild symptoms with one of them hospitalized and all quickly recovering. Four of the victims were students at California Polytechnical State University and three had their meals at the university cafeteria. These infections were reported during the first days of August 2002 (Prado and Huff, 2002; Huff, 2002a,b,c; Stone, 2002; Wootson, 2002). The source of the outbreak was traced back to the sprouts by interviewing the victims with no lab report incriminating the sprouts (Huff, 2002c).

C.- Generalities about *Escherichia coli* O157:H7

Escherichia coli O157:H7 is a gram-negative, non-sporeforming, motile rod-shaped organism, possessing the ability to ferment lactose with production of gas within 48 hours at 35 °C and showing optimal growth at 30 to 42 °C (Hitchins et al., 1995; Doyle, 1997; Ryser, 1998). *E. coli* O157:H7, also called enterohemmorhagic *E. coli* (EHEC), presents some specific characteristics which allow for differentiation from most other *E. coli*. These features include the inability to grow well, if at all at > 44.5 °C, poor growth at < 10 °C, inability to ferment sorbitol within 24 h unlike 80 to 93% of other *E. coli* strains, inability to produce β-glucuronidase (i.e., inability to hydrolyze 4-methyl-umbelliferyl-D-glucuronide) contrarily to 92 to 96% of all other *E. coli* strains, possession of an attaching and effacing (*eae*) gene, carriage of a 60-Mda plasmid and expression of an uncommon 5000 to 8000-molecular-weight outer membrane protein (Hitchins et al., 1995; Doyle et al., 1997; Ryser, 1998).

E. coli O157:H7 shows an exceptional tolerance for acidic conditions. It has been found to survive in apple cider at pH 3.6 to 4.0 for 10 to 31 days and 2 to 3 days at 8 or 25 °C, respectively (Conner and Kotrola 1995; Doyle et al. 1997). Concentrations of up to 1.5% acetic, citric, or lactic acid used as antibacterial sprays on beef were not effective in reducing *E. coli* O157:H7 populations (Brackett et al., 1994; Doyle, 1997). Although not fully understood, the mechanism of acid tolerance seems to be related to a protein (s) that can be induced by preexposing the organism to acid conditions (Doyle, 1997). *E. coli* O157:H7 is also known for its ability to form homogeneous biofilms on sprouts and other plants (Fett 2000).

D.- Basis for laboratory identification of *E. coli* O157:H7

Differentiation of *E. coli* O157:H7 using Cefixime - Tellurite Sorbitol McConkey Agar (CT-SMAC) is based on the inability of this serovar to ferment d-sorbitol, unlike most other strains of *E. coli*. On Sorbitol McConkey agar (SMAC) non-sorbitol fermenting bacteria produce pale gray or colorless colonies compared to the bright pink-red colonies produced by sorbitol fermenters such as non-O157:H7 *E. coli* strains and other enterics (Hitchins et al. / FDA-BAM, 1995; Difco, 1998). Two antibiotics, cefixime and potassium tellurite (CT Supplement), (Dynal, Lake Success, NY) added to Sorbitol McConkey agar increase the selectivity of the medium for *E. coli* O157:H7 by suppressing the growth of normal flora (Hitchins et al. / FDA-BAM, 1995). Bile salts and crystal violet are selective agents that inhibit growth of gram-positive organisms (Difco, 1998).

2.2.2.- *SALMONELLA* TYPHIMURIUM DT104

A.- Health significance of *Salmonella* Typhimurium DT104

Strains of *Salmonella* that are resistant to antimicrobial agents have become a world-wide problem (Glynn et al., 1998). *Salmonella* subsp. *enterica* serovar Typhimurium Definitive Type104 is a non-enteric fever (non-typhoid) *Salmonella* strain featuring a rare multiantibiotic-resistant plasmid. In the US, it has shown resistance to 5 antibiotics: ampicillin, chloramphenicol, streptomycin, sulfonamides and tetracyclines. This penta-antibiotic resistance pattern is used as a screening method for *S.* Typhimurium DT104 with chloramphenicol-resistance being the most specific marker for this pattern of resistance. Moreover, in England such strains have developed additional resistances to trimethoprim (24%), fluoroquinolones and ciprofloxacin (14%) (Glynn et al. 1998). This resistance is believed to be induced by sub-therapeutic use of these drugs by veterinarians in animal rations (D'Aoust, 1989; Glynn et al 1998), with the antibiotic resistant strains being transmitted to humans by a very healthy population of animal carriers (D'Aoust, 1997).

In the US, prevalence of penta-resistant *S.* Typhimurium DT104 isolates increased from 0.6% in 1979-1980 to 34 % in 1996, with the highest prevalence seen in western states. In 1998, *S.* Typhimurium DT104 accounted for *ca.* 75% of the multiresistant *Salmonella* infections. The highest attack rate occurred among children, the elderly, immuno-compromised individuals and antibiotic users (Glynn et al.,1998). These authors suggested that infections caused by antibiotic resistant *S.* Typhimurium DT104 might be associated with greater morbidity and mortality than other *Salmonella* infections.

The contamination route is mostly fecal to oral (D'Aoust, 1989). Symptoms of *Salmonella* infection usually appear after 8-72 hours of incubation (D'Aoust, 1997; Ryser, 1998). The clinical manifestations of non-typhoid *Salmonella* infections in humans can range from mild and self-limited gastroenteritis to septicemia and death.

In mild cases, nausea and vomiting are the first signs of gastroenteritis or enterocolitis, also named " *Salmonella* food poisoning ". These symptoms generally subside within a few hours. Development of mild fever, chills, prostration, myalgia and abdominal pain sometimes resembling acute appendicitis, is soon followed by diarrhea, the most prominent symptom, which can range from a few loose stools to overtly bloody and rice-water cholera-like stools in more severe cases (D'Aoust, 1989 and 1997; Ryser, 1998). The clinical condition is generally self-limiting with remission of diarrhea and abdominal pain typically occurring without intervention within 5 days of the onset of the symptoms (D'Aoust, 1997; Ryser, 1998). Another public health concern at this stage is the shedding of *Salmonella* in infected patients' stools at concentrations of 10^6 to 10^9 CFU/g (Ryser, 1998). In the case of uncomplicated enterocolitis, supportive therapy such as fluid and electrolyte replacement is usually sufficient (D'Aoust, 1997; Ryser, 1998). Antibiotics are not recommended for gastroenteritis as they will also disturb or destroy the normal gut microflora which normally competes with *Salmonella* for nutrients and intestinal binding sites and produces bacteriocins that limit the growth or survival of *Salmonella*. Administration of antibiotics at this stage tends to prolong the asymptomatic carrier state and the intermittent excretion of salmonellae possessing greater antibiotic resistance (D'Aoust, 1989 and 1997; Ryser,1997).

S. Typhimurium DT 104 enterocolitis may also proceed to septicemia among pre-disposed individuals and precipitate chronic conditions. The population at risk encompasses infants, young children, the elderly, people with a disturbed intestinal microflora and patients with pre-existing physiological, anatomical or immunological disorders, such as cancer, liver disease, sickle-cell anemia, gastric disorders, gallblader diseases and AIDS (D'Aoust, 1989 and 1997; Ryser, 1998). The defense mechanism possessed by these patients is unable to counteract the effect of invasive *Salmonella* (D'Aoust, 1997). Fever is the primary symptom of *Salmonella* septicemia. At this stage the action of an efficient antibiotic is strongly recommended.

Unusually virulent cases of *S.* Typhimurium DT104 infection, and prolonged and untreated septicemia can lead to serious complications such as osteomyelitis, brain abscesses, meningitis and other neural infections, pneumonia, pyelonephritis, endocarditis, suppurative arthritis and splenomegaly (D'Aoust 1989, Ryser, 1998).

In England. *S.* Typhimurium DT104 leads to a high percentage of hospitalizations (41%) and fatalities (3%) compared to less than 3% hospitalizations and 0.1% fatalities for other strains of *Salmonella* (D'Aoust 1989). Evidence from outbreaks suggests that as few as 1 to 10 cells can constitute a sufficient infectious dose, particularly for populations at risk (D'Aoust, 1997).

Despite an obvious need for immunogenic preparations against non-typhoid salmonellae, the development of prophylactics is hampered by the multiplicity of serovars, the rapid succession of serovars in the human population and the unpredictable pathogenicity of infective strains (D'Aoust, 1997).

B.- Evidence of *S.* Typhimurium DT104 in fresh produce

Salmonella is widely known as a contaminant of fresh produce (Beuchat,1996). Fruits and vegetables are one of the reservoirs for non-typhoid salmonellae. Global export, questionable hygienic conditions during production, harvesting and distribution, irrigation water and untreated fertilizers, all increase the risk of contamination. Cross-contamination with other foods or from infected workers is also an important means of spreading *Salmonella*. Alfalfa sprouts have been incriminated in at least 14 *Salmonella* outbreaks since 1994 (Table 2.5).

In a bacteriological survey of sixty "health" foods in the Baltimore-Washington, D.C. area, Andrews et al. (1979) found *Salmonella poona* at a level of 6.3×10^4 CFU/g after sampling a brand of organically labeled alfalfa seeds.

An outbreak of *Salmonella* Bovismorbificans occurred in Sweden (103 cases) and Finland (210 cases) in March, 1994. From May through November 1994, 282 additional cases were recorded in Sweden. The bacterium was isolated from alfalfa sprouts but not from the implicated seeds which came from the same importer and, probably, the same lot originating from Australia (Pönkä et al., 1995; Taormina and Beuchat, 1999a).

Salmonella Stanley was responsible for an international sprout-related outbreak which peak occurred during May - June 1995, and involved alfalfa sprouts in Finland and 17 US states, including Michigan. Isolates from Finland and the United States yielded the same DNA profile and one particular antibiotic resistance pattern (resistance to trimethoprim-sulfamethoxazole, tetracycline, sulfisoxasole, streptomycin and kanamycin) (Jaquette, 1996; Mahon et al., 1997; Tauxe et al., 1997; FDA/NACMCF, 1999). The implicated sprouts, eaten by 50 patients in 6 states were traced through 9

TABLE 2.5.- SUMMARY OF *SALMONELLA* OUTBREAKS ASSOCIATED WITH RAW ALFALFA SPROUT CONSUMPTION

STRAINS INVOLVED	DATE AND LOCATION	REPORTED CASES	REMARKS	REFERENCES
S. Bovismorbificans	Sweden and Finland, 1994	385 cases (Sweden) 210 cases (Finland)	Organism isolated from sprouts but not from seeds	Pönkä et al., 1995 Taormina and Beuchat, 1999a
S. Stanley	Finland and 17 US states. May-June 1995	≥ 242 culture-confirmed cases	*S.* Stanley PFGE pattern indistinguishable in US and Finland oubreaks. Same Dutch seed shipper.	Jaquette et al., 1996 Tauxe et al., 1997 Mahon et al., 1997 FDA/NACMCF, 1999 Van Beneden et al., 1999 Taormina and Beuchat, 1999a Taormina et al., 1999
S. Newport	Oregon and British Columbia in late 1995 and early 1996. Georgia and Vermont in late 1995 Denmark in June, 1995	133 cases in Oregon and British Columbia	*S.* Newport isolated from the seeds. Same Dutch seed shipper as in S. Stanley outbreak.	Tauxe et al., 1997 Van Beneden et al., 1999 Taormina and Beuchat, 1999a FDA/NACMCF, 1999 Taormina et al., 1999
S. Montevideo	Nevada and California from May through July, 1996	~ 417 culture-confirmed cases	Same strain isolated from patients and sprouts. Pathogen not isolated from seeds.	FDA/NACMCF, 1999 Mohle-Boetani et al., 2001

TABLE 2.5.- CONT'D

STRAINS INVOLVED	DATE AND LOCATION	REPORTED CASES	REMARKS	REFERENCES
S. Meleagridis	Nevada and California from May through July, 1996	~ 75 culture-confirmed cases	Same strain isolated from patients and sprouts. Pathogen not isolated from seeds.	FDA/NACMCF, 1999 Mohle-Boetani et al., 2001
S. Meleagridis	Canada in October, 1997	78 cases		Taormina et al., 1999
S. Infantis and Anatum	Kansas and Missouri in 1997	109 culture-confirmed cases	*S.* Anatum and Infantis recovered from sprouts.	FDA/NACMCF, 1999
S. Senftenberg	Nevada and California from late 1997 through July, 1998	~ 52 to 60 cases	Alfalfa sprouts mixed with clover sprouts. Pathogen not recovered from seeds.	FDA/NACMCF, 1999 Taormina et al., 1999 Mohle-Boetani et al., 2001
S. Havana	Arizona and California in May, 1998	18 cases	Indistinguishable sprout and patient isolates	FDA/NACMCF, 1999 Taormina et al., 1999 Mohle-Boetani et al., 2001
S. Cubana	Arizona, California, Maryland, New-Mexico, Utah from May through August 1998	22 cases	Indistinguishable sprout and patient isolates	FDA/NACMCF, 1999 Taormina et al., 1999 Mohle-Boetani et al., 2001
S. Mbandaka	Oregon, Washington, Idaho, California in January through March 1999	75 cases	Pathogen isolated from seeds.	FDA/NACMCF, 1999

TABLE 2.5.- CONT'D

STRAINS INVOLVED	DATE AND LOCATION	REPORTED CASES	REMARKS	REFERENCES
S. paratyphi B var. *java*	Alberta, British Columbia, Saskatchewan in August - September 1999	51 cases		FDA/NACMCF, 1999 Taormina et al., 1999
S. Muenchen	Wisconsin, California, Idaho, Michigan, Missouri, Nevada, Washington	157 cases	Indistinguishable sprout and patient isolates. First documented outbreak occurring despite proper treatment with FDA recommended 2% $Ca(OCl)_2$ for 15 min.	Kuenn, 2000; Proctor et al., 2001
S. Kottbus	California, Colorado, New Mexico, Arizona	32 cases	S. Kottbus isolates with indistinguishable PFGE pattern for patients seeds and production environment	Mohle-Boetani et al. 2002
S. bovismorbificans	Washington, Oregon	12 cases	-	FDA, 2004

growers and one distributor, to one Dutch shipper. The Finnish sprouts were also traced to seeds from the same Dutch shipper (Tauxe et al., 1997; Mahon et al., 1997; Taormina and Beuchat, 1999a; FDA/NACMCF, 1999). Approximately 242 culture-confirmed cases were reported in this outbreak (Tauxe, 1997;Taormina et al., 1999). Based on the rate of underreporting from other *Salmonella* outbreaks, Mahon et al., (1997) estimated the actual number of cases at 5,000 to 24,000.

A *S.* Newport outbreak of approximately 133 cases, linked to alfalfa sprouts, was reported in Oregon, USA and British Columbia, Canada, in late 1995 and early 1996. The pathogen was isolated from both alfalfa seeds and sprouts. Using PFGE, isolates from the aforementioned outbreaks were indistinguishable from each other (Tauxe et al., 1997; Van Beneden et al., 1999; Taormina and Beuchat, 1999a; FDA/NACMCF, 1999) and from isolates from previous *S.* Newport outbreaks in late 1995 in Georgia and Vermont, and in June 1995 in Denmark (Taormina et al., 1999). This observation is coherent with the fact that these seeds came from the same Dutch shipper also involved in the *S.* Stanley outbreak (Tauxe et al., 1997; Van Beneden et al., 1999; Taormina and Beuchat, 1999a; FDA/NACMCF, 1999). Cultures of the implicated seeds yielded *S.* Newport (Taormina et al., 1999).

Nevada and California were sites of a series of infections involving *Salmonella* Montevideo and Meleagridis. In May through July 1996, ~ 500 culture-confirmed cases were recorded. A case-control study showed that consumption of alfalfa sprouts was associated with the outbreak. Moreover, the same strain of *S.* Meleagridis was isolated from patients and from sprouts obtained from retail stores and the sprouting facilities, although the seed samples did not yield either serotype (FDA/NACMCF, 1999). In

October, 1997, *S.* Meleagridis was again the cause of 78 cases of illness linked to consumption of alfalfa sprouts in Canada (Taormina et al., 1999).

In 1997, alfalfa sprouts were incriminated in an outbreak of *Salmonella* Infantis and *Salmonella* Anatum in Kansas and Missouri that resulted in 109 culture-confirmed cases. *S.* Anatum was recovered from the seeds, whereas the sprouts yielded both *Salmonella* serovars (FDA/NACMCF, 1999).

From late 1997 through July 1998, 52 to 60 culture-confirmed patients showing infections with the same strain of *Salmonella* Senftenberg were reported in the states of Nevada and California and linked to consumption of an alfalfa / clover sprout mixture. Cultures of clover and alfalfa seeds used to grow the implicated sprouts did not yield the pathogen (FDA/NACMCF, 1999; Taormina et al., 1999).

Alfalfa sprouts were again incriminated in an outbreak of 18 cases of *Salmonella* Havana in Arizona and California in May 1998 and, also, in an outbreak of 22 cases of *Salmonella* Cubana in Arizona, California, Maryland, New-Mexico and Utah during May to August 1998. The sprouts were grown from the same seed lot yielding *S.* Havana, *S.* Cubana and *S.* Tennessee which were indistinguishable from the patient isolates. These sprouts came from one large California producer who claimed to soak the seeds in 2,000 ppm chlorine for 30 minutes, followed by a 300 ppm chlorine soak for several hours before sprouting (FDA/NACMCF, 1999; Taormina et al., 1999).

Alfalfa sprouts were associated with about 75 cases of *Salmonella* Mbandaka infection in Oregon, Washington, Idaho and California during January through March 1999. The pathogen was isolated from the traced seed lot which was grown in California and distributed to the implicated sprout growing facilities (FDA/NACMCF, 1999).

Health Canada (2001) reported laboratory-confirmed infections of *Salmonella paratyphi* B var. *java* which were potentially linked to consumption of alfalfa sprouts during August and September of 1999, in Alberta (43 cases), British Columbia (6 cases) and Saskatchewan (2 cases).

During September of 1999, a multistate outbreak of *Salmonella* Muenchen associated with eating raw alfalfa sprouts was identified in Wisconsin (Kuenn, 2000; Proctor, 2001). In this state, 62 case patients were observed with six hospitalized and no deaths reported. Ultimately, 95 additional outbreak-related cases were identified in 6 states other than Wisconsin (California, 23; Idaho,11; Michigan, 35; Missouri, 19; Nevada, 5; Washington, 2). Due to underreporting, the total number of cases was estimated at 3,500 to 16,200. Dates of illness for the Wisconsin patients were between August 20 and October 20 and onsets of non-Wisconsin cases occurred between July 8 and November 29. This is the first specific documentation (signed FDA affidavit) of an outbreak occurring despite the incriminated sprouts having been grown from seeds that were previously sanitized with the recommended FDA 2% calcium hypochlorite treatment for 15 minutes. Trace back procedures indicated that all contaminated sprouts were grown from the same seed lot. *Salmonella* Muenchen isolates from the 157 patients and from intact packages of alfalfa sprouts grown from the traced lot of seed were indistinguishable by PFGE (Proctor et al., 2001). Following trace back after the Wisconsin outbreak, an FDA Class I recall (Table 2.6) was issued for 32,900 lb of alfalfa seeds.

During February to April 2001, *Salmonella* Kottbus, a relatively rare strain of *Salmonella*, was implicated in a multi-state oubreak (California, 24 cases; Arizona, 6

cases, Colorado, 1 case and New-Mexico, 1 case) linked to consumption of alfalfa sprouts produced at a single facility. The *S.* Kottbus isolates from patients, seeds and the production environment were indistinguishable by pulsed-field gel electrophoresis. Twenty-one (21) patients developed an acute diarrheal illness, three patients had urinary tract infections and three patients were hospitalized (Mohle-Boetani et al., 2002).

In early June 2004, 12 cases of *Salmonella bovismorbificans* infection reported in the states of Oregon and Washington have been possibly linked to consumption of raw alfalfa sprouts. Following this outbreak, an undetermined amount of sprouts have been recalled in Washington, Oregon and North California (FDA, 2004).

These numerous sprout contaminations by various *Salmonella* strains have led to at least 2 Class II and 18 Class I recalls from 1990 to date, involving more than 83,000 pounds of sprouts and 58,000 pounds of seeds (Table 2.6). These numbers give a realistic account of the acute financial and public health concerns that underlies the critical need to find a more reliable alternative to the currently recommended 20,000 ppm calcium hypochlorite treatment.

C.- Generalities about *Salmonella* Typhimurium DT104

Salmonella are facultatively anaerobic gram-negative rods belonging to the family of Enterobacteriaceae. These bacteria grow optimally at 37 °C and catabolize D-glucose and other carbohydrates with the production of acid and gas, but do not utilize lactose and sucrose. Salmonellae are oxidase negative and catalase positive, grow on citrate as the sole carbon source, generally produce hydrogen sulfide, decarboxylate lysine and ornithine, and do not hydrolize urea (D'Aoust, 1997).

TABLE 2.6.- RECALLS OF SPROUT PRODUCTS AND SPROUTING SEEDS LINKED TO CONTAMINATION BY *SALMONELLA*.

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
1/29/1991	12/19/1990	Class II # F-117/127-1	Various vegetables sprouts including: alfalfa sprouts, salad sprouts, clover sprouts, bean sprouts, radish sprouts, sunflower greens, sprouted lentils, sprouted peas, alfalfa/onion sprouts, alfalfa seeds, assorted seeds	~ 45,000 pounds distributed. ~22,500 pounds estimated remaining on the market at the time of recall.	Nationwide	*Salmonella*
7/23/1996	6/30/1996	Class II # F-620-6	Alfalfa sprouts	~ 640 pounds	Nevada, California	*Salmonella* Montevideo
10/29/1997 1/28/1998	6/2-3/1997	Class I # F-058/063-8 # corrected # F-058/064-8	Alfalfa sprouts, mung bean sprouts, adzuki/lentil/green pea sprouts, radish sprouts, alfalfa/radish sprouts, green pea/chinese red bean/lentil sprouts, snow pea sprouts	Firm estimates none remains on the market.	Kansas, Missouri	*Salmonella* Anatum and/or *Salmonella* Infantis. Sprouts associated with outbreak.
11/5/1997	2/25/1997 3/7/1997	Class I # F-084/085-8	Alfalfa sprouts, alfalfa/radish sprouts	Firm estimates none remains on the market.	California, Arizona, Utah Colorado, New Mexico	*Salmonella* Oranienburg

TABLE 2.6.- CONT'D

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
11/25/1998	7/10/1998	Class I # F-049/051-9	Alfalfa sprouts, alfalfa/clover sprouts, alfalfa/clover/radish sprouts, clover sprouts	Firm estimates none remains on the market.	California, Nevada	*Salmonella* jointly with *E. coli* O157:NM
3/3/1999	2/12/1999	Class I # F-212/214-9	Alfalfa sprouts, alfalfa/radish/clover sprouts, onion/alfalfa sprouts	~ 3,000 to 3,500 units of 1 pond plastic trays, 2 pound cardboard cases but mostly 6 oz container distributed every other day	Oregon, Washington, California, Idaho	*Salmonella* Sprouts associated with outbreak
7/14/1999	6/11/1999	Class I # F-545-9	Clover sprouts	Firm estimates none remains on the market.	Florida	*Salmonella* Typhimurium Seeds pertain to the same lot which was implicated in an outbreak in Colorado
8/24/1999	5/28-29/1999	Class I # F-651/653-9	Clover sprouts, alfalfa/clover sprouts, clover/radish sprouts	Undetermined	Colorado, Utah	*Salmonella* Typhimurium. Sprouts associated with outbreak in Colorado
8/24/1999	5/28/1999 6/1-2/1998	Class I # F-654/656-9	Clover sprouts, alfalfa/clover sprouts, clover/radish sprouts	9,480 pounds distributed	Colorado	*S.* Typhimurium. Sprouts associated with outbreak in Colorado

TABLE 2.6.-CONT'D.

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
3/8/2000	10/25/1999	Class I # F-160-0	Alfalfa seeds	12,500 pounds distributed	Nationwide	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin
3/8/2000	10/24/1999	Class I # F-161-0	Grain sprouting seeds	~ 150 ponds	Nationwide	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin
3/8/2000	10/29/1999	Class I # F-162-0	Alfalfa seeds	40 pounds distributed	Pennsylvania, Louisiana, Kentucky	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin
3/8/2000	10/23/1999	Class I # F-163-0	Alfalfa sprouts	504 pounds distributed	Ohio	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin
3/8/2000	10/22/1999	Class I # F-164-0	Alfalfa sprouts	320 4 oz containers	Mississippi	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin
3/28/00	10/28/1999	Class I # F-165-0	Alfalfa sprouts	~ 2,200 pounds distributed	Idaho	*Salmonella* Muenchen. Seeds associated with outbreak in Wisconsin

TABLE 2.6.-CONT'D.

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
3/28/00	10/30-31/1999	Class I # F-170-175-0	Alfalfa sprouts, spicy sprouts, alfalfa/onion sprouts, alfalfa/garlic sprouts, alfalfa/radish sprout, sprout salad	30,000 pounds distributed	Michigan, Ohio	Salmonella Muenchen. Seeds associated with outbreak in Wisconsin
7/26/2000	4/18-19/2000	Class I # F-619-0	Mung bean sprouts	Firm estimates none remains on the market	Nevada, Oregon	Salmonella enteritidis. Sprouts epidemiologic-ally linked with outbreak
6/4/2003	3/15/2003	Class I # F-381-3 #F-382-3 #F-383-3	Alfalfa /sweetpea/lentil/broccoli sprouts, onion sprout	8,000 pound distributed	Washington, Oregon, Arkansas Idaho	*Salmonella* Saint Paul
Not available	11/26/2003	Not available	Alfalfa sprouts, salad sprouts, alfalfa/onion sprouts	Not available	Washington, Oregon, Alaska, Idaho	Salmonella Six cases detected in Oregon by 11/26/2003
Not available	06/03-10/2004	Not available	Alfalfa sprouts, spicy sprout mix	Not available	Washington, Oregon, North California	Salmonella bovismorbificans 12 cases detected in Washington and Oregon

Source: FDA (2004) Enforcement reports

* Class I : a situation in which there is a reasonable probability that the use of, or exposure to, a violative product will cause serious adverse health consequences or death.

Class II : a situation in which the use of, or exposure to a violative product may cause temporary or medically reversible adverse health consequences or where the probability of serious adverse health consequences is remote

Salmonella adapts well to extreme environmental conditions and actively grows at temperatures as high as 54 °C. Some strains also exhibit psychotrophic properties , as reflected by their ability to grow in foods stored at 2 to 4 °C (D'Aoust, 1997). *Salmonella* can form homogeneous biofilms on sprouts (Korber et al., 1997; Fett, 2000).

D.- Basis for laboratory identification of *S.* Typhimurium DT104

The use of Xylose Lysine Desoxycholate agar (XLD) as a specific media to select for *Salmonella* is based on the following principles. Xylose is fermented by most enteric organisms except *Shigella* and *Providencia.* Addition of lysine in the medium formulation allows for differentiation of *Salmonella.* As xylose is exhausted, *Salmonella* decarboxylates lysine to cadaverine causing a reversion to alkaline conditions. Alkaline reversion by other lysine-positive organisms is prevented by excess acid production from fermentation of lactose and sucrose. Sodium thiosulfate and ferric ammonium citrate allow for visualization of hydrogen sulfide production under alkaline conditions. After growth on XLD agar, *S.* Typhimurium cells appear red with black centers due to hydrogen sulfide production. Moreover, sodium desoxycholate inhibits the growth of gram-positive organisms (D'Aoust, 1997; Difco, 1998).

2.2.3.- *LISTERIA MONOCYTOGENES*

A.- Health significance of Listeria monocytogenes

Listeria monocytogenes emerged as one of the major foodborne pathogen during the 1980's with the first confirmed foodborne outbreak of listeriosis occurring in 1981, in Nova-Scotia, Canada (Schlech et al., 1983; Rocourt and Cossart, 1997). Listeriosis is a

major public health concern and may be the leading fatal foodborne disease in the United States (Farber and Peterkin, 1991; Fisher et al., 2000). Despite the availability of suitable antibiotic therapy, the treatment of listeriosis is complicated by growth of *L. monocytogenes* as an intracellular pathogen within macrophage cells of the spleen and liver with many antibiotics unable to effectively penetrate the blood-brain barrier (Ryser, 1998). The highly significant case-fatality rate (~ 20%), the severity of the illness and severe complications make listeriosis the second most costly foodborne disease in the United States (Rocourt and Cossart, 1997; Ryser, 1998).

Development of listeriosis in humans is affected by host susceptibility, gastric acidity, inoculum size, the strain of *L. monocytogenes* and various virulence factors of the organism (Ryser, 1998). Host susceptibility is a determinant factor in *Listeria* infections. Although *Listeria* is a ubiquitous pathogen, healthy individuals seldom acquire listeriosis (Ryser, 1998). Ingestion of food containing $\geq 10^2$ CFU/g poses a significant health risk for susceptible individuals including pregnant women and newborn infants, the elderly, immunocompromised adults including cancer patients, transplant patients under cortico-steroids, human immunodeficiency virus (HIV)-positive individuals and acquired immunodeficiency syndrome (AIDS) patients and also patients with underlying illnesses, such as heart disease, diabetes, cirrhosis of the liver and alcoholism (Rocourt and Cossart, 1997; Ryser, 1998)

L. monocytogenes produces very serious systemic infections in this high risk population. In non-pregnant adults, the bacterium has a particular tropism for the central nervous system but some cases of mild gastrointestinal illness have been observed (Rocourt and Cossart, 1997). In immunocompromised adults, *Listeria* can cause

meningitis, encephalitis and septicemia (Ryser, 1998). After an initial incubation period of 2 days to 3 months, the symptoms develop suddenly with severe headache, dizziness, stiff neck or back, incoordination and other disturbances of the central nervous system most often observed (Ryser, 1998). The prognosis of listeriosis depends on both the type of infection and the underlying conditions (Rocourt and Cossart, 1997). A favorable prognosis is influenced by a rapid diagnosis and appropriate antibiotic therapy (Ryser, 1998). Without proper treatment about 20 % of those infected will die (38 to 40% among immunocompromised adults, the elderly and patients with other predisposing conditions), with some survivors developing permanent neurologic complications (Rocourt and Cossart, 1997; Ryser, 1998). Oral administration of large doses of ampicillin or penicillin together with an aminoglycoside for 2 to 4 weeks is the recommended treatment.

Pregnant women are most frequently affected in the third trimester of pregnancy (Rocourt and Cossart, 1997; Ryser, 1998). In this group, *Listeria* infection may be either asymptomatic (Rocourt and Cossart, 1997) or flu-like leading to infection of the fetus. These patients experience symptoms such as sudden chills, fever, sore throat, headache, dizziness, myalgia, lower back pain, discolored urine and occasionally diarrhea. Pregnant women almost invariably recover without complications (Rocourt and Cossart, 1997; Ryser, 1998).

Infection of the fetus or the infant carries more serious consequences. Contamination from the mother can result in abortion, fetal death, stillbirth or the premature delivery of an infant with neonatal septicemia, a severe infection of the respiratory, circulatory and central nervous systems that can either terminate fatally or lead to permanent retardation (Rocourt and Cossart, 1997; Ryser, 1998).

The possibility that cold and prolonged storage may enhance virulence of some *L. monocytogenes* strains (Lou and Yousef, 1999) increases the acuteness of this public health problem. Finding *L. monocytogenes* in a ready-to-eat food is grounds for immediate rejection, as the US has adopted a policy of "zero tolerance" for this pathogen in all RTE or cooked foods (Ryser, 1998).

B.- Evidence of *L. monocytogenes* in fresh produce

Listeria monocytogenes is widely distributed on plant vegetation (Beuchat, 1996). Fruits and vegetables can be contaminated by soil, water, manure, decaying vegetation and fertilization with effluents from sewage treatment plants containing the pathogen (Al-Ghazali and Al-Azawi 1990; Reina et al., 1995; Rocourt and Cossart, 1997). The aforementioned 1981 listeriosis outbreak in Canada, with 41 cases reported, was linked to consumption of coleslaw prepared from cabbage fertilized with manure from a flock of *Listeria*-infected sheep (Schlech et al., 1983; Rocourt and Cossart, 1997). Fresh produce can also become contaminated during harvest and further processing (Zhang and Farber 1996).

This pathogen has been isolated from fresh cut vegetables and, being a "hardy" organism, survives well on produce (Zhang and Farber 1996). As a psychotrophic organism that can grow at refrigeration temperature, this pathogen can develop on refrigerated foods (Lou and Yousef, 1999). Carlin and Nguyen-the (1994) and Beuchat (1996) reported that *L. monocytogenes* grew on lettuce and endive during storage at 10 °C. Indeed, *Listeria* spp. has been found on a wide range of retail fruits and vegetables, including potatoes, radishes, cabbage, cucumbers, mushrooms and lettuce (Heisick et al.,

1989; Reina et al., 1995; Rocourt and Cossart, 1997). Raw celery, tomatoes and lettuce were incriminated in a listeriosis outbreak involving 23 patients from eight Boston hospitals in 1979 (Ho et al. 1986; Beuchat, 1996).

No *L. monocytogenes* outbreaks has thus far been linked to consumption of alfalfa sprouts. However, this pathogen have been detected on various types of sprouts, leading to two Class I recalls, in 1998 and 2002 (Table 2.7), as recorded in FDA enforcement reports (FDA, 2004).

C.- Generalities about *Listeria monocytogenes*

Listeria monocytogenes is a ubiquitous, gram-positive, non-spore-forming, aerobic to facultatively anaerobic, short diphteroid-like, rod-shaped bacterium (Ryser, 1998; Fisher, 2000). Biochemically, *Listeria* species produce catalase, ferment glucose to acid without gas and hydrolize esculin. Typical *L. monocytogenes* isolates ferment rhamnose but not xylose and are weakly β-hemolytic (Ryser, 1998). Hemolysis is used to differentiate between *L. monocytogenes* and *L. inocua,* the most frequently encountered non-pathogenic *Listeria* specie (Rocourt and Cossart, 1997). When grown at room temperature, *L. monocytogenes* exhibits a characteristic tumbling motility when viewed under the microscope (Ryser, 1998).

In general, temperatures > 50 °C are lethal to *L. monocytogenes*. In a suitable laboratory media, this pathogen will grow at 0 to 45 °C with optimal growth occurring at 30 to 38 °C. Frozen storage at temperatures as low as -18 °C, and even repeated freezing

TABLE 2.7.- RECALLS OF SPROUT PRODUCTS LINKED TO CONTAMINATION BY *L. MONOCYTOGENES*.

FDA ENFORCEMENT REPORT DATE	RECALL DATE	RECALL CLASS* AND NUMBER	RECALLED PRODUCT	QUANTITY	DISTRIBUTION	CONTAMINATING PATHOGEN
2/17/1999	9/4-5/1998	Class I # F-153/163-9	Alfalfa sprouts, clover sprouts, alfalfa/radish/wheat sprouts, alfalfa/dill sprouts, cabbage/fenugreek/clover sprouts, alfalfa/onion/mustard sprouts, onion/clover sprouts, alfalfa/radish sprouts, clover/radish sprouts, alfalfa/onion sprouts, radish sprouts,	Firm estimates none remains on the market	Illinois, Indiana, Michigan, Ohio, Wisconsin	*L. monocytogenes*
2/19/2003	11/8/2002	Class I # F-204-3	Alfalfa sprouts, clover sprouts,	6,780 4 oz-containers	Alabama, Tennessee, Georgia, Kentucky	*L. monocytogenes*

Source: FDA (2004) Enforcement reports

* Class I : a situation in which there is a reasonable probability that the use of, or exposure to, a violative product will cause serious adverse health consequences or death.
Class II : a situation in which the use of, or exposure to a violative product may cause temporary or medically reversible adverse health consequences or where the probability of serious adverse health consequences is remote.

and thawing have little negative impact, and these conditions are more likely to injure than inactivate *L. monocytogenes,* with cold storage possibly enhancing virulence of some strains isolated from refrigerated foods (Rocourt and Cossart, 1997; Lou and Yousef, 1999).

L. monocytogenes is also acid tolerant and able to grow at pH 4.3 -10. This bacterium exhibits a high salt tolerance, being able to grow in the presence of 10% salt and survive in a 23% brine solution (Rocourt and Cossart, 1997, Ryser 1998) and persist at nitrite concentrations allowed in foods (Rocourt and Cossart, 1997).

D.- Basis for laboratory identification of *L. monocytogenes*

Modified Oxford Agar (MOX), one of the most popular media for selective isolation of *Listeria* spp, is prepared by adding Bacto Modified Oxford Antimicrobic Supplement to Bacto Oxford Medium Base. The principle underlying the selectivity action of MOX is the hydrolysis of esculin by all *Listeria* species. The reaction between ferric ions present in MOX as ferric ammonium citrate, and the 6,7-dihydroxycoumarin produced by hydrolysis of esculin results in black colonies surrounded by a black halo (Difco, 1998). Lithium chloride is added to this medium to inhibit the growth of enterocci. Modified Oxford Antimicrobic Supplement contains moxalactam and colistin methane sulfonate or colistin sulfate. These antibiotics increase the selectivity of the medium by completely inhibiting gram-negative organisms and most gram-positive organisms after 24 hours of incubation (Difco, 1998).

2.3.- BACTERICIDAL AGENTS

Disinfection of seeds prior to germination is a possible method of eliminating pathogens, even though, it may not necessarily affect the final count on sprouts (Piernas and Guiraud, 1997). A major problem with disinfectants, in the case of ready to eat foods, is the retention of toxic residues. Since germination requires that seeds be frequently rinsed with water, such rinses could reduce toxic residues, thus allowing higher concentrations of sanitizer to be used (Piernas and Guiraud, 1997). However, as low levels of pathogens on seeds can grow to high numbers during sprouting, disinfection of the mature sprouts must also be considered.

2.3.1.- CLOROX ™ / SODIUM HYPOCHLORITE (NACLO)

A.- General characteristics

Chlorine is a well-known and potent microbicide (Reina et al., 1995) that is extensively used for treating water. Sodium hypochlorite (NaClO) is the active ingredient of Clorox™ which is a disinfectant as well as a bleaching and oxidizing agent. Sodium hypochlorite is available in either powder or liquid formulations.

When chlorine is added as calcium or sodium hypochlorite to water, a mixture of hypochlorous (HOCl) and hydrochloric acid is formed. HOCl has the highest oxidation potential of all chlorine species formed (Wei et al.,1985; Dychdala, 1991; Ong et al., 1996). These authors have shown that the percentage of HOCl, in solution, is greatest at low pH and decreases as the pH and temperature of the solution increase.

Environmental health communities have expressed concerns about residual chlorine by-products and the fact that they are returned to the environment through

wastewater (Xu, 1999). Important by-products found in chlorinated drinking water include chloroform, bromodichloromethane and MX [3-chloro-4-(dichloromethyl)-5-hydroxy-2(5H)-furanone] (Richardson et al. 1998). Trihalomethane formed in foods as a result of chlorination is carcinogenic (Wei et al., 1985; Kim et al., 1999). The produce industry is concerned about the possibility of future regulatory constraints on the use of chlorine-based sanitizing agents. Chlorine, even when used at a low concentration, may cause taste and odor defects in treated products (Kim et al., 1999).

B.- Mechanism of action

The mechanism of action of chlorine is not completely understood (Dychdala, 1991). It is suggested that hypochlorous acid is responsible for the antimicrobial potency of sodium hypochlorite:

$$NaClO + H_2O \rightarrow Na^+ + OCl^- + H2O$$

$$NaClO + H_2O \rightarrow NaOH + HOCL$$

$$HOCl \Leftrightarrow H^+ + OCl^-$$

The dissociation of hypochlorous acid, which depends on the pH and equilibrium between HOCL and OCL^-, is maintained even though HOCL is constantly consumed through its germicidal function (Dychdala, 1991). Several theories have been proposed to explain the mechanistic action of chlorine. It is generally believed that chlorine inhibits essential cell enzyme systems through oxidation (Dychdala, 1991; Alasri et al., 1992). HOCl liberates nascent oxygen, which in turn, combines with components of cytoplasm to destroy the cell. According to another theory, chlorine destroys bacteria by combining

with cell membrane proteins, forming N-chloro compounds, which change the cell membranes to allow the cell contents to diffuse outward (Dychdala, 1991).

C.- Antimicrobial performance

Low concentrations of chlorine are used to sanitize fresh produce. However, chlorine has a limited effectiveness in killing bacteria on fruit and vegetables surfaces due to its inactivation by other organic materials (Xu, 1999; Mari et al., 1999), with maximum reductions of 2 - 3 logs being reported (Park et al.,1991; Sapers, 1998; Xu, 1999).

Chlorine solutions prepared by adding appropriate amounts of 5% sodium hypochlorite stock solution to 0.05 M potassium phosphate buffer (pH 6.8) were used at 21 °C, by Jaquette et al. (1996), to reduce populations of *Salmonella* Stanley on alfalfa seeds. Dipping alfalfa seeds containing an initial population of 339 CFU/g for 10 minutes in a 100 ppm chlorine solution reduced the population to 197 CFU/g. Dipping in 290 ppm chlorine further reduced the population to 99 CFU/g. Dipping in 480 ppm caused a reduction to 64 CFU/g and a 1,010 ppm treatment reduced the microbial load to 37 CFU/g which means that no further appreciable reductions were found between 290 and 1,010 ppm chlorine. Jaquette et al. (1996) needed to use concentrations containing 2,040 and 3,990 ppm free chlorine to decrease a *Salmonella* Stanley population of 65 CFU/g to an undetectable level when analyzed by direct plating. However, these samples were not enriched. Jaquette et al. (1996) also reported a 1995 personal communication by D. Caudill stating that treatment of alfalfa seeds with up to 5,000 ppm chlorine did not substantially reduce seed viability. Jaquette et al. (1996) have suggested a 2,000 to 4,000 ppm chlorine treatment to reduce *Salmonella* populations on alfalfa seeds while not

adversely affecting germination. However, these authors also warned that because of the dramatic increase which occurs during sprouting, even 2,000 to 4,000 ppm chlorine can not guarantee that sprouts will be free of *Salmonella*.

Beuchat (1997) reported that *Salmonella* populations decreased from 3.9 logs to <1 CFU/g after soaking seeds for 30 seconds in 1,800 ppm $Ca(ClO)_2$ or 2,000 ppm NaClO. Piernas and Guiraud (1997) found that adding active chlorine as sodium hypochlorite to the wash water had little effect on the bacterial load of rice seeds until 1000 ppm, when aerobic plate counts were reduced by 2 to 3 logs over 20 minutes. Weissinger and Beuchat (2000) reported a 0.33 log reduction in *Salmonella,* after a 10 minute exposure to 200 ppm NaClO and a 0.72 log reduction using 2,000 ppm of NaClO. These authors indicated that chemical sanitizers, other than 20,000 ppm of chlorine, can be used to achieve similar reductions in populations of *Salmonella* without reducing the germination rate.

In the multi-state, February-April, 2001 *Salmonella kottbus* outbreak, the grower indicated that heated seeds were subjected to a 2,000 ppm sodium hypochlorite soak for 15 minutes The outbreak suggests that the aforementioned technique was inadequate to eliminate *Salmonella* from the seeds (Mohle-Boetani et al., 2002).

2.3.2.- TSUNAMI / PEROXYACETIC ACID

A.- General characteristics

Tsunami™ is the brand name of a commercial sanitizing solution that contains a combination of peracetic acid and hydrogen peroxide. This product is mainly used as sanitizer and oxidizing agent.

Peracetic acid (PAA) shows better stability than chlorine dioxide and acts faster as a biocide with its action not pH dependent. Low temperatures decrease the sanitizing effectiveness of PAA (Mari et al 1999). The toxicity level of PAA is lower than ClO_2 with PAA exhibiting an LD_{50} in rats of 1,540 mg/kg. The corrosiveness of PAA on stainless steel can be alleviated by using a commercial formulation containing a lower concentration of PAA without sulfuric acid (Mari et al., 1999).

B.- Mechanism of action

The mechanism of action of peracetic acid is covered in more detail in section 2.3.6.3. After contact with organic substrates, peracetic acid decomposes to yield oxygen and acetic acid (Doores, 1983). The antibacterial action of acetic acid is partially due to the lowering of pH below the optimum level for growth. Indeed, proteins, nucleic acids, phospholipids can be structurally altered by pH changes (Doores, 1983).

C.- Antimicrobial performance

Various concentrations of PAA were tested by Mari et al (1999) for their fungistatic effect on fruits and vegetables and may be used as references to evaluate the

bacteriostatic effect of PAA. The nature and pH of the fruit surface influence the decomposition rate of PAA and may explain differences observed in the antimicrobial action of PAA (Mari et al. 1999).

Two concentrations of Tsunami™ (40 and 80 ppm) were applied to alfalfa seeds as 20 minute soaking treatments and also sprayed at regular intervals during sprouting and subsequent storage of the sprouts. Using 80 ppm Tsunami™, a 1.61 log reduction in numbers of *E. coli* O157:H7 was observed after a 20 minute soak in 80 ppm Tsunami™. The antimicrobial action of Tsunami™ 40 or 80 ppm did not lead to reductions >1 log. Thus, treatment with Tsunami™ can not guarantee the safety of sprouts (Taormina and Beuchat, 1999a,b).

2.3.3.- VEGI CLEAN™ / ACID-ANIONIC SURFACTANT

A.- General characteristics

Vegi-Clean™ is the brand name for a chemical sanitizer manufactured by Microcide, Inc. (Detroit, MI) under US Patent # 5,143,720 and 5,280,042. This highly water soluble white powder is intended as a sanitizer for fruits and vegetables. Active ingredients in Vegi-Clean™ include citric acid, sodium acid phosphate and sodium dodecyl benzene sulfonate with the latter acting as an anionic surfactant. Acid-anionic surfactants combine the advantages of strong bactericidal action and low toxicity. The antimicrobial properties of acid-anionic surfactants are better expressed at low pH (pH 2 to 3) (Dychdala and Lopes, 1991).

B.- Mechanism of action

The mechanism of action of acid-anionic surfactants has not yet been fully elucidated (Dychdala and Lopes, 1991). However, these sanitizers may function by inhibiting key enzymes, disorganizing the cell membrane, interrupting cellular transport and/or denaturating cellular proteins (Dychdala and Lopes, 1991).

C.- Antimicrobial performance

The efficacy of 1 and 2% Vegi-Clean™ during sprouting of alfalfa seeds and storage of the sprouts was studied by Taormina and Beuchat (1999b). Seeds were soaked for 20 minutes in Vegi-Clean™ which was, then, sprayed at regular intervals on the germinating and growing sprouts. Although populations of *E. coli* O157:H7 decreased 0.67 logs after spraying, this treatment was unable to eliminate the pathogen. Other than the aforementioned germination step, spraying of Vegi-Clean™ during sprout production and storage failed to significantly reduce the pathogen load.

2.3.4.- COPPER ION SOLUTION

A.- General characteristics

Copper is well known, for centuries, for its antimicrobial properties (Yeager, 1991). Copper ions generated through an electrolytic process can be dispersed into a circulating water stream with this copper ion solution intended as a disinfectant. As stated by copper ion system manufacturers, copper ion water is environmentally safe, user friendly and cost effective. These previously mentioned systems are also registered, as

meeting all requirements, with the Environmental Protection Agency (Superior Water Solutions, Inc., advertising booklet).

B.- Mechanism of action

Copper ion water is believed to form electrostatic bonds between positively charged copper ions and negatively charged sites on the bacterial cell surface. Reactions at the cell surface allow the metal ion to penetrate the cell membrane and induce copper toxicity (Superior Water Solutions Inc., advertising booklet).

In 1989, Yayha et al. acknowledged that the bactericidal mechanism of copper ion was not fully understood. At that time, copper ion was assumed to bind to the sulfhydril groups of respiratory enzymes and therefore, impair respiratory activity. It is also believed that copper, like other metals, can take up key functional thiol groups from enzymes (Lukens, 1991).

Years later, these assumptions have been supported by Riggle and Kunamoto (2000) who reported that copper, an essential cofactor for enzymes, is implicated in respiration, destruction of free radicals, iron homeostasis and neurological development. Nevertheless, copper, at excess levels can induce a high toxicity by generating reactive oxygen species via the Fenton reaction, disrupting metal ion binding and homeostasis and binding macromolecules, such as proteins. Like other prokaryotes, bacteria resist copper toxicity by reducing influx and/or utilizing efflux mechanisms by way of ATPase transporters to control intracellular copper levels.

C.- Antimicrobial performance

As a bactericide, copper ion acts slower than chlorine. However, copper appears to injure coliforms more effectively than chlorine, requiring longer recovery times (Yayha et al., 1989). Concentrations of 250 ug/L can induce 90% injury in *E. coli*, in less than 24 hours at 4 °C with more rapid injury occuring at room temperature. (Yayha et al., 1989). Landeen et al. (1989) reported a 3.3 log reduction in *E. coli* after a 1 hour exposure to 302 μg/L of copper ions. The same authors also reported that a contact time of at least 24 hours was necessary for a solution containing 400 μg/L and 40 μg/L of copper and silver ion, respectively, to achieve a 3 log reduction in numbers of *Legionella pneumophila.* In contrast, a solution of 0.4 mg/L of free chlorine was able to achieve a 2.6 log reduction in 1.5 minutes.

No studies assessing the efficacy of copper ion water solution for seed or sprout disinfection have appeared in the literature. Nevertheless, Rodrick and Hultstrand in a November 1998 correspondence to Superior Aqua Enterprises Inc., acknowledged a 2 log reduction in total surface bacteria and at least a 1 log reduction in yeasts and molds on tomatoes, after a 5 minute exposure to a copper ion concentration of 0.5 - 1.0 mg /L. Superior Water Solutions Inc. claims reductions of ~ 3.5 logs for microorganisms on raw fruits and vegetables when their Superior Aqua Agriculture System was used in fruit and vegetable packing stations. A letter by Debra A. Sanders to James Mulha of Superior Aqua Enterprises Inc. reports an experiment involving the washing of scallions containing 1 x 10^5 CFU/g of *E. coli*. The scallions were dipped in an aqueous solution 1 ppm copper ion produced by a Superior Aqua Agricultural System installed on a

recirculated tank. The microbial load fell to less than 100 CFU/g which means approximately a 3.5 log reduction.

Yayha et al.(1989) reported a reduction of only 0.12 log for *E.coli* in an aqueous model system after 6 minutes of exposure to a copper-silver ion solution having a concentration of 433ug/L : 43ug/L. In a similar model system, these authors observed a 2.8 log reduction in *E.coli,* after 0.5 minutes, using 0.20 mg/ L of free chlorine and a 3.5 log decrease in 0.5 minutes when the previously mentioned concentration of copper-silver ion and free chlorine were combined. Thus, the combination treatment decreased *E. coli* populations an additional 0.7 log. Inactivation by combined copper-silver ion is relatively slow compared with to free chlorine (Yahya et al., 1989). However, when these metal ions are added to low levels of free chlorine, inactivation rates for indicator organisms, usually used to judge water quality were greater than those for free chlorine alone, at comparable levels. In the letter previously mentioned, Debra Sanders also suggested that the use of chlorine in conjunction with Superior Aqua Agricultural System achieved additional reductions in microorganisms, with the work of Landeen et al. (1989) supporting this observation. With the aqueous model system previously mentioned, these authors reported a 3.7 log reduction in *Legionella pneumophila* after 1.5 minutes when 0.4 mg/L of free chlorine was combined with a 400 ug and 40 ug copper and silver ion solution, compared to a reduction of 2.6 log using free chlorine alone and 3 logs, using copper and silver ion alone.

2.3.5.- SONICATION

A.- Characterization of the technique

Sonication is a physical technique involving the use of ultrasound. Shukla (1992) and Lillard (1994) indicated that the active force in microwave ultrasonics (sound waves pitched above the level of human hearing at 16 kHz or 16,000 cycles/sec) is mechanical as opposed to heat in microwave heating. Large stresses, strains and high shear forces are induced during cavitation when high frequency vibrations result in alternate compressions and expansions of microscopic bubbles which implode violently, releasing large amounts of energy and generating high temperatures and pressures.

B.- Mechanism of action

Cavitation is the mechanical effect responsible for the destruction of bacterial cells. The magnitude of ultrasonic waves is sufficiently high to produce protein breakdown and hydrolysis, simple cell lysis and protein particulation (Shukla, 1992).

Sonication can be used to break up bacterial cell clumps and to dislodge bacteria from solid surfaces and possibly from the surface of fruits and vegetables. Sonication at higher magnitudes plays a major role in disrupting the bacterial cell wall. Hence, the aforementioned effects may help to improve the usefulness of sanitizers.

C.- Antimicrobial performances of sonication

Lillard (1993) showed that bactericides lethal to salmonellae in processing water, do not access bacteria that are firmly attached or entrapped. Bacteria are not easily removed from poultry skin. Sonication of poultry skin for 15 or 30 minutes in a chlorine

solution containing 0.5 ppm free residual chlorine increased the numbers of dislodged bacteria and reduced the microbial load by 2.44 to 3.93 logs. This author also reported that ultrasound in combination with chlorine is more effective in reducing bacterial counts than either ultrasound or chlorine alone.

Sonication was used by Walker et al. (1998) in combination with other techniques to recover bacteria from peas and beans. Sonication from a 40 kHz ultrasonic generator also was used by Burleson et al. (1975) alone or in combination with ozone or oxygen against *Salmonella* Typhimurium and enteropathogenic *E. coli* O126:B16 suspended in phosphate buffer solution or in a secondary effluent from a wastewater treatment plant.

Lillard (1993) reported experiments involving the use of sonication to reduce microbial populations in milk. Cell destruction occured at high frequencies, whereas increases in total counts were observed at low frequencies and were probably due to declumping of bacteria normally found in milk. Ultrasonic waves (80 kHz/sec) were reported to remove most bacteria from milk films on metal surfaces.

2.3.6.- NOVEL FDA-APPROVED FATTY ACID-BASED SANITIZER

A.- Characterization of the product

The major antimicrobial product examined in this study is a novel FDA-approved sanitizer concentrate which is diluted 1:200 in water so as to contain 250 ppm peroxyacid, 1000 ppm fatty acid (caprylic and capric acids), 500 ppm glycerol monolaurate and 1000 ppm lactic acid as active ingredients.

This fatty acid-based sanitizer contains peroxyacetic acid conjugated to peroxycaprylic and peroxycapric acids (Guthery, 2001). Peracetic acid is bactericidal at fairly high concentrations, generally greater than 100 ppm. Similarly, peroxyfatty acids are also biocidal at concentrations greater than 200 ppm (Oakes et al., 1993). The combination of these acids produces a synergistic effect, providing a much more potent biocide than that obtained when these components are used separately and offers the unique advantage of having antimicrobial or biocidal activity at substantially lower concentrations (Oakes et al., 1993). These lower concentrations minimize cost, odor and potential toxic effects to the user. An effective antimicrobial solution is formed when the concentrate is diluted in water at a pH of 2 to 8. At pH < 5, peroxyacids, including peroxyfatty acids, are very potent biocides at low levels. This solution may be used at temperatures ranging from 4 to 60 °C (Oakes et al., 1993).

Peroxyacetic acid is obtained after reacting glacial acetic acid with hydrogen peroxide (50%) overnight, in the dark (Guthery, 2001). It is formed when an oxygen molecule is bound to the carboxyl atom of acetic acid (Doores, 1983). Peroxyacetic acid is reportedly superior to other forms used in this type of sanitizer (Oakes et al., 1993). Acetic acid is a monocarboxylic acid with a pungent odor and taste which limits its use. It is the principal component of vinegar and is highly soluble in water (Doores, 1983). Acetic acid is generally regarded as safe (GRAS) for miscellaneous and general purpose use (21 CFR 182.1005). The acceptable daily intake for human consumption is not limited (Doores, 1983). Peracetic acid is effective as a biocide in aqueous solutions. It is not time dependent, making it a fast disinfectant (Alasri et al., 1992).

Peroxyfatty acids are formed by reacting hydrogen peroxide (50% v/v) with a mixture of 60% caprylic acid and 40% capric acid, commercially available under the brand name Emery 658 (Guthery, 2001). Caprylic acid (octanoic, C_8) and capric acid (decanoic, C_{10}) are structurally represented by the formula R_1-CO_3H. These linear, monoperoxy aliphatic fatty acids are particularly appropriate and preferred among other eligible fatty acids for this type of sanitizer (Oakes et al., 1993). Caprylic acid is a colorless liquid which has a slightly unpleasant odor and a burning rancid taste. It is slightly soluble in water and has a pk_a of 4.89. The mouse LD_{50} mouse is estimated at 600 mg/kg of body weight after intravenous administration. It is approved as a GRAS substance for miscellaneous and general purpose use. Caprylic acid may be used to flavor many foods at levels ranging from 0.001% to 0.16% (21 CFR 184.1025). It is also approved as an antimicrobial agent for indirect use in cheese wraps (21 CFR 186.1025) (Doores, 1983).

The diluted sanitizer contains an equilibrium mixture of hydrogen peroxide, peroxyacetic acid, peroxycaprylic acid, peroxycapric acid, free acetic acid, free caprylic and free capric acid and water. Moreover, the sanitizer also contains glycerol monolaurate, lactic acid and some additional function enhancing components with sulfuric acid added as a catalyst (Guthery, 2002).

Hydrogen peroxide, generally used as an antiseptic in dilute solutions (Baldry, 1983; Alasri et al.,1992) is GRAS-approved for use at concentrations of 0.05% as an antimicrobial agent in cheese-making and whey processing (Davidson et al. 1983; Venkitanarayanan et al., 1999). However, the actual concentration of hydrogen peroxide

present in this sanitizer, as a result of the equilibrium reaction, falls below the level where microbicidal action could be expected (Guthery 2002).

Glycerol monolaurate, also called monolaurin and sold under the brand name Lauricidin® (Lauricidin, Inc., Okemos, MI), is the monoacyl derivative resulting from esterification of lauric acid to glycerol. This food-grade monoester is approved in the United States as a food emulsifier by the FDA (21 CFR GRAS 182.4505). In addition to its emulsification properties, glycerol monolaurate also possess antimicrobial activity which has been extensively investigated (Oh and Marshall 1992, 1993; Kabara, 1972, 1975, 1979; Kabara et al., 1977). The addition of an acidulant can increase the antimicrobial spectrum and activity of glycerol monolaurate (Oh and Marshall, 1992).

Lactic acid (pk_a, 3.8) is a hygroscopic, syrupy liquid having a moderately strong acid taste. It is one of the most widely distributed acids in nature as it is the primary acid produced during natural fermentation (Doores, 1983 and 1993). Lactic acid is GRAS approved (21 CFR 182.1061) for miscellaneous and general purpose (Doores, 1983, 1993; Anderson and Marshall,1990; Greer and Dilts, 1992). The oral LD_{50} for rats and guinea pigs is, respectively, 3,730 and 1,810 mg/kg of body weight. The acceptable human daily intake is unlimited (Doores, 1983). Lactic acid is inhibitory to various pathogenic and spoilage organisms (Anderson and Marshall,1990; Greer and Dilts, 1992). The antimicrobial activity of organic acids can be increased or potentiated when combined with other food preservatives (Bøgh-Sørensen 1994; Venkitanayaranan et al., 1999; Marshall and Kim, 1996).

Other components can be added to this sanitizer, such as hydrotrope coupling agents, stabilizers and chelating agents (Oakes et al., 1993). The hydrotrope solubilizer

acts to solubilize the fatty acids in both the concentrate and the ready-to-use solution (Oakes et al., 1993). Propylene glycol is present as a solvent in this sanitizer. Although propylene glycol can exhibit antimicrobial properties at concentrations >70%, the concentration used in this sanitizer (40%) is too low for this purpose (Guthery, 2002, personal communication).

The components of this FDA-approved fatty acid-based sanitizer are characterized as GRAS. Fatty acids and their esters are naturally occurring substances in foods and are non-toxic, unless used at "heroic doses" (sic. Kabara, 1975), that are not applicable in food processing (Freese et al., 1973; Kabara, 1975, 1984; Shibasaki and Kato, 1978; Oh and Marshall, 1993). Organic acids are metabolized by the body primarily through lipid oxidation and via the tricarboxylic acid cycle (Doores, 1983). Therefore, they offer a considerable advantage over other types of chemical sanitizers to control microorganisms. The adverse impact of chemical sanitizers on humans and the environment has been often overlooked. Nowadays, safety and environmental concerns surrounding chemical products are being raised by governments, industry and consumers. The concern is about being able to control microbial pathogens while minimizing the side effects created by these powerful agents which have become more a part of the problem than the solution(e.g., resistance of microorganisms to germicides) (Kabara, 1984). Some chemical agents are being seriously considered for removal from the market because of their potential or perceived toxic or carcinogenic effects (Oh and Marshall, 1993). The solution to this problem is to consider natural, non-toxic, agents, such as fatty acids, that inactivate bacteria and fungi without harming humans and the environment.

B.- Mechanism of action

Peroxyfatty acids have the ability to form peroxides and other free radicals that inhibit bacterial growth (Kabara, 1979; Hinton and Ingram, 2000). Peroxyacetic acid, like other peroxides and oxidizing agents, is assumed to oxidize sensitive sulfhydryl and sulfur bonds in proteins, enzymes and other metabolites. It is suggested that peroxyacetic acid disrupts the chemiosmotic function of the cytoplasmic membrane and transport through dissociation or rupture of cell walls (Baldry and Fraser, 1988; Block, 1991).

Other than sulfuric acid, most of the remaining components of this sanitizer are weak organic acids. In aqueous solution, weak acids are only slightly ionized and do not readily give up their proton(s) to water (Doores, 1983). At a pH lower than the pk_a, the equilibrium reaction shifts toward a higher concentration of undissociated acid. Based on experiments reported by Doores (1983) comparing the antimicrobial performance of weak and strong acids at higher pH, toxicity was due, not to hydrogen ion alone, but was a function of the undissociated molecule. Bacterial membranes are less permeable to charged molecules than uncharged molecules (Doores, 1983). Therefore, the inhibition by organic acids used as antimicrobial agents increases with decreasing pH, in agreement with the pk_a values (Freese et al., 1973; Doores, 1983). According to Galbraith and Miller (1973a,b,c) and Oh and Marshall (1992, 1993), lowering the pH of the suspending medium increased fatty acid uptake by *Bacillus megaterium* and reduced the interfacial tension at the bacterial lipid membrane-aqueous medium interface. Hunter and Segel (1973) and Doores (1983) suggest that weak acids at or below their pk_a could discharge the proton gradient and ionize within the cell to acidify the interior. It was postulated that the rate of proton leakage into a cell versus proton ejection would determine inhibition

(Freese et al., 1973; Doores, 1983). Peracetic acid, upon contact with organic substrates, decomposes to yield oxygen and acetic acid (Doores, 1983). Antibacterial action of acetic acid is partially due to the lowering of pH below the optimum level for growth. The same is also true for lactic acid. Indeed, proteins, nucleic acids and phospholipids can be structurally altered by pH changes (Doores, 1983).

In general, fatty acids function as surface-active anionic detergents (Kabara et al., 1972). Fatty acids uncouple both substrate transport and oxidative phosphorylation from the electron transport system (Freese et al., 1973). They act by disrupting the bacterial cell membrane and lysing protoplasts as evidenced by the leakage of 260nm-absorbing material, protein and other internal metabolites from both bacteria and protoplasts. Thus, fatty acids prevent bacterial growth by modifying cell membrane permeability which leads to changes and/or inhibition in oxygen, aminoacid, nucleic acid, organic acid, phosphate and other substrate molecule uptake (Freese et al., 1973; Kabara, 1979; Oh and Marshall, 1992, 1993; Galbraith and Miller, 1973 a,b,c; Doores,1983; Hinton and Ingram, 2000). Although saturated fatty acids inhibit cellular oxygen consumption, they do not inhibit NADH oxidation by isolated membranes, that is by the cytochrome-linked electron transport system. Therefore, inhibition of oxygen consumption in whole cells must result from the deficiency of compounds that donate electrons to the electron transport chain. This deficiency results from the inability to transport necessary substrates into cells (Freese et al., 1973). Inhibition of cellular uptake, however, does not necessarily prove that transport itself is inhibited; it may merely reflect inhibition of metabolism with unmetabolized compounds preventing their own uptake (Freese et al., 1973).

Long chain fatty acids show greater antibacterial activity against Gram positive organisms (Galbraith and Miller, 1973a; Sheu and Freese,1973; Kabara et al., 1977). Except for certain short chains, gram-negative organisms are generally not affected by fatty acids (Sheu and Freese,1973; Kabara et al., 1977). When compared to the concentrations of short chain fatty acids up to C_6 needed for inhibition of *E. coli*, twice the concentration of C_8 and about 50 times the concentration of C_{10} is required with no inhibition observed for longer chain fatty acids (Freese et al., 1973). As a possible explaination, the lipopolysaccharide layer that typically surrounds the wall of gram-negative organisms could screen out the larger chain fatty acids (Freese et al.,1973, Doores, 1983). Another theory proposes that gram-negative organisms can rapidly metabolize these fatty acids, thereby preventing their accumulation within the cell (Kabara et al. 1972; Freese et al., 1973; Doores, 1983 and 1993). The concentration of fatty acids, up to C_8 that completely inhibits the growth of *E. coli* does not reduce the amount of ATP/A_{600} measured after 20 or 40 minutes of incubation. Apparently, in *E. coli*, some other factor acts to limit microbial viability (Freese et al., 1973).

Vadehra et al. (1985) and Oh and Marshall (1992) reported that glycerol monolaurate causes extensive leakage of 260 nm-absorbing intracellular proteins from *Staphylococcus aureus*. Lower pH may also increase the uptake of glycerol monolaurate (Oh and Marshall, 1993).

C.- Antimicrobial performance

Peer-reviewed publications regarding the performance of this novel FDA-approved fatty acid-based sanitizer are not yet available. A patent process is currently ongoing for this disinfectant.

Peracetic acid exhibits excellent antimicrobial properties, especially under acidic conditions. It is less effective at pH 8 because of its pk_a of 8.2 (Baldry, 1983). When peracetic acid was tested against populations of 10^6 CFU/ml of *Pseudomonas aeruginosa* ATCC 15442, *Klebsiella pneumoniae* ATCC 4352, *Streptococcus faecalis* 10541 or *Staphylococcus aureus* ATCC 538 in aqueous solutions, 66 μmol/L failed to produce a complete kill within 30 minutes; whereas 330 μmol/L and 1.3 m mol/L w ere e ffective after10 and < 1 minutes, respectively (Baldry, 1983).

Acetic acid is more effective in limiting bacterial and yeast growth than mold growth (Doores, 1983). As gram-negative bacteria were more inhibited than gram-positive bacteria at pH 6 and 5, acetic acid may effectively select for gram-positive organisms (Doores,1983).

Peroxycaprylic and peroxycapric acids reportedly provide antibacterial activity against a wide variety of gram-positive (e.g., *Staphylococcus aureus*) and gram-negative (e.g., *Escherichia coli*) bacteria as well as yeasts, molds and bacterial spores (Oakes et al., 1993). The present blend of peroxyacetic, peroxycaprylic and peroxycapric acids reduced microbial populations 5 logs after 30 seconds of exposure to concentrations ranging between 100 ppm and 20 ppm of the peracid blend (Oakes et al., 1993).

Inhibition by fatty acids generally increases about 10-fold when the concentration is increased by a factor of 10, indicating an essentially linear concentration dependence

(Freese et al.1973). As an anionic surfactant, fatty acids are less potent at physiological pH values (Kabara et al., 1972). The inhibitory action of fatty acids can be largely diminished by the presence of reversing agents including Ca^{2+} and Mg^{2+}. The reversing action of other cations such as Fe^{3+} is also a possibility (Galbraith and Miller, 1973a). These authors studied the effect of cations on bactericidal activity of lauric and linoleic acid against fatty acid sensitive strains of *Bacillus megaterium* and *Micrococcus lysodeictikus* at pH 7.0. With lauric acid, considerable reversal of inhibition occurred with Ba^{2+} and Sr^{2+} at equimolar concentrations, but Fe^{3+} and Sn^{2+} were usually ineffective.

When tested against various gram-positive and gram-negative organisms, caprylic acid was not inhibitory at concentrations up to 7.8 μmol/mL (Kabara et al.1972; Doores, 1983). The antimicrobial performance of caprylic acid at pH 5.0 and 6.0 appears to support the observation that, whereas acids with fewer than 7 carbons are more effective at lower pH, acids with 8 to 12 carbons are more effective at neutrality or above (Doores, 1983).

Another fatty acid, oleic acid at concentrations of 2, 4, 6, 8 and 10% (w/v) was reported to decrease the bacterial population on poultry skin (Hinton and Ingram, 2000). *Listeria monocytogenes* was least resistant to the antibacterial activity in vitro while *Escherichia coli* showed higher resistance. *Salmonella* Typhimurium was most resistant to oleic acid (Hinton and Ingram, 2000).

When used at a concentration of 0.1%, hydrogen peroxide alone was reportedly ineffective (Venkitanarayanan et al.,1999) against *E. coli* O157:H7. However, Alasri et al. (1992) reported a 5 log reduction in numbers of *E. coli* ATCC 8739 in an aqueous

model system after 5 minutes of contact with 0.625 % hydrogen peroxide. A synergistic biocidal effect (5 log reduction in 5 minutes) was also found between 391 ppm of hydrogen peroxide and 2.5 ppm of peroxyacetic acid. The degree of synergistic action was similar on the species studied : *E.coli* ATCC 8739, *Pseudomonas aeruginosa* ATCC 9027 and *Staphylococcus aureus* ATCC 6538 P (Alasri et al., 1992).

Optimum antimicrobial activity was found for fatty acids and their corresponding monoglycerides when the chain length was C_{12} (Kabara et al. 1977), such as for lauric acid (Nawar, 1996). Esterification of a fatty acid to glycerol to form monoacyl derivatives generally results in greater biocidal activity. Monolaurin, the most active ester is indeed more potent than free lauric acid (Kabara et al., 1972; Conley and Kabara, 1973; Kabara et al., 1977). The minimal inhibitory concentration (MIC) is defined as the lowest concentration of a compound at which no macroscopic evidence of growth is observed after an appropriate period of incubation (Kabara et al. 1972; Kabara et al., 1977). Beuchat (1980) demonstrated that the MIC at which inhibition of *V. parahaemolyticus* could be detected was lower for glycerol monolaurate (5μg/ml) than for lauric (60 μg/ml), capric (60 μg/ml) or caprylic acid (100 μg/ml) in laboratory media at pH 6.7.

According to Kabara (1979) and Oh and Marshall (1992), glycerol monolaurate has a broad spectrum of antimicrobial activity in culture media against gram-positive microorganisms. Kato and Shibasaki (1976) and Oh and Marshall (1992) showed that glycerol monolaurate is also effective against Gram-negative bacteria in culture media containing citric and polyphosphoric acid. Kabara (1979), Kato (1981) and Oh and

Marshall (1992) also reported on the antimicrobial effects of glycerol fatty acid esters against pathogenic and spoilage organisms.

Oh and Marshall (1992, 1993) demonstrated that the antimicrobial effect of glycerol monolaurate on *L. monocytogenes* is strongly influenced by pH, temperature and their interaction. However, differences in susceptibility between four different strains of *L. monocytogenes* treated with glycerol monolaurate were negligible. By reducing the pH from 7.0 to 5.0, the minimum inhibitory concentration (MIC) of glycerol monolaurate decreased from 10 to 3 μg/mL for three of four strains. The contribution of temperature to glycerol monolaurate effectiveness showed that inactivation occurred more rapidly at higher temperatures (Oh and Marshall, 1993). *Listeria* was rapidly inactivated by glycerol monolaurate at lower pH values and higher temperatures (Oh and Marshall, 1993). These authors also assumed that, in addition to pH and temperature, other environmental factors, such as initial inoculum level, competing microflora and food composition may influence the effectiveness of glycerol monolaurate. Glycerol monolaurate was more effective than sorbic acid and sodium benzoate against *Vibrio parahaemolyticus* with MIC values of 5, 70 and 300 μg/mL, respectively (Beuchat,1980; Oh and Marshall, 1992). At a concentration of 0.005%, glycerol monolaurate was unable to reduce populations of *E. coli* O157:H7 suspended in 0.1% peptone by 5 log CFU/ml (Venkitanayaranan et al., 1999).

Lactic acid is inhibitory to a wide range of microorganisms. However Venkitanarayanan et al. (1999) emphasized that *E. coli* O157:H7 is unusually acid tolerant. Conner and Kotrola (1995) and Venkitanarayanan et al.(1999) also reported that *E. coli* O157:H7 was able to survive up to 56 days in Tryptic Soy Broth acidified to pH

4.7 with lactic acid. Abdul-Raouf et al. (1993) determined that *E. coli* O157:H7 survived well in beef slurries containing lactic acid. Lactic acid at 1.5% was unable to reduce populations of *E.coli* O157:H7 suspended in 0.1% peptone by 5 log CFU/mL (Venkitanayaranan et al., 1999). Lactic acid showed marked inhibitory capacity against *Mycobacterium tuberculosis* which increased as the pH decreased (Doores, 1983).

In a model system, evaluating the effectiveness of 1.5% lactic acid plus 0.005% glycerol monolaurate against *E. coli* O157:H7 resulted in a microbial reduction of > 5.0 log CFU/mL in 20 minutes at 22 °C (Venkitanarayanan et al., 1999).

3.- SYNERGISTIC EFFECTS BETWEEN COMMERCIAL CHEMICAL SANITIZERS (CLOROX™, TSUNAMI™, VEGI-CLEAN™) AND SONICATION OR COPPER IONS FOR REDUCTION OF *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT104 AND *LISTERIA MONOCYTOGENES* ON INOCULATED ALFALFA SEEDS AND SPROUTS.

ABSTRACT

Alfalfa seeds and sprouts were inoculated to contain 10^6 to 10^8 CFU/g *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104 or *Listeria monocytogenes* and exposed to the following sanitizers for 30 seconds to 10 minutes : Clorox™ (sodium hypochlorite, 200 to 20,000 ppm), Tsunami™ (peroxyacetic acid / hydrogen peroxide, 80 and 800 ppm) or Vegi-Clean™ (anionic surfactant, 1%, 2%, 5%). Appropriate dilutions in 0.1% peptone were spiral plated on Sorbitol Mc Conkey Agar (SMAC) or Cefixime Tellurite Sorbitol McConkey Agar (CT-SMAC) for *E. coli* O157:H7, Xylose Lysine Desoxycholate agar (XLD) for *S.* Typhimurium DT104 and Modified Oxford Agar (MOX) for *L. monocytogenes*. Only sodium hypochlorite (> 1,000 ppm) was able to reduce pathogens on sprouts by > 5 logs. Ultrasound (20 kHz) generated by a sonicating water bath or copper ions generated by an electrolytic process and dispersed into a circulating water stream to a concentration of 1 ppm, were tested alone or in combination with the previous sanitizers to assess possible synergistic effects. No appreciable differences in pathogen reduction were observed using either sonication or copper ions in combination with the previous sanitizers.

3.1.- INTRODUCTION

Alfalfa sprouts have been incriminated in several countries as the source of numerous outbreaks involving *E. coli* O157:H7 (Taormina and Beuchat, 1999a,b) and *Salmonella* sp. (Mahon et al., 1997; Glynn, 1997; Taormina and Beuchat, 1999a,b).

Research efforts have focused on identifying various strategies that can reduce the microbial load on alfalfa seeds by 5 logs while maintaining the germination rate at a commercially acceptable level and being non toxic for consumers. Some of these methods investigated include heat (Jaquette et al., 1996), gamma irradiation (Rajkowski and Thayer, 2000), volatile compounds (Park et al., 2000) and chemical sanitizers (Piernas and Guiraud, 1997; Beuchat, 1997; Taormina and Beuchat, 1999a; Bari et al., 1999; Weissinger and Beuchat, 2000). The antimicrobial efficacy of Clorox™ (sodium hypochlorite) for reducing pathogens on alfalfa seeds and sprouts has been investigated by Jacquette et al. (1996) and Weissinger and Beuchat (2000). Similarly, Taormina and Beuchat (1999b) have studied the effect of Tsunami™ (peroxyacetic acid / hydrogen peroxide) and Vegi-Clean™ (acid-anionic surfactant) in controlling microbial growth during alfalfa seed sprouting. Although, these studies have expanded our knowledge concerning pathogen inactivation, none of these bactericidal treatments will effectively guarantee the microbiological safety of organoleptically acceptable alfalfa sprouts. Therefore, much further work still remains.

Sonication is a cell disruption technique involving the use of ultrasound. Large stresses and strains produced during cavitation create a mechanical effect that ultimately destroys bacterial cells. The magnitudes of ultrasonic waves are sufficiently high to cause protein breakdown, protein hydrolysis, simple cell lysis, protein particulation and even

high temperature biocatalysis if the enzymes are resistant to ultrasonic waves (Shukla, 1992; Lillard, 1993). In addition to causing bacterial cell wall damage, sonication may also help to declump and dislodge bacteria from the surface of alfalfa seeds and sprouts, and more readily expose these weakened cells to the harmful effects of chemical sanitizers, thus leading to greater microbial reduction.

Copper has been long known for its anti-microbial properties (Yeager 1991). Riggle and Kunamoto (2000) reported that copper, as an essential cofactor for many enzymes, is vital to a wide range of biological processes including respiration, destruction of free radicals, iron homeostasis. Nevertheless, excess levels of copper can induce a high toxicity by generating reactive oxygen species via the Fenton reaction, thereby disrupting metal ion binding and homeostasis and binding macromolecules, such as proteins. Like other prokaryotes, bacteria resist copper toxicity by reducing the influx and/or utilizing efflux mechanisms by way of ATPase transporters to control intracellular copper levels. According to Yahya (1989) and Sanders (correspondence to Superior Water Solutions, Inc.), the combination of copper ion and sodium hypochlorite showed stronger antimicrobial activity than either copper ion or sodium hypochlorite alone. Thus copper ions will exert a certain level of toxicity towards bacterial pathogens on alfalfa seeds and sprouts that might be extentuated by chemical sanitizers. A synergistic antimicrobial effect is expected from the combination of copper ion with Clorox ™, Tsunami ™ or Vegi-Clean ™. It is thought that the formation of electrostatic bonds between positively charged ions and negatively charged sites on the microbes (Superior Water Solutions, Inc.) promotes greater sanitizer contact which leads to enhanced antimicrobial activity.

The objective of this study is to carry out an exploratory assessment of the various antimicrobial combinations previously mentioned in order to identify some promising schemes toward the 5 log reduction targeted.

3.2.- MATERIALS AND METHODS

Bacterial strains

Three *Escherichia coli* O157:H7 strains (AR, AD 305 and AD 317) and 3 *Listeria monocytogenes* strains (LM Brick silage, LM T3BL, LM F5027) were obtained from Dr. Catherine W. Donnelly (Dept. of Nutrition and Food Sciences, University of Vermont, Burlington, VT). Three *Salmonella* Typhimurium DT104 strains (G1601, G1074 and G10931) were obtained from Dr. Peggy Hayes (Centers for Disease Control and Prevention). Laboratory stock cultures were maintained at – 80 °C in Tryptic Soy Broth (TSB, pH 7.3) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol. Individual strains were separately activated by transferring a loop of the frozen stock culture into 9 ml of sterile TSB containing 0.6% (w/v) yeast extract (YE) (Difco) then incubated at 35°C for 18-24 hours. Thereafter, for each strain, 5 tubes containing 35 ml each of TSB-YE were inoculated and similarly incubated before use.

Preparation of inoculum

The aforementioned cultures were harvested by centrifugation (Sorvall Super T21, Newtown, CT) at 10,000 rpm for 15 minutes at 4 °C, resuspended in 0.1 % peptone (Difco) and combined in equal volumes to prepare the 3-strain inoculum cocktails containing ~ 10^9 CFU/ml.

Inoculation of alfalfa seeds and sprouts

Alfalfa seeds used in this study (originating from Australia and identified by the batch serial # AUS / S / DM / 71) were purchased in bags of 25 kg from Dan Caudill (Louisville, KY).

Seeds were sprouted at room temperature (22 – 25 °C), in a Kitchen Crop sprouting device manufactured by NK Lawn and Garden (Chattanooga, TN). According to the manufacturer's instructions, 10 to 15 g of seeds were layered on the plastic trays and watered twice daily using tap water. The overall process took approximately 4 to 5 days. Then, the sprouts were rinsed several times in tap water and stored at 5 °C prior to inoculation.

Alfalfa seeds and sprouts were inoculated so as to contain 10^5 to 10^7 CFU/g and 10^8 to 10^9 CFU/g, respectively. Seeds (500 g) and sprouts (300 g) were separately immersed in a 525 ml 3-strain cocktail of *E. coli* O157:H7, *S.* Typhimurium DT104 or *L. monocytogenes* and gently agitated in this mixture for approximately 10 minutes. The seeds were dried for 4 hours under laminar flow in a biosafety cabinet and stored at room temperature. The sprouts were strained and stored at 5 °C. Seeds and sprouts were stored

at least 24 hours before use in order to allow for adequate pathogen attachment. Unused inoculated seeds and sprouts were discarded after 5 days.

Inactivation of pathogens on alfalfa seeds and sprouts

Chemical sanitizers

The following chemical sanitizers were investigated and compared to a water control:

1.- Clorox™ (The Clorox Company, Oakland, CA), (sodium hypochlorite as active ingredient) at concentrations of 200, 1000, 2000, 10,000 and 20,000 ppm chlorine.

2.- Tsunami™ (Ecolab, Mendota Heights, MN), (peracetic acid and hydrogen peroxide (PAH) as active ingredients) at concentrations of 80 and 800 ppm.

3.- Vegi-Clean™ (Microcide, Inc., Detroit, MI, U.S. Patents # 5,143,720 and # 5,280,042), (citric acid, sodium acid phosphate and sodium dodecyl benzene sulfonate as active ingredients) at concentrations of 1%, 2% and 5%.

Clorox™ solutions were prepared by diluting the commercial concentrate with sterile deionized water and adjusting the concentration with a chlorine test kit (La Motte Chemical Products Co., Inc., Chestertown, MD). Preparation of Tsunami™ solutions, according to label instructions, consisted of adding 47.8 µl and 478 µl of sanitizer concentrate to 100 ml of sterile deionized water to obtain concentrations of 80 and 800 ppm, respectively. To prepare the Vegi-Clean™ 1, 2 and 5% experimental solutions, 1, 2 and 5 g of powdered product, respectively, were dissolved in 100 ml of sterile deionized water. All sanitizer solutions were freshly prepared just prior to use.

Sonication

A Fisher Scientific (Chicago, IL) FS 220 water bath sonicator was used to generate ultrasound (20 kHz). Sterile 24 oz stomacher bags (Whirl-Pak, Nasco, USA) containing 10 g of seeds or sprouts in 40 or 90 ml treatment solution, respectively, were immersed in the water bath sonicator for 30 seconds to 10 minutes.

Copper ion water

Copper ions were generated through an electrolytic process (Superior Water Solutions, Inc). A pilot plant-sized copper ion generator (Superior Water Systems, Inc., Fort Wayne, Indiana) dispersed copper ions into a recirculating water stream so as to reach a concentration of 1 ppm. The copper ion concentration was determined prior to treatment using a colorimetric copper ion test kit (model EC-20; La Motte Chemical Products Co., Inc., Chestertown, MD), with this solution used as the disinfectant.

Hurdle approach treatment

Sonication and copper ion were combined with the aforementioned sanitizers in order to increase the antimicrobial efficacy of the latter. When combined with copper ion, the sanitizer working solutions were prepared using copper ion water (1 ppm) rather than deionized water.

Experimental procedure

In order to assess the effect of treatment on microbial load of the pre-inoculated seeds and sprouts, an experimental procedure, modified from Taormina and Beuchat (1999a) was adopted. Forty ml of sanitizer were added to a 24 oz. sterile stomacher bag containing 10 g of inoculated seeds and manually agitated for 30 seconds, 1, 3, 5 or 10 minutes. When combined with sonication, the stomacher bag containing the seeds and

sanitizer was held in sonicating water bath for the same durations. After treatment, the sanitizer was discarded and replaced by 40 ml of neutralizing buffer after which the sample was homogenized at high speed in a stomacher (Model SD-45 Tekmar Co., Cincinnati, OH)) for 2 minutes.

Alternatively, 90 ml of sanitizer were added to a sterile stomacher bag containing 10 g of inoculated sprouts and manually agitated during the chosen duration of treatment. The sanitizer was discarded and replaced by 90 ml of appropriate neutralizing buffer with the sample similarly homogenized at high speed for 2 minutes in a stomacher.

A water control was also prepared by replacing the sanitizer with a corresponding amount of water and applying the procedure previously described for the seeds and the sprouts. For calculating microbial load reduction, the inoculated seeds or sprouts were analyzed by adding 40 ml or 90 ml of buffer, respectively, in a stomacher bag containing a 10 g sample, followed by processing in a stomacher.

Microbial Analysis

In all cases, appropriate dilutions in 0.1% peptone were spiral-plated (Autoplate 4000, Spiral Biotech Inc., Bethesda, MD) on Sorbitol Mc Conkey Agar (SMAC) or Cefixime Tellurite Sorbitol McConkey Agar (CT-SMAC) for *E. coli* O157:H7, Xylose Lysine Desoxycholate agar (XLD) for *Salmonella* Typhimurium DT 104, Modified Oxford Agar (MOX) for *Listeria monocytogenes* and Plate Count Agar (PCA) for Mesophilic Aerobic Bacteria with the latter counts being reported in Appendix A.

Neutralizing buffers were 0.1% peptone (Difco) for the inoculated seeds and the water control; 0.1%, 1% and 5% sodium thiosulfate (JT Baker, Phillipsburg, NJ) for

Clorox ™ 200 ppm, 1000 to 2000 ppm and 10,000 to 20,000 ppm, respectively, and 1% and 5% peptone for Tsunami™ 80 ppm and 800 ppm, respectively. The buffer for Vegi-Clean™ was prepared by dissolving lecithin (2,2g) (Sigma, St Louis, MO), Tween 80 (15.5ml) (Sigma), and KH_2PO_4 (4.6 g) (JT Baker, Phillipsburg, NJ) in 100 ml of sterile deionized water. This stock solution was diluted 1:10, 1:5, 1:2 to neutralize concentrations of 10,000, 20,000 and 50,000 ppm. The pH of these buffers were adjusted to 7.0 / 7.4 with sterile 0.1 N NaOH solution.

Reduction in the microbial load was calculated by subtracting the microbial count after treatment from the corresponding initial microbial count on the untreated inoculated seeds. In absence of replications, statistical analysis was not applied to these results.

3.3.- RESULTS AND DISCUSSION

3.3.1.- WATER WASHING

A reduction in bacterial load on alfalfa seeds previously inoculated with pathogens was always observed after rinsing with water. Reductions in *E. coli* O157:H7 population averaged 0.32 $\log_{10}$ CFU/g on alfalfa seeds previously inoculated with *E. coli* O157:H7 (Table 3.1). After washing alfalfa seeds previously inoculated with *Salmonella,* the pathogen population fluctuated between an apparent increase of 0.23 log and a reduction of 0.92 $\log_{10}$ CFU/g (Table 3.2). These results are consistent with the work of Piernas and Guiraud (1997) who reported that washing rice seeds with sterile water decreased aerobic plate counts <1 log.

TABLE 3.1.- REDUCTION OF *ESCHERICHIA COLI* O157:H7 (log_{10} CFU/g) ON INOCULATED ALFALFA SEEDS USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.84		0.88		0.30
Water control *	0.68		0.68		0.58
Clorox 200 ppm	1.36		1.57		0.60
Water control	0.68		0.68		0.58
Clorox 20,000 ppm	1.32	1.18	1.53	1.23	1.49
Water control	0.23	0.28	0.06	0.06	0.03
Clorox 200 ppm + sonication	1.91		1.56		1.15
Water control	0.68		0.68		0.58
Tsunami 80 ppm	0.91		0.61		1.11
Water control	0.68		0.68		0.58
Tsunami 800 ppm	0.46	0.50	0.62	0.73	0.63
Water control	0.23	0.28	0.06	0.06	0.03
Tsunami 80 ppm + sonication	1.91		1.79		1.28
Water control	0.68		0.68		0.58
Vegi-Clean 1%	1.60		1.41		1.24
Water control	0.68		0.68		0.58
Vegi-Clean 2%	0.44	0.66	0.71	0.57	1.06
Water control	0.23	0.28	0.06	0.06	0.03
Vegi-Clean 1% + sonication	1.51		1.70		1.60
Water control	0.68		0.68		0.58
Copper ion 1 ppm	0.52				0.29
Water control	0.68		0.68		0.58
Copper ion + sonication	1.07				0.86
Water control	0.68		0.68		0.58

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

TABLE 3.2.- REDUCTION OF *SALMONELLA* TYPHIMURIUM DT104 (log_{10} CFU/g) ON INOCULATED ALFALFA SEEDS USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.04	0.18	0.28	0.39	+0.06
Water control *	0.32	+0.23	0.21	0.09	+0.09
Clorox 200 ppm	1.42	>1.92	1.42	0.98	0.94
Water control	0.32	+0.23	0.21	0.09	+0.09
Clorox 20,000 ppm	+ 0.07	+0.16	+0.16	+0.10	+0.10
Water control	0.19	1.11	0.60	0.92	0.49
Clorox 200 ppm + sonication	>1.92	1.73	0.70	1.65	1.21
Water control	0.32	+0.23	0.21	0.09	+0.09
Tsunami 80 ppm	2.02	>3.32	>3.32	>3.32	>3.32
Water control	0.32	+0.23	0.21	0.09	+0.09
Tsunami 800 ppm	1.07	1.32	0.62	1.79	1.61
Water control	0.19	1.11	0.60	0.92	0.49
Tsunami 80 ppm + sonication	>1.92	>1.92	0.98	>1.92	1.94
Water control	0.32	+0.23	0.21	0.09	+0.09
Vegi-Clean 1%	1.32	1.00	1.09	0.96	0.34
Water control	0.32	+0.23	0.21	0.09	+0.09
Vegi-Clean 2%	1.07	1.43	1.79	1.94	1.60
Water control	0.19	1.11	0.60	0.92	0.49
Veg.-Clean 1% + sonication	0.72	0.72	1.15	1.02	0.54
Water control	0.32	+0.23	0.21	0.09	+0.09
Copper ion 1 ppm	+0.18	0.24	0.40	0.24	0.47
Water control	0.32	+0.23	0.21	0.09	+0.09
Copper ion + sonication	0.46	0.94	0.21	0.78	0.24
Water control	0.32	+0.23	0.21	0.09	+0.09

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

+ This sign placed before a data inside a column refer to an increase instead of a decrease of the microbial load.

When inoculated sprouts were washed in water, populations of *E. coli* O157:H7, *Salmonella* DT 104 and *L. monocytogenes* decreased by an average of 0.60, 0.76 and 0.45 logs, respectively (Tables 3.3, 3.4, 3.5).

The microbial reductions obtained after washing the alfalfa seeds or sprouts with water, were not a function of treatment duration. Since water was assumed to have no bactericidal effect, similar microbial reductions should have been expected independently of the duration of treatment.

Using either seeds or sprouts showed that microbial reductions were generally lower for the longest washing treatments. This observation is consistent with previous reports from Jaquette et al. (1996) and Taormina and Beuchat (1999a). In the case of a longer water-seed contact time, the seed imbibes water and therefore releases bacterial cells that were previously trapped or hidden in crevices or between the testae and cotyledons. A similar explanation is also applicable for sprouts. Moreover, the process of drying seeds after inoculation would protect bacteria that had entered the seeds through cracks and crevices (Jaquette et al.,1996, Taormina and Beuchat 1999a; Mohle-Boetani et al., 2002).

Water washing exerted a stronger effect on bacterial reduction in sprouts than in seeds. Sprouts are usually kept in a very moist environment. Therefore, stems and leaves are very turgid and, subsequently, offer a larger contact surface for water action. In contrast, seeds are covered with a waxy material that repels water (Jaquette et al.,1996), suggesting that the seed coat might be responsible for the lower microbial reductions observed during washing.

TABLE 3.3.- REDUCTION OF *ESCHERICHIA COLI* O157:H7 (log_{10} CFU/g) ON INOCULATED ALFALFA SPROUTS USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Clorox 1,000 ppm	3.58		4.48	5.44	5.96
Water control*			0.99	0.63	0.40
Clorox 2,000 ppm	3.54		5.31		>7.92
Water control			0.99	0.63	0.40
Clorox 10,000 ppm	4.79	4.57	5.44	>5.97	>7.37
Water control	0.52	0.39	0.39	0.45	0.55
Clorox10,000 ppm+Cu ion	4.44	4.07	5.69	>5.97	>7.37
Water control	0.52	0.39	0.39	0.45	0.55
Vegi-Clean 5%	2.24	2.99	2.60	3.00	3.20
Water control	0.60		0.78	0.76	0.68
Vegi-Clean 5%+copper ion	2.35	2.52	2.73	2.74	3.12
Water control	0.60		0.78	0.76	0.68

* All water control data in this table have to be compared to the treatment results reported on the row placed above them

TABLE 3.4.- REDUCTION OF *SALMONELLA* TYPHIMURIUM DT104 (log_{10} CFU/g) ON INOCULATED ALFALFA SPROUTS USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.62		0.49		0.98
Water control *	0.86	0.56	0.88	0.68	0.89
Clorox 200 ppm	0.51	1.13	1.68	2.34	2.34
Water control	0.86	0.56	0.88	0.68	0.89
Clorox 20,000 ppm	4.26	4.68	4.36	4.60	>6.36
Water control	0.68	0.73	0.88	0.66	0.75
Clorox 200 ppm + sonication	1.27	1.24	2.53	2.63	>3.98
Water control	0.86	0.56	0.88	0.68	0.89
Tsunami 80 ppm	1.00	1.27	1.34	1.46	1.48
Water control	0.86	0.56	0.88	0.68	0.89
Tsunami 800 ppm	2.11	2.75	3.44	3.27	3.62
Water control	0.68	0.73	0.88	0.66	0.75
Tsunami 80 ppm + sonication	1.21	1.10	2.38	1.68	1.86
Water control	0.86	0.56	0.88	0.68	0.89
Vegi-Clean 1%	0.74	1.24	1.47	1.76	2.08
Water control	0.86	0.56	0.88	0.68	0.89
Vegi-Clean 2%	1.81	1.75	2.13	2.20	2.43
Water control	0.68	0.73	0.88	0.66	0.75
Veg.-Clean 1% + sonication	0.72	1.46	1.74	2.02	2.74
Water control	0.86	0.56	0.88	0.68	0.89
Copper ion 1 ppm	0.70	0.46	0.54	0.64	0.92
Water control	0.86	0.56	0.88	0.68	0.89
Copper ion + sonication		0.45	0.58	0.60	0.84
Water control	0.86	0.56	0.88	0.68	0.89

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

TABLE 3.5.- REDUCTION OF *LISTERIA MONOCYTOGENES* (log_{10} CFU/g) ON INOCULATED ALFALFA SPROUTS USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Clorox 20,000 ppm	2.42	2.87	3.11	4.66	5.46
Water control*	0.46	0.42	0.51	0.49	0.38
Vegi-Clean 2%	0.91	1.11	1.44	1.64	2.08
Water control	0.46	0.42	0.51	0.49	0.38

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

The general trends of these results support the assertion of Fett (2000) who stated that the common occurrence of natural biofilms on the surface of fruits and vegetables may account for the fact that rinsing with water usually reduces total bacterial counts by ≤1 log.

3.3.2 .- SONICATION

Sonicating seeds previously inoculated with *E. coli* O157:H7 and *S.* Typhimurium DT 104 did not noticeably alter the microbial populations. After sonication, *E. coli* populations decreased an average of 0.67 log compared to reductions averaging 0.65 log, after washing inoculated seeds in water. On alfalfa seeds previously inoculated with *Salmonella*, microbial reductions averaged 0.16 log, after sonication, compared to 0.06 log for the water control.

Sonication alone was also applied to alfalfa sprouts previously inoculated with *Salmonella*, with reductions of 0.49 to 0.98 log. The corresponding water control decreased *Salmonella* population 0.56 to 0.89 log.

As for the seeds, sonicating sprouts did not offer any benefit over washing. Microbial reductions were not a function of the duration of treatment. The phenomenon described for water soaking was also seen using with sonication with the release of previously imbedded bacteria giving the appearance of a lower bacterial reduction after 10 minutes of treatment.

Our results with sonication alone are consistent with the work of Burleson et al. (1975) who reported that sonicating alone for 10 minutes did not inactivate *Salmonella* Typhimurium or enteropathogenic *E. coli* O126:B16 in phosphate saline buffer or

secondary effluent from a wastewater treatment. While most bacteria present in milk films on metal surfaces were removed using 80 kHz (Daufin and Saincliviert, 1967; Lillard, 1993), our sonicating water bath which generated 20 kHz was likely too weak to remove bacteria from alfalfa seeds or sprouts.

3.3.3.- COPPER ION

A 1 ppm copper ion solution was generally unable to decrease populations of *E. coli* O157:H7 and *S.* Typhimurium DT104 > 0.50 logs on previously inoculated alfalfa seeds, with these reductions usually no greater than water alone (Tables 3.1, 3.2).

As for alfalfa seeds, the microbial reduction resulting from the action of copper ion on alfalfa sprouts inoculated with *S.* Typhimurium DT104 was similar to that seen for water. *Salmonella* reductions ranged from 0.46 to 0.92 and 0.56 to 0.89 logs for copper ion and water, respectively. Thus, copper ion alone did not result in any additional antimicrobial activity against either pathogen.

In aqueous model systems, Yahya et al. (1989) reported an *E. coli* reduction of 0.12 $\log_{10}$CFU/g following a 6 minute exposure to a 433 μg/L : 43 μg/L copper-silver ion solution. These findings more closely reflect our results with seeds and sprouts than those of Rodrick and Hultstrand (1998) who reported a 2 log reduction in total surface bacteria on tomatoes after a 5 minute exposure to a 0.5 to 1.0 mg/L copper ion solution. Similarly, our results are not in accordance with the 3.5 logs CFU/g reduction in the microbial load of scallions claimed by Sanders (Superior Water Solutions, Inc., in a non-dated correspondence) after a 1 ppm copper ion water treatment.

When combined with sonication, 1 ppm copper ion reduced populations of *E. coli* O157:H7 (Table 3.1) and *S.* Typhimurium DT104 (Table 3.2) 0.86 to 1.07 and 0.24 to 0.94 logs, respectively, on inoculated alfalfa seeds. Compared to copper ion alone, the combined use of copper ion and sonication did not result in greater microbial reduction.

3.3.4.- CLOROX ™

When alfalfa seeds previously inoculated with *E. coli* O157:H7 were treated with 200 ppm, populations of *E. coli* O157:H7 decreased 0.60 to 1.57 logs (Table 3.1). Using 20,000 ppm NaClO, microbial reductions were more constant over the treatment times, ranging from 1.18 to 1.53 logs for *E. coli* O157:H7 (Table 3.1).

Taormina and Beuchat (1999a) obtained a 2 to 3 log reduction in *E. coli* using 20,000 ppm of $Ca(OCl)_2$. They reported that treatment with 20,000 ppm $Ca(OCl)_2$ reduced *E. coli* O157:H7 populations from 2.33 to 3.46 $\log_{10}$ CFU/g to less than 0.3 $\log_{10}$ CFU/g, after 3 or 10 minutes. These data show that a higher bacterial reduction was achieved with $Ca(OCl)_2$ than what we observed with NaClO. However, their inability to achieve > 3 log reduction with a concentration of 20,000 ppm of $Ca(OCl)_2$ is consistent with our findings.

Results obtained after treatment with 200 ppm and 20,000 ppm of NaClO show that bactericidal activity is not related to exposure time. For greater exposure times, bacteria hidden in crevices located on the seed surface were released by the action of the aqueous solution. This phenomenon led to bacterial reductions that appeared lower than those obtained for shorter times. When active chlorine comes in contact with high levels of organic matter, such as alfalfa seeds, its potency is quickly diminished (Jaquette et al.,

1996). The comparatively lower bacterial reduction obtained for the 10 minute / 200 ppm treatment could be due to insufficient levels of free chlorine remaining available in solution after 10 minutes of soaking, to destroy organisms previously hidden in crevices. In contrast, sufficient free chlorine likely remained available after 10 minutes using 20,000 ppm. This assumption is supported by the work of Taormina and Beuchat (1999a) who showed that, although the amount of active chlorine decreased in the presence of organic matter, 80% of the initial available chlorine remained in solution, after holding 10 g of alfalfa seeds in 40 ml of 20,000 ppm active chlorine for 12 minutes. When alfalfa seeds were inoculated with *S.* Typhimurium DT104, exposure to 200 ppm NaClO decreased populations 0.94 to at least 1.92 logs (Table 3.2). Hence, the sanitizer effect resulted in higher bacterial reductions than those obtained by washing in water for the same length of time.

In contrast, comparing counts from all treatments to initial populations present on inoculated seeds, Weissinger and Beuchat (2000) calculated the reduction in microbial load in reference to the water control. After plating on TSAN (Trypticase Soy Agar + Nalidixic acid), *Salmonella* populations decreased 0.33 and 0.72 log after a 10 minute exposure to 200 and 2,000 ppm NaClO, respectively. Their results for reduction of *Salmonella* using 200 ppm NaClO were less impressive than ours (0.94 to 1.3 logs compared with water control). The main conclusion remains that both their results and ours are far from the 5 log reduction targeted.

The aforementioned reductions in *Salmonella* populations reported by Weissinger and Beuchat (2000) after 10 minute treatments with 200 and 2000 ppm NaClO were in a similar range (0.33 versus 0.72 log). Our findings with *Salmonella* and *E. coli* are

consistent with these authors, in that none of these treatments completely destroyed the pathogen on seeds. This observation explains why Jaquette et al (1996) needed to use concentrations of 2,040 and 3,990 ppm free chlorine to reduce *Salmonella* Stanley from 65 CFU/g to undetectable levels when analyzed by direct plating. Beuchat (1997) observed a < 3.9 log reduction for *Salmonella* when seeds were soaked in 1,800 ppm $Ca(ClO)_2$ or 2,000 ppm NaClO for 30 seconds. Both our results and those of Weissinger and Beuchat (2000) fell in the lower range compared to those of Beuchat (1997).

According to Piernas and Guiraud (1997), a 20 minute treatment in 1000 ppm NaClO was able to reduce the bacterial load 2 to 3 logs on rice seeds. Decontamination efficacy was not improved by extending the contact time, nor by increasing the chlorine concentration to 10,000 ppm as observed in our work. Piernas and Guiraud (1997) also reported that the greater bactericidal activity using 10,000 ppm NaClO was due to the higher pH. A buffer at the same pH had an equivalent effect on the aerobic plate count, which was not seen using the 1,000 ppm concentration.

Clorox™, at a concentration of 200 ppm chlorine was also combined with sonication with populations of *E. coli* O157:H7 on inoculated seeds decreasing 1.15 to 1.91 logs (Table 3.1). Similarly, *Salmonella* counts decreased 0.70 to >1.92 logs on *Salmonella*-inoculated seeds (Table 3.2). This combination did not bring the superior microbial load reduction expected.

Using 200 ppm NaClO, populations of *Salmonella* on inoculated alfalfa sprouts decreased 0.51 to 2.34 logs (Table 3.4). Slightly greater reductions (1.24 to >3.98) were seen when 200 ppm NaClO was combined with sonication. In contrast, reductions of

4.26 to > 6.36 logs in *Salmonella* were reached after a 10 minute exposure to 20,000 ppm NaClO.

A progressive decrease of the microbial load, although not proportional with the duration of treatment, was observed with alfalfa sprouts that were previously inoculated with *L. monocytogenes* and then treated with 20,000 ppm NaClO (Table 3.5). Populations of *L. monocytogenes* decreased 2.42 to 5.46 logs, with the targeted 5 log reduction again being reached. However, exposing the sprouts to 20,000 ppm NaClO resulted in producing an obviously inedible product.

Based on these results, the efficacy of 10,000 ppm NaClO against *E. coli* O157:H7 was also assessed (Table 3.3). This treatment reduced population of *E. coli* 4.57 to >7.37 logs, with the greatest reductions observed after the longest exposure. A > 5 log reduction was again seen after 10 minutes of soaking; however, sprout quality was undesirable.

Some researchers (Yahya, 1989; Sanders in a Superior Water Solutions, Inc. correspondence) reported that the combination of copper ion and sodium hypochlorite exhibited stronger antimicrobial activity than when either was used alone. Consequently, 10,000 ppm sodium hypochlorite was added to a 1 ppm copper ion solution. However, no noticeable differences were observed in reductions of *E. coli* O157:H7 using copper ion / sodium hypochlorite compared to sodium hypochlorite alone (Table 3.3). Populations of *E. coli* decreased 4.07 to > 7.37 logs after the combined copper ion / sodium hypochlorite treatment, whereas reductions of 4.79 to > 7.37 logs were found with sodium hypochlorite alone.

Given that the sprouts were damaged following exposure to 10,000 ppm NaClO, the concentration was lowered to 2,000 and 1,000 ppm with both of these concentrations proving effective against *E. coli* O157:H7 (Table 3.5). The 2,000 ppm and 1,000 ppm treatments decreased the *E. coli* load by 3.54 to >7.92 and 3.58 to 5.96 logs, respectively. However, the sprouts' appearance remained unsatisfying.

When NaClO was used in combination with either sonication or copper ion, the reduction in microbial populations present on seeds and sprouts was primarily due to the effect of NaClO rather that of the other treatments.

3.3.5 .- TSUNAMI ™

After inoculated seeds were treated with 80 and 800 ppm Tsunami™, populations of *E. coli* O157:H7 decreased 0.61 to 1.11 and 0.46 to 0.73 logs, respectively (Table 3.1). Using Tsunami™ at 80 ppm, Taormina and Beuchat (1999a) reportedly obtained a >1.70 log reduction in microbial load with their findings similar to ours. In combination with sonication, Tsunami™ 80 ppm yielded reductions of 1.28 to 1.91 logs for *E. coli* O157:H7 on inoculated seeds (Table 3.1). Hence, sonication did not markedly increase the effectiveness of Tsunami™.

Tsunami™ 80 and 800 ppm reduced populations of *S.* Typhimurium 2.02 to >3.32 and 0.62 to 1.79 logs, respectively (Table 3.2), on previously inoculated seeds. When Weissinger and Beuchat (2000) exposed *Salmonella*-inoculated alfalfa seeds to Tsunami™ concentrations of 270 ppm, 530 ppm and 1,060 ppm, *Salmonella* populations decreased 0.79, 1.12 and 1.50 logs, respectively (water control taken as basis for the calculations). Their results for reductions in *Salmonella* were similar to ours. Our

observations from both Tables 3.1 and 3.2 agree with Weissinger and Beuchat's (2000) findings in that a higher concentration of Tsunami™ did not lead to a proportional decrease in microbial load. When sonication was combined with 80 ppm Tsunami™ (Table 3.2), populations of *Salmonella* decreased 0.98 to > 1.94 logs with sonication failing to enhance the effectiveness of Tsunami™. Reductions in *E. coli* populations were usually less than those noticed for *Salmonella*.

When alfalfa sprouts were inoculated with *S.* Typhimurium DT104 and treated with 80 ppm Tsunami™, populations of *Salmonella* decreased 1.00 to 1.48 logs (Table 3.4). Taormina and Beuchat (1999b) did not soak their inoculated sprouts in Tsunami™ 80 ppm solution, as was done in our study. Instead, these authors sprayed inoculated seeds, at various steps throughout the sprouting process, with Tsunami™ 80 ppm. Despite these spray applications, *E. coli* populations increased from 2.62 $\log_{10}$ CFU/g on seeds to 6.61 $\log_{10}$ CFU/g in the mature sprouts. When combined with sonication, 80 ppm Tsunami™ decreased populations of *Salmonella* 1.10 to 2.38 logs (Table 3.4), with sonication failing to enhance the effectiveness of Tsunami™.

3.3.6.- VEGI-CLEAN™

Use of 1 and 2% Vegi-Clean™ generally reduced bacterial populations < 1.50 logs (Table 3.1). A concentration of 1%, as recommended by the manufacturer for washing fruit and vegetables, decreased populations of *E. coli* O157:H7 on inoculated seeds only 1.24 to 1.60 logs. Results obtained with the 2% concentration were inferior, with reductions of 0.44 to 1.06 logs for *E. coli* O157:H7. When sonication was

combined with 1% Vegi-Clean™, *E. coli* O157:H7 decreased 1.51 to 1.60 logs. Hence, sonication also failed to enhance the effectiveness of Vegi-Clean™.

Taormina and Beuchat (1999a) studied the effect of 1 and 2 % Vegi-Clean™ on alfalfa seeds inoculated with *E. coli* O157:H7. These authors obtained slightly greater microbial reductions with 1.77 to 2.10 logs for 1% and 1.50 to 1.72 logs for 2% (compared to the water control). These microbial reductions were not a function of treatment time with 0.10 log and 0.26 log remaining on the seeds, respectively, after treaments of 3 and 10 minutes with 1% Vegi-Clean™. The same trend was observed using 2% Vegi-Clean™ (0.48 log present after 3 minutes versus < 0.30 log after 10 minutes). As in our study, microbial reductions were usually greater using 1% rather than 2% Vegi-Clean™.

Reductions in numbers of *S.* Typhimurium DT104 on inoculated alfalfa seeds were also minimal using 1 and 2% Vegi-Clean™ (Table 3.2). With a 1% solution, *S.* Typhimurium DT104 decreased 0.34 to 1.32 logs. Increasing the concentration to 2% resulted in decreases of 1.07 to 1.94 logs. Thus, somewhat greater reductions were seen at the higher concentration. Length of treatment exposure had a greater impact using 2% as compared to 1% Vegi-Clean™. When combined with sonication, 1% Vegi-Clean™ reduced *Salmonella* population 0.72 to 1.15 logs (Table 3.2). As for the other sanitizers, sonication also failed to enhance the efficacy of Vegi-Clean™.

Two concentrations of Vegi-Clean™ (1% and 2%) were applied to alfalfa sprouts inoculated with *S.* Typhimurium DT104 (Table 3.4). Results for both concentrations were generally similar, with populations of *S.* Typhimurium decreasing 0.74 to 2.08 logs

for the 1% solution, and 1.81 to 2.43 logs for the 2% application. The combination of 1% Vegi-Clean™ and sonication failed to enhance the reduction in *Salmonella,* with decreases of 0.72 to 2.58 logs. For all Vegi-Clean™ treatments with sprouts, a trend toward greater microbial reduction was observed as the duration of treatments increased.

The results obtained after treating *Listeria*-inoculated sprouts with 2% Vegi-Clean™ were similar to those previously recorded for *Salmonella* with reductions of 0.91 to 2.08 logs (Table 3.5).

Sprouts previously inoculated with *E. coli* O157:H7 were treated with a higher concentration of Vegi-Clean™ (5%) in order to maximize the chance for obtaining a 5 log reduction. Under these conditions, *E. coli* O157:H7 reductions of 2.24 to 3.20 logs were observed with these decreases only slightly higher than those seen using 2% Vegi-Clean™ against *Salmonella* and *Listeria* (Tables 3.4 and 3.5). Adding 5% Vegi-Clean™ to 1 ppm copper ion water did not enhance antimicrobial activity, as reductions ranged from 2.35 to 3.12 logs (Table 3.3).

Considering the effect of the various Vegi-Clean™ concentrations applied alone or in combination with sonication or copper ion, reductions in microbial populations on seeds and sprouts were mainly due to Vegi-Clean™. Although the mechanism of action of acid-anionic surfactants has not been fully elucidated, it is assumed that acid-anionic surfactants play a role in inhibiting key enzyme activities, disrupting the cell membrane, interrupting cellular transport and / or denaturating cellular proteins (Dychdala and Lopes, 1991). In most cases, these microbial reductions were usually greater than those obtained using water alone.

3.4.- CONCLUSIONS

When used alone, sonication or copper ion were no better than water for decreasing pathogen populations on inoculated alfalfa seeds and sprouts. In addition, sonication or copper ion failed to enhance the efficacy of Clorox™, Tsunami™ or Vegi-Clean™. Only NaClO at concentrations higher than 1,000 ppm decreased the microbial load on alfalfa sprouts by more than 5 logs. However, this treatment produced sprouts that were unacceptable due to osmotic dehydration and bleaching. None of the other treatments tested were able to reduce microbial populations to commercially acceptable levels. Therefore, replications of the individual experiments were not carried out.

Fett (2000) documented the presence of natural biofilms on the surface of fruits and vegetables. Moreover, bacterial pathogens can enter plant tissues with organisms remaining protected in seeds and sprouts. Itoh et al.(1998), using immunofluorescence microscopy and scanning electron microscopy, demonstrated the presence of viable and culturable *E. coli* O157:H7 cells in the inner tissues and stomata of cotyledons of radish sprouts grown from experimentally contaminated seeds. These observations help to explain the inability of sonication to efficiently detach bacteria from seeds and sprouts. The ionization state of the copper ion solution was expected to increase contact between surface pathogens and the chemical sanitizers. Biofilm formation and internalization within seeds and sprouts likely played a role in protecting these organisms from the harmful effects of these sanitizers. In contrast, NaClO at concentrations higher than 1,000 ppm were able to enter the sprout tissues by means of a strong osmotic pressure. This was inferred by the fact that, after soaking, the sprouts appeared thoroughly dehydrated and as thin as a strand of hair. This observation

combined with complete bleaching may explain how this sanitizer was able to enter the tissues to destroy these pathogens in sprouts as opposed to seeds.

4.- EFFICACY OF A FATTY ACID-BASED SANITIZER TO INACTIVATE *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT104 AND *LISTERIA MONOCYTOGENES* ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS.

ABSTRACT

Alfalfa seeds were inoculated with a 3-strain cocktail of *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104 or *Listeria monocytogenes* by immersion so as to contain ~ 6 to 8 log CFU/g, then sanitized with an FDA-approved fatty acid based-sanitizer containing 250 ppm peroxyacid (PA), 1,000 ppm caprylic and capric acid (Emery 658), 1,000 ppm lactic acid (LA) and 500 ppm glycerol monolaurate (GM) for a reference concentration of 1x. Concentrations of 5x, 10x and 15x were used for 1, 3, 5 and 10 minutes. The lowest concentration to decrease all three 3 pathogens > 5 logs was 15x. Following a 3 minute immersion, populations of *E. coli* O157:H7, *S.* Typhimurium DT 104 and *L. monocytogenes* decreased >5.45, >5.62 and >6.92 logs, respectively, with no injury and no significant loss in seed germination rate or sprout yield. The components of this 15 x concentration (Treatment A) were assessed alone and in various combinations to optimize inactivation of the three pathogens. During 3 and 5 minutes of exposure, Treatment C (15,000 ppm E 658, 15,000 ppm LA and 7,500 ppm GM) decreased *Salmonella* 6.23 and 5.57 logs, respectively, and *E. coli* 4.77 and 6.29 logs, respectively. Treatment D (15,000 ppm E 658 and 15,000 ppm LA) reduced *Salmonella* > 6.90 logs after all exposures and *E. coli* 4.60 and > 5.18 logs after 3 and 5 minutes, respectively. No significant differences were found between Treatments A, C and D. Overall, Treatment D was most effective in reducing *E. coli* and *Salmonella* populations ~ 5 logs and may provide a viable alternative to the recommended 20,000 ppm chlorine.

4.1.- INTRODUCTION

Alfalfa seeds and sprouts have been incriminated as the source of at least 20 *Escherichia coli* O157:H7 and *Salmonella* sp. outbreaks worldwide and the target of at least 19 Class I recalls (Pönkä et al., 1995; Jaquette et al., 1996; Tauxe et al., 1997; Mahon et al., 1997; Glynn, 1997; Gutierrez, 1997; Como-Sabetti et al., 1997; Hara-Kudo et al., 1997; Itoh et al., 1998, Van Beneden et al., 1999; Watanabe et al., 1999; Taormina, 1999; Taormina et al., 1999; Taormina and Beuchat, 1999a; FDA/NACMCF, 1999; Proctor et al., 2001; Mohle-Boetani et al., 2001 and 2002; Huff, 2002a,b,c; FDA, 2004). In one such *E. coli* O157:H7 outbreak involving 108 cases, 30,000 pounds of sprouts were recalled by a Michigan grower. In a similar outbreak involving *Salmonella* Muenchen in Wisconsin and six other states that was traced to 157 cases, over 12,500 pounds of alfalfa seeds and 2,700 pounds of sprouts were recalled. Thus far, *Listeria monocytogenes* has not been linked to any outbreaks involving the consumption of raw alfalfa sprouts. However, the pathogen has been detected on various vegetable sprouts, leading to two Class I recalls, in 1998 and 2002 as recorded in FDA Enforcement Reports (FDA, 2004). Since 1998, the US has maintained its policy of "zero tolerance" for *L. monocytogenes* in all ready-to-eat and cooked foods (Ryser, 1998).

Numerous microbial reduction strategies including heat (Jaquette et al., 1996), gamma irradiation (Rajkowski and Thayer, 2000), 20,000 ppm calcium hypochlorite (Taormina and Beuchat, 1999a), acidified sodium hypochlorite (Weissinger and Beuchat, 2000), hydrogen peroxide (Beuchat, 1997; Taormina and Beuchat, 1999a; Weissinger and Beuchat, 2000), trisodium phosphate (Taormina and Beuchat, 1999a; Weissinger and Beuchat, 2000), calcium hydroxide (Weissinger and Beuchat, 2000), calcinated calcium

(Weissinger and Beuchat, 2000), active oxygen (Taormina and Beuchat, 1999a; Weissinger and Beuchat, 2000), organic acids (Weissinger and Beuchat, 2000) and allyl isothiocyanate (Park et al., 2000) have been assessed for their ability to decrease bacterial populations > 5 logs on alfalfa seeds while still maintaining a commercially acceptable seed germination rate. However, none of those treatments have proven to be completely successful.

A novel fatty acid-based sanitizer containing peroxyacid, fatty acids (caprylic and capric acids), lactic acid and glycerol monolaurate was recently developed as an antimicrobial dip for ready-to-eat foods. When caprylic, capric and acetic acids are reacted with hydrogen peroxide overnight, peroxycarboxylic acids possessing particularly strong antimicrobial activity are formed (Guthery, 2001, personal communication). Peracetic acid is bactericidal at fairly high concentrations, generally greater than 100 ppm. At pH 3.5, 5 and 10 ppm of peroxycaprylic and peroxycapric acid, respectively, decreased *E. coli* populations 5 logs, in an aqueous model system (Oakes et al., 1993). However, at pH 5, 5 ppm peroxycaprylic and 6 ppm peroxycapric acid, respectively, were noninhibitory to *E. coli* when suspended in distilled water (Oakes et al., 1993). The combination of these acids produced a much more potent biocide than when used individually with antimicrobial activity observed at far lower concentrations (Oakes et al., 1993).

Lactic acid is inhibitory to a wide range of bacterial pathogens (Anderson and Marshall, 1990; Greer and Dilts, 1992). Glycerol monolaurate also possesses antimicrobial activity which has been extensively investigated (Oh and Marshall 1992,1993; Kabara, 1972, 1975, 1979; Kabara et al., 1977). More importantly, the

biocidal activity of these individual antimicrobial agents is greatly enhanced when combined with other food preservatives (Shibasaki and Kato, 1978; Kabara, 1984; Oakes et al., 1993; Bøgh-Sørensen 1994; Marshall and Kim, 1996; Venkitanarayanan et al.,1999).

All components of this novel fatty acid-based sanitizer are GRAS with these non-toxic fatty acids and their esters occurring naturally in foods (Kabara, 1984; Oh and Marshall, 1 993; A ndrews, 1 996). T herefore, they carry a considerable advantage over other types of chemical sanitizers developed to control microorganisms.

The two-fold objective of this study is to (1) determine the optimal concentration and length of exposure to this fatty acid-based sanitizer for decreasing *E. coli* O157:H7, *Salmonella* Typhimurium DT104 and *Listeria monocytogenes* populations 5 logs on alfalfa seeds while maintaining an acceptable germination rate and sprout yield, and (2) optimize the sanitizer formulation and exposure time, by testing its individual components alone and in combination, for inactivation of the aforementioned pathogens with the end-result being a custom-designed antimicrobial formulation for alfalfa seeds that will reduce pathogen populations at least 5 logs without negatively impacting germination rate and sprout yield.

4.2.- MATERIAL AND METHODS

Bacterial strains

Three *Escherichia coli* O157:H7 strains (AR, AD 305 and AD 317) and three *Listeria monocytogenes* strains (LM Brick silage, LM T3BL, LM F5027) were obtained from Dr. Catherine W. Donnelly (Department of Nutrition and Food Sciences, University of Vermont, Burlington, VT). Three *Salmonella* Typhimurium DT104 strains (G1601, G1074 and G10931) were obtained from Dr. Peggy Hayes (Centers for Disease Control and Prevention). Laboratory stock cultures were maintained at – 80 °C in Tryptic Soy Broth (TSB, pH 7.3) (Difco Laboratories, Detroit, MI) containing 10% (v/v) glycerol. Individual strains were separately activated by transferring a loop of the frozen stock culture into 9 ml of sterile TSB containing 0.6% (w/v) yeast extract (YE) (Difco) then incubated at 35°C for 18-24 hours. Thereafter, for each strain, 5 tubes containing 35 ml each of TSB-YE were inoculated and similarly incubated before use.

Preparation of inoculum

The aforementioned cultures were harvested by centrifugation (Sorvall Super T21, Newtown, CT) at 10,000 rpm for 15 min at 4 °C, resuspended in 0.1% peptone (Difco) and combined in equal volumes to prepare three separate 3-strain cocktails containing approximately 10^9 CFU/ml of each pathogen.

Inoculation of alfalfa seeds

Alfalfa seeds originating from Australia were purchased in 25 kg-bags (batch serial # AUS / S / X / 2017) from Dan Caudill (Louisville, KY).

The seeds (500 g) were separately immersed in 525 ml of the 3-strain cocktail and gently agitated for approximately 10 minutes so as to contain 10^6 to 10^8 CFU/g of *E. coli* O157:H7, *S.* Typhimurium DT104 or *L. monocytogenes.* Thereafter, the seeds were dried for 4 h in a laminar flow biosafety cabinet and stored at 22 - 24 °C for at least 24 h before use. All seeds were used within 5 days of inoculation.

Inactivation of pathogens on alfalfa seeds

Sanitizer formulations

Two separate solutions, Asep I and Asep II, were obtained from Dr. B. Eugene Guthery (Broken Bow, OK). Asep I contained the following ingredients (v/v) : 49% propylene glycol (VWR, Chicago, IL), 15% Emery 658 (a mixture of 60% w/w caprylic (octanoic,C_8) and 40% w/w capric (decanoic,C_{10}) acids) (Cognis Corporation, Cincinnati, OH), 10% glacial acetic acid (VWR), 1% sulfuric acid and, added just before use, 25% hydrogen peroxide (50% v/v) (Solvay-Interox, Houston, TX) to activate the solution overnight, in the dark. After 24 h, activated ASEP I contained 10% peroxycarboxilic acid (PA) (a combination of peroxyacetic acid, peroxycaprylic acid and peroxycapric acid). Asep II contained the following ingredients (v/v) : 40% Emery 658, 40% lactic acid (88% v/v) (LA) (JT Baker, Phillipsburg, NJ) and 20% glycerol monolaurate (GM) (Lauricidin, Inc., Okemos, MI).

To obtain 100 ml of the various working solutions, ASEP I and II were diluted as shown in Table 4.1, with concentrations of active components in these solutions provided

in Table 4.2.[1]. Asep I and Asep II were added to rapidly stirring sterile deionized water to produce an emulsion. Aliquots of the sanitizer were taken from the stirring solution and immediately used for inactivation experiments.

TABLE 4.1.- COMPOSITION (ML) OF THE VARIOUS SANITIZER WORKING SOLUTIONS.

SANITIZER CONCENTRATION	ASEP I	ASEP II	WATER
1 X (reference conc.)	0.25	0.25	99.50
5 X	1.25	1.25	97.50
10 X	2.50	2.50	95.00
15 X	3.75	3.75	92.50

TABLE 4.2.- ACTIVE ANTIMICROBIAL COMPONENTS (PPM) OF VARIOUS CONCENTRATION OF THE DILUTED FATTY ACID-BASED SANITIZER WORKING SOLUTIONS

SANITIZER CONC.	ASEP I	ASEP II		
	(PA)	(E 658)	(LA)	(GM)
1 X (ref. conc.)	250	1,000	1,000	500
5 X	1,250	5,000	5,000	1,000
10 X	2,500	10,000	10,000	5,000
15 X	3,750	15,000	15,000	7,500

After identifying the minimal sanitizer concentration needed to decrease any of the three pathogens ≥ 5 logs on inoculated alfalfa seeds, PA, E 658, LA and GM were assessed individually and in various combinations to determine the most efficacious

[1] Some components of this sanitizer theoretically do not possess antimicrobial activity in the concentration given. After activation of ASEP I, the concentration of hydrogen peroxide decreases to non-inhibitory levels. Propylene glycol is used as a solvent in the sanitizer. Although propylene glycol can exhibit antimicrobial activity at concentrations > 70%, the concentration used in this sanitizer (40%) is too low for this purpose. Moreover, a small amount of free fatty acid remains unreacted after ASEP I activation and is not taken in account in Table 4.2 (Guthery, 2002).

formulation for inactivating *E. coli* O157:H7, *S.* Typhimurium DT104 and *L. monocytogenes* ≥ 5 logs. Formulations for these nine antimicrobial solutions tested, designated A through I, are shown in Table 4.3. All nine solutions were prepared by adding the component(s) to stirring sterile deionized water as previously described. GM was melted by heating to approximately 70 °C and was added to stirring water at the same temperature, after which the stirring solution was allowed to return to ambient temperature before use.

TABLE 4.3.- ANTIMICROBIAL SOLUTIONS FORMULATIONS (PPM) INCLUDING PEROXY ACID (PA), EMERY 658 (E658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN VARIOUS COMBINATIONS.

TREATMENT	PA	E 658	LA	GM	pH
A (Asep 1+2)	3,750	15,000	15,000	7,500	2.30
B (Asep 1)	3,750	-	-	-	2.30
C (Asep 2)	-	15,000	15,000	7,500	2.52
D (E658+LA)	-	15,000	15,000	-	2.50
E (E658+GM)	-	15,000	-	7,500	3.40
F (GM+LA)	-	-	15,000	7,500	2.50
G (E658)	-	15,000	-	-	3.22
H (LA)	-	-	15,000	-	2.19
I (GM)	-	-	-	7,500	4.60

Experimental procedure

In order to assess the treatment effect on microbial load of pre-inoculated seeds, an experimental procedure, modified from Taormina and Beuchat (1999a) was adopted. Forty ml of any given sanitizer formula, or sterile deionized water as a control, were added to a 24 oz. sterile stomacher bag (Whirl-pack, Nasco, USA) containing 10 g of

inoculated seeds and manually agitated for 1, 3, 5 and/or 10 minutes. The treatment solution was then discarded and replaced by 40 ml of an appropriate neutralizing buffer after which the sample was homogenized at high speed in a stomacher (Model SD-45 Tekmar Co., Cincinnati, OH) for 2 min. Inoculated control seeds were analyzed by adding 40 ml of buffer to a stomacher bag containing 10 g of seeds, followed by processing in a stomacher.

The neutralizing buffer solution (Guthery, 2001) was prepared by adding 5 g peptone (Difco Laboratories, Detroit, MI), 1 g sodium thiosulfate (JT Baker, Phillipsburg, NJ), 0.25 g mono-potassium phosphate (JT Baker), 0.25 g catalase (Sigma, St Louis, MO), 30 g tween 80 (Sigma) , 10 g lecithin (Sigma) to 1 liter of Letheen broth (Difco).

Microbial Analysis

Determination of bacterial counts

Appropriate dilutions in 0.1% peptone were spiral-plated (Autoplate 4000, Spiral Biotech Inc., Bethesda, MD) in duplicate on Cefixime Tellurite Sorbitol McConkey Agar (CT-SMAC) (SMAC Difco) (CT supplement Dynal, Lake Success, NY) for enumeration of *E. coli* O157:H7, Xylose Lysine Desoxycholate Agar (XLD) (Difco) for *S.* Typhimurium DT104 and Modified Oxford Agar (MOX) (Difco) for *L. monocytogenes.* and Tryptic Soy Agar (TSA) for Mesophilic Aerobic Bacteria with the latter counts being reported in Appendix B.

Reduction in microbial load was calculated by subtracting the microbial count after treatment from the corresponding initial microbial count obtained from the untreated inoculated seed control.

Detection of injured cells

After homogenization in the stomacher, aliquots of the supernatant were also spiral-plated in quadriplicate on Tryptic Soy Agar containing 0.6% (w/v) yeast extract (TSA-YE) (Difco), incubated 4 to 6 h and then overlaid with CT-SMAC for *E. coli* O157:H7, XLD for *S.* Typhimurium DT104 and MOX for *L. monocytogenes.* Numbers of injured cells were determined by subtracting the number of colonies on the selective media (non-injured cells)(S) from those enumerated on overlaid TSA-YE (total pathogen population composed of repaired and non-injured cells)(O).

Germination and Yield Tests

Forty ml of the aforementioned sanitizer formulas were added to a stomacher bag containing 10 g of inoculated seeds. The bag contents were agitated for 1, 3, 5 and / or 10 min after which the sanitizer was discarded and the seeds washed three times by agitating in 150 ml of tap water. The washed alfalfa seeds (treated samples and untreated controls) were placed on moistened 5 x 5 cm cotton squares (Meijer Distribution, Inc, Grand Rapids, MI) in 150 mm-diameter Petri dishes (40 seeds per Petri dish x 3 dishes per replicate x 3 replicates per treatment) and kept moist for 6 days at 22 - 24 °C. Percent germination and sprout yield were then calculated as followed:

$$\% \text{ Germination} = \frac{\text{Number of sprouts per treatment}}{\text{Number of seeds per treatment}} \times 100$$

$$\text{Sprout yield} = \frac{\text{Weight of sprouts per treatment}}{\text{Weight of seeds per treatment}}$$

Statistical analysis

Two-way Analysis of Variance (ANOVA) was done on microbial, germination and yield data using the Statistical Analysis System (Proc Anova, SAS, Version 8, SAS© Institute Inc., Cary, NC) to determine the effect of time and concentration. Data in the tables are means from duplicate or quadriplicate samples from three replicates and were compared using the Tukey-Kramer adjustement at the 95% confidence level ($P = 0.05$).

4.3.- RESULTS

Minimal concentration for reducing of *E. coli* O157:H7, *S.* Typhimurium DT104 and *L. monocytogenes* ≥ 5 logs on alfalfa seeds.

***E. coli* O157:H7.** The effect of three concentrations of this novel fatty acid-based sanitizer (5x, 10x and 15x) on the microbial load of alfalfa seeds previously inoculated with *E. coli* O157:H7 was assessed (Tables 4.4 and 4.5). No bacterial survivors were observed after seeds inoculated with *E. coli* O157:H7 were exposed to the 10x and 15x concentrations for 1 to 10 minutes. Both concentrations decreased *E. coli* populations > 4.90 logs, in a first set of experiments (Table 4.4) and > 5.45 logs (Table 4.5), in a second set of experiments. When the concentration was reduced to 5x, 3.23 and 2.68 log reductions were observed in the *E. coli* O157:H7 population after 5 and 10 minutes of exposure, respectively (Table 4.4). Significant differences ($p < 0.05$) were found between the 5x concentration and the other concentrations and controls. The interaction between concentration and time was not significant for any of the treatments applied to seeds inoculated with *E. coli* O157:H7.

***S.* Typhimurium DT104**. Populations of *S.* Typhimurium DT104 also decreased > 5 logs following 1 to 5 minutes of exposure to the 15x concentration (Table 4.6). However, in contrast to *E. coli* O157:H7, the 10x concentration was significantly less effective against *S.* Typhimurium DT104 ($p < 0.05$) with a maximum reduction of 4.28 logs achieved after a 5 minute exposure. Inactivation of this bacterial population was a function of treatment duration as expressed by a significant concentration-time interaction.

TABLE 4.4.- INACTIVATION OF *E. COLI* O157:H7 ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS INOCULATED TO CONTAIN 6.20 ± 0.11[a] LOGS USING 5X, 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

Sanitizer Concentration	Exposure Time (min)	*E. coli* O157:H7 (log CFU/g)	
		After Treatment	Reduction
0x (Water control)	5	6.24 ± 0.17[a]	- 0.04 ± 0.17[a]
	10	6.32 ± 0.04[a]	- 0.12 ± 0.04[a]
5x	5	2.97 ± 0.17[b]	3.23 ± 0.17[b]
	10	3.52 ± 0.24[b]	2.68 ± 0.24[b]
10x	5	< 1.30 ± 0.00[c]	> 4.90 ± 0.00[c]
	10	< 1.30 ± 0.00[c]	> 4.90 ± 0.00[c]
15x	5	< 1.30 ± 0.00[c]	> 4.90 ± 0.00[c]
	10	< 1.30 ± 0.00[c]	> 4.90 ± 0.00[c]

- Stands for an increase of the microbial load compared to the unwashed inoculated seeds control.
Means ± standard deviation (n = 3)
Means with different letters are significantly different ($p < 0.05$)

TABLE 4.5.- INACTIVATION OF *E. COLI* O157:H7 ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS INOCULATED TO CONTAIN 6.15 ± 0.08^{a} USING 5X, 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		E. coli O157:H7 (log CFU/g)			
		Healthy cells		Healthy / Injured cells	Injured cells
Sanitizer Concentration	Exposure Time (min)	After Treatment (CT-SMAC) (S)	Reduction	After Treatment (TSA-YE/CT-SMAC) (O)	After Treatment (O-S)
0x (Water control)	1	5.94 ± 0.04^{a}	0.20 ± 0.04^{a}		
	3	5.86 ± 0.05^{a}	0.28 ± 0.05^{a}		
	5	6.00 ± 0.05^{a}	0.15 ± 0.05^{a}		
	10	6.04 ± 0.04^{a}	0.10 ± 0.04^{a}		
10x	1	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	3	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	5	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	10	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
15x	1	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	3	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	5	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND
	10	$< 0.70 \pm 0.00^{b}$	$>5.45 \pm 0.00^{b}$	< 0.70	ND

Means ± standard deviation (n = 3). Means with different letters are significantly different ($p < 0.05$) / ND Not detected

TABLE 4.6.- INACTIVATION OF *S.* TYPHIMURIUM DT104 ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS INOCULATED TO CONTAIN 6.32 ± 0.14[a] LOGS USING 5X, 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		Salmonella Typhimurim DT104 (log CFU/g)			
		Healthy cells		Healthy / Injured cells	Injured cells
Sanitizer Concentration	Exposure Time (min)	After Treatment (XLD) (S)	Reduction	After Treatment (TSA-YE / XLD) (O)	After Treatment (O-S)
0x (Water control)	1	5.87 ± 0.23[a]	0.45 ± 0.23[a]		
	3	5.97 ± 0.20[a]	0.35 ± 0.20[a]		
	5	6.04 ± 0.16[a]	0.28 ± 0.16[a]		
10x	1	4.79 ± 0.23[b]	1.53 ± 0.23[b]	4.89	0.10
	3	2.61 ± 0.31[c]	3.71 ± 0.31[c]	2.61	ND
	5	2.04 ± 0.09[c]	4.28 ± 0.09[c]	2.11	0.07
15x	1	< 0.70 ± 0.00[d]	>5.62 ± 0.00[d]	< 0.70	ND
	3	< 0.70 ± 0.00[d]	>5.62 ± 0.00[d]	< 0.70	ND
	5	< 0.70 ± 0.00[d]	>5.62 ± 0.00[d]	< 0.70	ND

Means ± standard deviation (n = 3) Means with different letters are significantly different (p <0.05) / ND Not Detected

L. monocytogenes. Results observed after disinfecting *Listeria*-inoculated alfalfa seeds with a 15x concentration of the fatty-acid based sanitizer (Table 4.7) were similar to *E. coli* O157:H7 and *S.* Typhimurium DT104 (Tables 4.4, 4.5 and 4.6) with populations decreasing > 6.92 logs. The 10x concentration yielded significantly lower ($p < 0.05$) *Listeria* reductions of 2.16, 3.48 and 3.79 logs after 1, 3 and 5 min of exposure, respectively. The concentration-time interaction was not significant.

Extent of cell injury. Regardless of exposure time, no injured cells were detected after applying the 15x concentration to seeds inoculated with the three pathogens. Similar results were obtained using the 10x concentration on alfalfa seeds inoculated with *E. coli* O157:H7.

Optimization of the 15x fatty acid-based sanitizer for inactivation of *E. coli* O157:H7, *S.* Typhimurium DT104 and *L. monocytogenes* on alfalfa seeds.

Treatment A. After identifying 15x (Treament A) as the minimum sanitizer concentration needed to reduce the populations of all three pathogens at least 5 logs on inoculated alfalfa seeds, the sanitizer components (PA, E 658, LA and GM) were assessed, at their 15x concentration, individually and in various combinations to optimize this sanitizer for inactivation of *S.* Typhimurium DT104, *E. coli* O157:H7 and *L. monocytogenes.*

Treatment B. When Treatment B (3,750 ppm PA) was applied for 3 and 5 min, populations of *S.* Typhimurium DT104 and *E. coli* O157:H7 (Tables 4.8 and 4.9) were reduced similarly to the water controls with decreases ranging from 1.01 to 1.91 logs for both pathogens.

TABLE 4.7.- INACTIVATION OF *LISTERIA MONOCYTOGENES* ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS INOCULATED TO CONTAIN 7.62 ± 0.11[a] LOGS USING 5X, 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		L. monocytogenes (log CFU/g)			
		Healthy cells		Healthy / Injured cells	Injured cells
Sanitizer Concentration	Exposure Time (min)	After Treatment (MOX) (S)	Reduction	After Treatment (TSA-YE / MOX) (O)	After Treatment (O-S)
0x (Water control)	1	6.91 ± 0.06[a]	0.71 ± 0.06[a]		
	3	6.99 ± 0.05[a]	0.63 ± 0.05[a]		
	5	6.96 ± 0.01[a]	0.66 ± 0.01[a]		
10x	1	5.46 ± 0.40[b]	2.16 ± 0.40[b]		
	3	4.15 ± 0.38[b]	3.48 ± 0.38[b]		
	5	3.83 ± 0.31[b]	3.79 ± 0.31[b]		
15x	1	< 0.70 ± 0.00[c]	>6.92 ± 0.00[c]	< 0.70	ND
	3	< 0.70 ± 0.00[c]	>6.92 ± 0.00[c]	< 0.70	ND
	5	< 0.70 ± 0.00[c]	>6.92 ± 0.00[c]	< 0.70	ND

Means ± standard deviation (n = 3)

Means in the same column with different letters are significantly different (p <0.05) . / ND Not detected

TABLE 4.8.- INACTIVATION OF *S.* TYPHIMURIUM DT 104 ON ALFALFA SEEDS INOCULATED TO CONTAIN 7.60 ± 0.50 [a] LOGS USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		Salmonella (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment (log CFU/g) (S)	Reduction (log CFU/g)
Water control	3	6.80 ± 0.17 [ab]	0.80 ± 0.17 [ab]
	5	6.91 ± 0.15 [ab]	0.69 ± 0.15 [ab]
A.- (ASEP 1+2)	3	< 0.70 ± 0.00 [c]	> 6.90 ± 0.00 [c]
	5	< 0.70 ± 0.00 [c]	>6.90 ± 0.00 [c]
B.- (ASEP 1)	3	5.87 ± 0.38 [b]	1.73 ± 0.38 [b]
	5	6.00 ± 0.17 [b]	1.80 ± 0.17 [b]
C.- (ASEP 2)	3	1.37 ± 1.15 [c]	6.23 ± 1.15 [c]
	5	2.03 ± 1.15 [c]	5.57 ± 1.15 [c]
D.- (E658 + LA)	3	< 0.70 ± 0.00 [c]	> 6.90 ± 0.00 [c]
	5	< 0.70 ± 0.00 [c]	> 6.90 ± 0.00 [c]
E.- (E658 + GM)	3	6.78 ± 0.69 [ab]	0.82 ± 0.69 [ab]
	5	6.32 ± 0.28 [ab]	1.28 ± 0.28 [ab]
F.- (GM + LA)	3	6.41 ± 0.20 [ab]	1.19 ± 0.20 [ab]
	5	6.50 ± 0.13 [ab]	1.10 ± 0.13 [ab]
G.- (E658)	3	7.16 ± 0.14 [ab]	0.54 ± 0.14 [ab]
	5	7.15 ± 0.07 [ab]	0.55 ± 0.07 [ab]
H.- (LA)	3	6.48 ± 0.17 [ab]	1.12 ± 0.17 [ab]
	5	6.26 ± 0.23 [ab]	1.34 ± 0.23 [ab]
I.- (GM)	3	7.16 ± 0.14 [ab]	0.44 ± 0.14 [ab]
	5	7.15 ± 0.07 [ab]	0.45 ± 0.07 [ab]

Means ± standard deviation (n = 3).
Means with different letters are significantly different ($p < 0.05$)

TABLE 4.9.- INACTIVATION OF *E. COLI* O157:H7 ON ALFALFA SEEDS INOCULATED TO CONTAIN 7.66 ± 0.42 [a] LOGS USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		E.coli O157:H7 (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment	Reduction
Water control	3	7.38 ± 0.51^{ab}	0.28 ± 0.51^{ab}
	5	7.09 ± 0.11^{ab}	0.57 ± 0.11^{ab}
A.- (ASEP 1+2)	3	$< 0.70 \pm 0.00^{f}$	$> 6.96 \pm 0.00^{f}$
	5	$< 0.70 \pm 0.00^{f}$	$>6.96 \pm 0.00^{f}$
B.- (ASEP 1)	3	5.75 ± 0.60^{bc}	1.91 ± 0.60^{bc}
	5	6.65 ± 0.27^{ab}	1.01 ± 0.27^{ab}
C.- (ASEP 2)	3	2.89 ± 0.21^{de}	4.77 ± 0.21^{de}
	5	1.37 ± 1.15^{ef}	6.29 ± 1.15^{ef}
D.- (E658 + LA)	3	3.06 ± 0.42^{de}	4.60 ± 0.42^{de}
	5	$< 2.48 \pm 1.68^{def}$	$> 5.18 \pm 1.68^{def}$

Means ± standard deviation (n = 3).
Means with different letters are significantly different ($p < 0.05$)

Treatment C. In contrast, Treatment C (15,000 ppm E 658, 15,000 ppm LA and 7,500 ppm GM) drastically reduced the microbial load on inoculated alfalfa seeds (Tables 4.8, 4.9 and 4.10). On seeds previously inoculated with *S.* Typhimurium DT104, populations decreased 6.23 and 5.57 logs after 3 and 5 min of exposure, respectively, with these reductions not significantly different ($p < 0.05$) from that observed using Treatment A (Table 4.8). Three and 5 min of exposure to Treatment C led to 4.77 and 6.29 log reductions in *E. coli* O157:H7. Pathogen reductions observed after 5 min of exposure to Treatments C and A were not significantly different (Table 4.9). Exposing *Listeria*-inoculated seeds to Treatment C for 3 and 5 min reduced the pathogen populations 3.86 and 4.21 logs, respectively. These reductions were significantly different ($p < 0.05$) from both Treatment A and the controls (Table 4.10).

Treatment D. Treatment D (15,000 ppm E 658 and 15,000 ppm LA) produced microbial reductions that were similar to or higher than those observed using Treatment C. Treatment D reduced *Salmonella* populations > 6.90 logs, regardless of exposure time, with those reductions similar to those obtained using Treatments A and C (Table 4.8). When *E. coli* O157:H7-inoculated seeds were exposed to Treatment D for 3 and 5 min, reductions of 4.60 and > 5.18 logs, respectively, were observed (Table 4.9). These results were statistically similar to those obtained using Treatment C on *E. coli* O157:H7-inoculated seeds with no statistical differences seen between the 5 minute exposure for Treatments A, C and D. Given the 3.55 and 3.17 log reduction observed after 3 and 5 min exposures, respectively, the effect of Treatment D on alfalfa seeds previously inoculated with *L. monocytogenes* was statistically similar to that of Treatment C, but significantly different from Treatment A (Table 4.10).

TABLE 4.10.- INACTIVATION OF *L. MONOCYTOGENES* ON ALFALFA SEEDS INOCULATED TO CONTAIN 7.31 ± 0.09[a] LOGS USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		L. monocytogenes (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment	Reduction
Water control	3	6.94 ± 0.28^{a}	0.36 ± 0.28^{a}
	5	7.03 ± 0.12^{a}	0.27 ± 0.12^{a}
A- (Asep 1+2)	3	$< 0.70 \pm 0.00^{c}$	$> 6.61 \pm 0.00^{c}$
	5	$< 0.70 \pm 0.00^{c}$	$> 6.61 \pm 0.00^{c}$
C- (ASEP 2)	3	3.45 ± 0.66^{b}	3.86 ± 0.66^{b}
	5	3.10 ± 0.41^{b}	4.21 ± 0.41^{b}
D- (E658 + LA)	3	3.76 ± 0.58^{b}	3.55 ± 0.58^{b}
	5	4.13 ± 0.74^{b}	3.17 ± 0.74^{b}

Means ± standard deviation (n = 3).
Means with different letters are significantly different ($p < 0.05$)

Other Treatments. The remaining treatments (E through I) were generally ineffective, yielding results that were statistically similar to the water control (Table 4.8). In addition, Treatment B and Treatments E through I were equally ineffective and significantly different from Treatments A, C and D.

Washing effect of water

For alfalfa seeds inoculated with the three pathogens, microbial reductions resulting from washing the seeds in water were significantly different ($p < 0.05$) from those obtained using the 5x, 10x and 15x sanitizer concentrations and also from those produced by Treatments A, C and D. No statistical differences were found between the water controls and the corresponding inoculated seeds.

Effect of the fatty acid-based sanitizer on alfalfa seed germination

The impact of the 15x, 10x and 5x sanitizer concentrations on seed germination rate is presented in Tables 4.11 and 4.12. For each treatment time, germination rate decreased as the sanitizer concentration increased; however, significant differences were only seen when seeds were exposed to the 15x concentration for 5 and 10 minutes. No statistically significant differences were found between the treatments after 1 and 3 minutes of soaking; whereas significant differences were observed between some treatments after 5 and 10 minutes. When Treatment A was applied for 3 minutes, a non-statistically significant germination loss of 15.98% was observed (Tables 4.11 and 4.12) indicating that this treatment will ensure a > 5 log reduction in pathogens and an

acceptable germination rate. In addition, a 3 minute exposure to Treatment C reduced the germination rate by only 11.0% (Table 4.12).

Effect of the fatty acid-based sanitizer on alfalfa sprout yield

Sprout yields were usually acceptable following treatment as they did not significantly differ from the untreated controls (Table 4.13). A 3 minute exposure to Treatment A led to a non-significant yield loss of 8.5% ($p < 0.05$). Use of the 15x concentration for 10 minutes was the only treatment that decreased sprout yield greater than 10% with this treatment significantly different from the others (4.13).

TABLE 4.11.- EFFECT OF 3 CONCENTRATIONS OF A FATTY ACID- BASED SANITIZER ON THE GERMINATION RATE OF ALFALFA SEEDS.

Exposure Time (min)	5x		10x		15x	
	% germ.	% loss	% germ	% loss	% germ	% loss
1	95.99 ± 0.89^{a}	$\sim 0 \pm 0.89^{a}$	84.64 ± 2.56^{a}	11.28 ± 2.56^{a}	84.81 ± 1.71^{a}	11.11 ± 1.71^{a}
3	93.54 ± 0.95^{a}	2.38 ± 0.95^{a}	89.09 ± 1.05^{a}	6.83 ± 1.05^{a}	79.94 ± 1.81^{ab}	15.98 ± 1.81^{ab}
5	92.16 ± 1.50^{a}	3.76 ± 1.50^{a}	85.68 ± 2.08^{a}	10.24 ± 2.08^{a}	67.47 ± 6.01^{b}	28.45 ± 6.01^{b}
10	84.49 ± 2.08^{ab}	11.43 ± 2.08^{ab}	79.37 ± 2.85^{ab}	16.55 ± 2.85^{ab}	50.17 ± 8.13^{c}	45.75 ± 8.13^{c}

Percent germination of untreated seeds = $95.92\% \pm 0.55^{a}$

Means ± standard deviation

Means in the same column with different letters are significantly different ($p<0.05$)

TABLE 4.12.- GERMINATION RATE (%) FOR ALFALFA SEEDS AFTER A 3-MINUTE EXPOSURE TO TREATMENTS A AND C.

Treatment	Untreated Seeds	Treated seeds	Germination loss
A	95.92 ± 0.55 [a]	79.94 ± 1.81 [a]	15.98 ± 1.81
C	90.41 ± 0.72 [a]	79.44 ± 1.54 [a]	10.97 ± 1.54

Means ± standard deviation (n = 4)
Means in the same row with similar letters are not significantly different ($p<0.05$)

TABLE 4.13.- EFFECT OF 3 CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER ON ALFALFA SPROUT YIELD.

Treatments	10x			15x		
Exposure time	Sprout yield	Sprout production compared to the individual control (%)	Production loss compared to control (%)	Sprout yield	Sprout production compared to the individual control (%)	Production loss compared to control (%)
Control	10.11	100.00	-	10.52	100.00	-
1 minute	9.81	97.03 ± 5.96^{a}	2.93	10.50	99.80 ± 5.96^{a}	0.20
3 minutes	9.17	90.70 ± 2.81^{a}	9.30	9.63	91.54 ± 2.81^{a}	8.46
5 minutes	9.14	90.40 ± 3.48^{a}	9.60	9.58	91.06 ± 3.48^{a}	8.94
10 minutes	9.17	90.70 ± 6.18^{a}	9.30	7.37	70.05 ± 6.18^{b}	29.95

Means ± standard deviation (n = 3)
Means in the same column with different letters are significantly different ($p < 0.05$)

4.4.- DISCUSSION

This novel FDA-approved fatty acid-based sanitizer was evaluated, for the first time, for inactivating bacterial pathogens on alfalfa seeds. Therefore, scientific publications comparing the performance of this produce sanitizer to others are not yet available.

Overall, the bactericidal performance of the 15x concentration, against the 3 pathogens studied, on inoculated alfalfa seeds, was far superior compared to chlorine and other commonly used chemical sanitizers. No other chemical treatment including 20,000 ppm chlorine (as currently recommended by FDA for the sprout growing industry), Vegi-Clean™, Tsunami™, Clorox™ or Vortexx™ are currently able to achieve a 5 log reduction for the pathogens studied. The 10x concentration demonstrated superior efficacy with *E. coli* O157:H7 populations decreasing > 4.90 logs. Treatments C and D were also exceptional with *E. coli* O157:H7 and *Salmonella* reductions ranging from 4.60 to 6.29 logs and 5.57 to 6.90 logs, respectively.

As presented in Table 4.14, the efficacy of the 5x concentration against *E. coli* O157:H7 is comparable to that seen using calcium hypochlorite (20,000 ppm), hydrogen peroxide (1%), trisodium phosphate (4%), Vortexx™ (40 and 80 ppm) and allyl isothiocyanate (AIT) (2 g wet seeds exposed to an atmosphere containing 53 ppm AIT). The 5x concentration also led to higher microbial reductions than those seen with NaClO (200 and 20,000 ppm), Tsunami™ (80 ppm) and Vegi-Clean™ (1 and 2%) (Table 4.15).

TABLE 4.14.- INACTIVATION OF 2 TO 3 LOGS *E. COLI* O157:H7 ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	REFERENCE
$Ca(OCl)_2$ (20,000 ppm)	~ 2 to 3 in 3 and 10 minutes	Taormina and Beuchat (1999a)
Hydrogen peroxide (1%)	~ 3 in 3 and 10 minutes	Taormina and Beuchat (1999a)
Trisodium phosphate (4%)	~ 2 in 0.5 and 2 minutes	Taormina and Beuchat (1999a)
Vortexx™ (40 and 80 ppm)	~ 2 in 3 and 10 minutes	Taormina and Beuchat (1999a)
AIT (2g wet seeds / 53 ppm)	~ 2 in 24 hours	Park et al. (2000)

TABLE 4.15.- INACTIVATION OF ≤ 1.70 LOGS *E. COLI* O157:H7 ON ARTIFICIALLY CONTMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	REFERENCE
NaClO (200 and 20,000 ppm)	< 1.50 in 0.5 to 10 minutes	Ch 3 section 3.3.4.- Table 3.1
Tsunami™ (80 ppm)	> 1.70 in 3 and 10 minutes	Taormina and Beuchat (1999a)
Tsunami™ (80 ppm)	~ 0.61 to 1.1 in 0.5 to 10 minutes	Ch 3 section 3.3.5.- Table 3.1
Tsunami™ (800 ppm)	~ 0.46 to 0.7 in 0.5 to 10 minutes	Ch 3 section 3.3.5.- Table 3.1
Vegi-Clean™ (1 and 2%)	~ 1.50 to 2.1 in 3 and 10 minutes	Taormina and Beuchat (1999a)
Vegi-Clean™ (1 and 2%)	< 1.50 in 0.5 to 10 minutes	Ch 3 section 3.3.6.- Table 3.1

TABLE 4.16.- INACTIVATION OF ~1 TO 2 LOGS *SALMONELLA* ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	REFERENCE
$Ca(OCl)_2$ (20,000 ppm)	1.95 in 10 minutes	Weissinger and Beuchat (2000)
Tsunami™ (530 and 1,060 ppm)	1.12 and 1.50, respec. / 10 min.	Weissinger and Beuchat (2000)
Tsunami™ (800 ppm)	1.61 in 10 minutes	Ch 3 section 3.3.5.- Table 3.2
Trisodium phosphate (2 and 5%)	0.90 and 1.99, respec. / 10 min.	Weissinger and Beuchat (2000)
Acid. NaClO (500 & 1,200 ppm)	1.26 and 1.43, respec. / 10 min.	Weissinger and Beuchat (2000)
Vortexx™ (530 and 1,060 ppm)	1.29 and 1.62, respec. / 10 min.	Weissinger and Beuchat (2000)
Lactic acid (2%)	1.19 in 10 minutes	Weissinger and Beuchat (2000)
Acetic acid (5%)	1.74 in 10 minutes	Weissinger and Beuchat (2000)

TABLE 4.17.- INACTIVATION OF ~ 2 .8 TO 3.2 LOGS *SALMONELLA* ON ARTIFICIALLY CONTAMINATED ALFALFA SEEDS USING CHEMICAL SANITIZERS.

SANITIZERS	REDUCTION (Logs)	REFERENCE
Hydrogen peroxide (8%)	3.22 in 10 minutes	Weissinger and Beuchat (2000)
Calcium hydroxide (1%)	2.84 in 10 minutes	Weissinger and Beuchat (2000)
Calcinated calcium (1%)	2.88 in 10 minutes	Weissinger and Beuchat (2000)
Lactic (5%)	2.98 in 10 minutes	Weissinger and Beuchat (2000)
Citric acid (5%)	2.98 in 10 minutes	Weissinger and Beuchat (2000)

Reductions for *E. coli* O157:H7 on alfalfa seeds using Treatment B (3,750 ppm peroxyacid) are similar to those obtained by Taormina and Beuchat (1999a) using peroxyacid-based sanitizers such as Tsunami™ (80 ppm) and Vortexx™ (40 and 80 ppm) (Tables 4.14 and 4.15). However, as evident from Table 4.15, increasing the peroxyacid concentration exponentially does not result in higher reductions.

The bactericidal efficacy of the 10x sanitizer concentration against *Salmonella* on alfalfa seeds increased progressively with duration of exposure. A 1-minute exposure yielded a 1.50 log reduction in *Salmonella* which is similar to that seen using calcium hypochlorite (20,000 ppm), trisodium phosphate (2 and 5%), acidified NaClO (500 and 1,200 ppm), Vortexx™ (530 and 1,060 ppm), acetic acid (5%) and lactic acid (2%) (Table 4.16). Similar efficacy was also demonstrated by Treatment B with reductions comparable to those seen using Tsunami™ (530 and 1,060 ppm) and Vortexx™ (530 and 1,060 ppm) (Table 4.16). Reductions obtained after 3 and 5 minutes of exposure to Treatment H (1.5% lactic acid) agree with those obtained by Weissinger and Beuchat (2000) after applying 2% lactic acid to alfalfa seeds contaminated with *Salmonella* (Table 4.16).

When the 10x concentration of this fatty-acid based sanitizer was applied for 3 minutes, *Salmonella* populations decreased 3.71 logs which is equal to or greater than the reductions obtained after treatment with hydrogen peroxide (8%), calcium hydroxide (1%), calcinated calcium (1%), lactic (5%) and citric acid (5%) (Table 4.17). Similar reductions were obtained using heat (57 to 60 °C) on alfalfa seeds, with *Salmonella* populations decreasing < 3 logs, after 5 minutes (Jaquette et al., 1996).

Given the 4.28 log reduction observed for *Salmonella*, efficacy of the 10x concentration, when applied for 5 minutes, exceeded that of the other treatments in Table 4.17.

Jaquette et al. (1996) stated that seeds are covered with a waxy material that repels water. This waxy covering limits the efficacy of the aforementioned chemical sanitizers (Tables 4.14 to 4.17) when compared to the potency of this fatty acid-based sanitizer. While readily soluble in water, bactericidal potency of these other chemical sanitizers is partly based on the extent of contact with surface and internalized bacteria. This may explain why, after a certain dose, higher sanitizer concentrations do not lead to higher microbial reductions. Fatty acids are non-polar and, as such, have more affinity with the waxy material covering seeds. The components of this sanitizer are insoluble in water and, upon rapid agitation, form an emulsion, with microscopic droplets adhering to the seed surface. Thus, this more intimate contact allows the active ingredients to more easily penetrate the seed and disrupt the bacterial cell membranes.

Peroxyfatty acids form peroxides and other free radicals which have the ability to inhibit bacterial growth (Kabara, 1979; Hinton and Ingram, 2000). Peroxyacetic acid, like other peroxides and oxidizing agents, is assumed to oxidize sensitive sulfhydryl and sulfur bonds in proteins, enzymes and other metabolites. Peroxyacetic acid may also disrupt the chemiosmotic function of the lipoprotein cytoplasmic membrane and transport through dislocation or rupture of cell walls (Baldry and Fraser, 1988; Block, 1991).

Fatty acids typically function as surface-active anionic detergents (Kabara et al., 1972) and are capable of uncoupling both substrate transport and oxidative phosphorylation from the electron transport system (Freese et al., 1973). They act by

disrupting the bacterial cell membrane and lysing protoplasts as evidenced by the leakage of 260 nm-absorbing material, protein and other internal metabolites. Thus, fatty acids prevent bacterial growth by modifying cell membrane permeability which leads to uptake changes and/or inhibition of oxygen, amino-acids, nucleic acids, organic acids, phosphates and other substrate molecules (Freese et al., 1973; Kabara, 1979; Oh and Marshall, 1992, 1993; Galbraith and Miller, 1973 a,b,c; Doores, 1983, 1993; Hinton and Ingram, 2000). Although saturated fatty acids inhibit cellular oxygen consumption, they do not inhibit NADH oxidation by isolated membranes which is controlled by the cytochrome-linked electron transport system. Therefore, inhibition of oxygen consumption in whole cells must result from the deficiency of compounds that yield electrons entering the electron transport chain (Freese et al., 1973). Inhibition of cellular uptake, however, does not necessarily prove that transport itself is inhibited; it may merely reflect inhibition of metabolism with the unmetabolized compound preventing its own uptake (Freese et al., 1973).

Only certain short chains fatty acids generally affect gram-negative organisms (Sheu and Freese, 1973; Kabara et al., 1977). Fatty acids, up to eight carbon in length which completely inhibit *E. coli* do not reduce the concentration of ATP/A_{600} measured after 20 or 40 minutes of incubation (Freese et al., 1973). Apparently, in *E. coli*, some other compound is exhausted before ATP (Freese et al., 1973). When compared to C_6 fatty acids, twice the concentration of C_8 and about 50 times the concentration of C_{10} is required to inhibit *E. coli* with no inhibition observed with longer chain fatty acids (Freese et al., 1973). As a possible explanation, the lipopolysaccharide (LPS) layer that typically surrounds the cell wall of gram-negative organisms may screen out the larger

compounds (Freese et al.,1973, Doores, 1983). The short chain fatty acids (caprylic C_8 and capric C_{10}) used in this sanitizer are within the lethal range for gram-negative bacteria such as *E. coli* O157:H7 and *Salmonella.* However, application of 15,000 ppm E 658 on alfalfa seeds artificially contaminated with *S.* Typhimurium DT104 did not result in statistically significant reductions when compared to the water control (Table 4.8). These observations appear more in accordance with another theory proposing that gram-negative organisms can rapidly metabolize fatty acids with little or no accumulation within the cell (Kabara et al. 1972; Freese et al., 1973; Doores, 1983 and 1993).

Most components of this antimicrobial sanitizer including the fatty acids are organic acids, and as such, are weak acids. In aqueous solution, weak acids are only slightly ionized and do not readily give up their proton(s) to water (Doores, 1983 and 1993). At a pH lower than the pk_a, the equilibrium shifts toward the undissociated state. Freese et al. (1973), Woolford (1975) and Doores (1983 and 1993) reported that the antibacterial efficacy of weak acids was a function of the undissociated molecule. Membranes are less permeable to charged as compared to uncharged molecules (Doores, 1983 and 1993). Therefore, bacterial inhibition by organic acids increases with decreasing pH, in agreement with the pk_a values (Freese et al., 1973; Doores, 1983 and 1993).

According to Galbraith and Miller (1973) and Oh and Marshall (1992, 1993), lowering the pH of the suspending medium increases fatty acid uptake and reduces the interfacial tension at the bacterial lipid membrane/aqueous medium interface. This observation suggests that addition of lactic acid to Emery 658 which resulted in a pH decrease from 3.2 to 2.5 (Table 4.3), may partially account for the dramatic reduction in

microbial load on alfalfa seeds previously inoculated with *S.* Typhimurium DT104 (Table 4.8).

Hunter and Segel (1973) and Doores, (1983 and 1993) reported that weak acids at or below their pk_a could discharge the proton gradient and ionize within the cell to acidify the interior. It was postulated that the rate of proton leakage into the cell versus proton ejection would determine the extent of inhibition (Freese et al., 1973; Doores, 1983). Peracetic acid, upon contact with organic substrates, decomposes to yield oxygen and acetic acid (Doores, 1983). Antibacterial action of acetic acid is partially due to lowering of the intracellular pH below that which is optimal for growth. The same is also true for lactic acid used alone as a microbicide. Indeed, cellular proteins, nucleic acids and phospholipids can be structurally altered by pH changes (Doores, 1983 and 1993).

Lactic acid also acts as a chelating agent in this fatty acid-based sanitizer. The cation chelating properties of lactic acid are comparable to that of other metal chelators such as ethylenediaminetetraacetic acid (EDTA), citric acid, sodium acid pyrophosphate and polyphosphoric acid (Guthery, 1993 and 1994; Andrews, 1996).

Antimicrobial activity of the fatty acids and fatty acid esters increases dramatically when calcium or magnesium chelators are used to lower the pH to < 4.0 (Andrews, 1996; Guthery, 1993 and 1994). Although active against gram-positive bacteria, fatty acids are not effective by themselves against gram-negative bacteria at a neutral pH (Guthery, 1993). However, according to Guthery (1993), the effectiveness of fatty acids against gram-negative bacteria, such as *Salmonella,* is enhanced in the presence of citric acid.

Gram-negative bacteria have a lipopolysaccharide (LPS) layer which acts as a permeability barrier to prevent some compounds from penetrating the cell membrane. When added to foods, chelating compounds can sequester trace metals such as magnesium, calcium, iron, sodium and potassium cations present in the LPS layer of gram-negative bacteria (Boland et al., 2003). Chelation of metal ions from microorganisms, specifically gram-negative bacteria, increases the antimicrobial spectrum of bactericidal compounds by apparently destabilizing the LPS layer and increasing cell sensitivity by allowing these aforementioned compounds to penetrate the LPS layer, resulting in cell lysis (Boland et al., 2003). Magnesium assists in cell division and many membrane-bound enzyme reactions (Knabel et al., 1991; Boland et al., 2003). Magnesium aids calcium in cross-linking negatively charged groups to form salt bridges to bind polysaccharides on the surface of gram-negative bacteria. Formation of metal complexes with magnesium and calcium can result in leakage of cell solutes and loss of viability. Iron is essential for growth, replication, respiration and DNA synthesis in bacterial cells. Chelation of such essential cations from the LPS layer may disrupt the structural or functional integrity of the LPS, thus allowing other antimicrobial compounds to penetrate the cell wall (Boland et al., 2003).

The theory of lactic acid acting as a chelator of metal cations, increasing synergistically the antimicrobial effect of short chain fatty acids (Emery 658) against gram-negative bacteria is supported by the dramatic reduction observed when alfalfa seeds inoculated with *S.* Typhimurium DT104 and *E. coli* O157:H7 were exposed to Treatment D (Tables 4.8 and 4.9). This chelation mechanism appears to be primarily responsible for antimicrobial activity since the outcome of Treatment D was not

significantly different from Treatments A and C for *S.* Typhimurium and, generally, not significanly different from those of Treatments A and C for *E. coli* O157:H7. Knabel et al., (1991) observed that chelators, such as polyphosphates, can inhibit gram-positive bacteria by removing essential metals from cation-binding sites within their walls. This explaination may also account for the reduction, although more modest, in the bacterial load present on alfalfa seeds artificially contaminated with *L. monocytogenes* (Table 4.10).

Glycerol monolaurate was reported by Vadehra et al. (1985) and Oh and Marshall (1992) to cause extensive leakage of 260 nm-absorbing intracellular proteins from bacterial cells. However, in our study, 7,500 ppm GM did not result in statistically significant reductions in *S.* Typhimurium DT104 when compared to the inoculated seed and water controls. These results agree with those of Venkitanayaranan et al. (1999) in that 50 ppm GM did not yield a 5 log reduction for *E. coli* O157:H7 when suspended in 0.1% peptone. Although a lower pH may increase the uptake of glycerol monolaurate (Oh and Marshall, 1993), efficacy of GM was not increased by adding 15,000 ppm lactic acid to 7,500 ppm GM or by lowering the pH of the treatment solution from 4.6 to 2.5.

According to Kabara (1979) and Oh and Marshall (1992), GM has broad spectrum antimicrobial activity in culture media against gram-positive microorganisms. Therefore, the poor performance of GM (ester of C_{12} fatty acid) against gram-negative bacteria, including *S.* Typhimurium DT104 and *E. coli* O157:H7, agrees with the aforementioned observation in that inhibition of gram-negative bacteria is increasingly inefficient as the fatty acid chain length increases from C_6 to C_{10} and higher (Freese et al., 1973). In contrast, Kato and Shibasaki (1976) and Oh and Marshall (1992) showed that GM is also

effective against gram-negative bacteria in culture media containing citric and polyphosphoric acid, both of which are chelators of metallic ions (Andrews, 1996). Given that the combination GM and lactic acid (Treatment F) was less than optimal against *Salmonella* on alfalfa seeds (Table 4.8), being an ester of a C_{12} chain fatty acid, it may have been less effective against this gram-negative pathogen due to its longer chain length compared to Emery 658. Moreover, organic components of the seed itself may have neutralized the antimicrobial potency of GM since Shibasaki and Kato (1978) stated that the activity of GM was neutralized by addition of starch and gelatin.

Fatty acids and their related compounds disrupt bacterial respiration (Freese et al., 1973). However, they may also adversely affect the respiratory mechanism in seeds with the low pH of the sanitizer solution (Table 4.3) perhaps accounting for the loss in germination.

Chemical sanitizing of alfalfa seeds usually results in a lower germination rate. *Salmonella* populations decreased 1.62 logs using 1,060 ppm Vortexx™ (Table 4.16) and 3.22 logs using 8% hydrogen peroxide (Table 4.17) with no effect on germination (Weissinger and Beuchat, 2000). The same authors experienced germination losses of 45.6%, 35.5% and 10.9% after application of 5% of acetic acid, lactic acid or citric acid, respectively, for corresponding *Salmonella* reductions of 1.74, 2.98 and 2.98 logs. Weissinger and Beuchat (2000) also reported a 1.95 log decrease in *Salmonella* using 20,000 ppm $Ca(ClO)_2$ (Table 4.4.3) with a significantly lower germination rate of 91.6% compared to the control. Beuchat et al. (2001) observed a germination loss of 10 to 12% using Fit™ or 20,000 ppm chlorine for 30 minutes which led to a 2.3 log reduction in *Salmonella*. After treating alfalfa seeds with 20,000 ppm $Ca(ClO)_2$, Taormina and

Beuchat (1999a) reported a 2.93 log reduction in *E. coli* after 3 minutes (Table 4.14) along with a 70.3 % germination rate compared to an initial rate of 78.3%. A 10 minute exposure to 70% ethanol reduced the numbers of mesophilic aerobic bacteria 2 to 4 logs on rice, but only 11.5 % of rice seeds germinated after treatment, producing abnormal seedlings (Piernas and Guiraud, 1997).

These observations lead to the conclusion that the non statistically significant 15.98% germination loss observed after a 3 minute exposure to the 15x concentration of our fatty acid-based sanitizer (Treatment A) was acceptable considering that this treatment reduced populations of all three pathogens ~ 5 to 7 logs. However, the 3 minute exposure to Treatment C, resulting in a non statistically significant 10.97% germination loss, led to reductions of 6.23, 4.77 and 3.86 logs for *S.* Typhimurium DT104, *E. coli* O157:H7 and *L. moncytogenes*, respectively. Hence, Treatment C may be a reasonable compromise between pathogen inactivation and germination rate preservation, since a pathogen population of 2.5 logs on alfalfa seeds would be considered large in a commercial setting and, in reality, would be unlikely to occur. On alfalfa seeds, pathogen populations would likely be at least 100-fold lower (Jaquette et al., 1996). Considering that microbial population on alfalfa seeds are usually < 2 log CFU/g and that *Listeria* has never been involved in any alfalfa sprout related outbreaks, the 3 minute exposure to Treatment D which decreased *S.* Typhimurium DT104, *E. coli* O157:H7 and *L. monocytogenes* populations > 6.90, 4.60 and 3.55 logs, respectively, may be the best option for sprout growers.

Given that no statistical difference in pathogen reduction was seen for Treatments A, C and D, the combination of Emery 658 and lactic acid is primarily responsible for

inactivating *S.* Typhimurium DT104 and *E. coli* O157:H7. Therefore, further investigations should be done to determine the optimum ratio between the aforementioned compounds for attaining a > 5 log reduction in *S.* Typhimurium DT 104 and *E. coli* O157:H7 on alfalfa seeds while maintaining a germination loss of < 10%. Moreover, it is surmised that applications of this novel FDA-approved fatty acid-based sanitizer, as a whole, and the combination of Emery 658 and lactic acid, in particular, should prove to be of great interest to sprout growers.

5.- CONCLUSION

Exposing alfalfa seeds and sprouts previously inoculated with *Escherichia coli* O157:H7, *Salmonella* Typhimurium DT104 and *Listeria monocytogenes* to Clorox™ (sodium hypochlorite, 200 to 20,000 ppm), Tsunami™ (peroxyacetic acid / hydrogen peroxide, 80 and 800 ppm) or Vegi-Clean™ (anionic surfactant, 1%, 2%, 5%), applied for 30 seconds to 10 minutes did not offer a commercially desirable alternative (> 5 log reduction) to the currently FDA-recommended 20,000 ppm calcium hypochlorite. Maximum reductions in *E. coli* O157:H7 and *S.* Typhimurium populations on the seeds were, respectively, 1.57 and 1.92 logs after treatment with Clorox™, 1.11 and 3.32 logs, after treatment with Tsunami™ and 1.60 and 1.32 logs after treatment with Vegi-Clean™ compared to ~ 2 to 3 and 1.95 logs after treatment with 20,000 ppm chlorine. Only sodium hypochlorite (> 1,000 ppm) was able to reduce the three pathogens on sprouts >5 logs, however, these sprouts were deemed to be organoleptically unacceptable due to osmotic dehydration and bleaching.

When used alone, ultrasound (20 kHz) generated by a sonicating water bath or copper ions(1ppm) generated by an electrolytic process and dispersed into a circulating water stream were no better than water for decreasing pathogen populations on inoculated alfalfa seeds and sprouts. In addition, sonication or copper ion failed to enhance the efficacy of Clorox™, Tsunami™ or Vegi-Clean™.

Presence of hidden bacteria in crevices and internalization of bacterial pathogens in inner tissues with organisms remaining protected in seeds and sprouts likely played a role in protecting these organisms from the harmful effects of these sanitizers. These observations help to explain the inability of sonication to efficiently

detach bacteria from seeds and sprouts, as well as the failure of the ionization state of the copper ion solution to increase contact between surface pathogens and the chemical sanitizers. In contrast, NaClO at concentrations higher than 1,000 ppm was able to enter the sprout tissues by means of a strong osmotic pressure. This was inferred by the fact that, after soaking, the sprouts appeared thoroughly dehydrated and completely bleached. This observation may explain how this sanitizer was able to enter the tissues to destroy these pathogens in sprouts as opposed to seeds.

Further research indicated that a novel FDA-approved fatty acid based-sanitizer containing a combination of 3,750 ppm peroxyacid (PA), 15,000 ppm caprylic and capric acid (Emery 658), 15,000 ppm lactic acid (LA) and 7,500 ppm glycerol monolaurate (GM) provided a successful alternative to the currently recommended 20,000 ppm calcium hypochlorite with populations of *E. coli* O157:H7, *S.* Typhimurium DT 104 and *L. monocytogenes* decreasing >5.45, >5.62 and >6.92 logs, respectively, after 3 minutes of exposure. Additionally no injury and no significant loss in seed germination rate or sprout yield was seen.

When the components of this sanitizer were assessed alone and in various combinations to optimize inactivation of the three pathogens, the combination of 15,000 ppm Emery 658, 15,000 ppm LA and 7,500 ppm GM, applied for 3 and 5 minutes, decreased *S.* Typhimurium DT104 population 6.23 and 5.57 logs, respectively, and *E. coli* O157:H7 population 4.77 and 6.29 logs, respectively, on inoculated alfalfa seeds. The combination of 15,000 ppm Emery 658 and 15,000 ppm LA reduced *S.* Typhimurium DT104 > 6.90 logs after all exposures and *E. coli* O157:H7 4.60 and > 5.18 logs after 3 and 5 minutes, respectively. No significant differences were found between these 3

sanitizer combinations. Overall, the E 658 and LA combination was most effective in reducing *E. coli* and *Salmonella* populations ~ 5 logs and represents the most viable alternative to the recommended 20,000 ppm chlorine. Lactic acid likely acts as a chelator of metal cations, increasing synergistically the antimicrobial effect of short chain fatty acids (E 658) by destabilizing the bacterial cell lipopolysaccharide layer and allowing the fatty acid to penetrate the wall.

Based on these findings, this novel FDA-approved fatty acid-based sanitizer and the combination of Emery 658 and lactic acid, in particular, should be of great interest to sprout growers, in particular and to the produce industry, in general.

Future research goals focusing on the microbial safety of alfalfa sprouts would include the following:

- Determination of the most efficacious ratio between lactic acid and Emery 658 to improve inactivation of *E. coli* O157:H7, *S.* Typhimurium DT104 and *L. monocytogenes* while maintaining an acceptable germination rate for alfalfa seeds.
- Evaluation of the microbial load in alfalfa sprouts grown from seeds previously inoculated to several levels of pathogen and disinfected with treatment the optimized lactic acid and Emery 658 mixture.
- Scale-up of the FDA-approved sanitizer treatment for commercial sprout growers.
- Assessment of pathogen penetration into alfalfa seeds and subsequent inactivation by the FDA-approved sanitizer using confocal microscopy.

APPENDIX A

REDUCTION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS AND SPROUTS PREVIOUSLY INOCULATED WITH *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT104 AND *LISTERIA MONOCYTOGENES* USING COMMERCIAL CHEMICAL SANITIZERS (CLOROX™, TSUNAMI™, VEGI-CLEAN™) AND SONICATION OR COPPER IONS.

TABLE A.1.- REDUCTION OF MESOPHILIC AEROBIC BACTERIA (log_{10} CFU/g) ON ALFALFA SEEDS PREVIOUSLY INOCULATED WITH *ESCHERICHIA COLI* O157:H7 USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.71		0.80		0.28
Water control *	0.63		0.68		0.30
Clorox 200 ppm	1.15		1.17		0.51
Water control	0.63		0.68		0.30
Clorox 20,000 ppm	1.41	1.15	1.41	1.45	1.67
Water control	0.17	0.32	0.21	0.24	0.19
Clorox 200 ppm + sonication	1.59		1.33		0.94
Water control	0.63		0.68		0.30
Tsunami 80 ppm	1.75		0.57		0.52
Water control	0.63		0.68		0.30
Tsunami 800 ppm	0.71	0.67	0.72	0.82	0.78
Water control	0.17	0.32	0.21	0.24	0.19
Tsunami 80 ppm + sonication	1.68		1.39		1.07
Water control	0.63		0.68		0.30
Vegi-Clean 1%	1.41		1.31		0.97
Water control	0.63		0.68		0.30
Vegi-Clean 2%	0.71	0.78	0.87	0.74	1.01
Water control	0.17	0.32	0.21	0.24	0.19
Vegi-Clean 1% + sonication	1.23		1.57		1.50
Water control	0.63		0.68		0.30
Copper ion 1 ppm	0.43				0.17
Water control	0.63		0.68		0.30
Copper ion + sonication	1.07				0.75
Water control	0.63		0.68		0.30

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

TABLE A.2.- REDUCTION OF MESOPHILIC AEROBIC BACTERIA (log_{10} CFU/g) ON ALFALFA SEEDS PREVIOUSLY INOCULATED WITH *SALMONELLA* TYPHIMURIUM DT104 USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.42	0.39	0.41	0.58	0.39
Water control *	0.39	0.20	0.28	0.16	0.28
Clorox 200 ppm	1.45	1.69	1.66	1.16	1.33
Water control	0.39	0.20	0.28	0.16	0.28
Clorox 20,000 ppm	0.59	0.56	0.58	0.67	0.73
Water control	0.46	0.91	0.81	0.93	0.76
Clorox 200 ppm + sonication	1.47	1.45	1.24	1.85	1.61
Water control	0.39	0.20	0.28	0.16	0.28
Tsunami 80 ppm	1.68	2.98	2.98	2.98	2.98
Water control	0.39	0.20	0.28	0.16	0.28
Tsunami 800 ppm	1.42	1.46	1.08	1.03	1.73
Water control	0.46	0.91	0.81	0.93	0.76
Tsunami 80 ppm + sonication	2.49	1.47	0.76	1.85	1.86
Water control	0.39	0.20	0.28	0.16	0.28
Vegi-Clean 1%	1.48	1.47	1.47	1.36	0.98
Water control	0.39	0.20	0.28	0.16	0.28
Vegi-Clean 2%	1.55	1.00	2.55	2.61	2.35
Water control	0.46	0.91	0.81	0.93	0.76
Veg.-Clean 1% + sonication	1.20	1.51	2.02	1.63	1.35
Water control	0.39	0.20	0.28	0.16	0.28
Copper ion 1 ppm	1.13	0.49	0.47	0.42	0.42
Water control	0.39	0.20	0.28	0.16	0.28
Copper ion + sonication	0.39	0.85	0.49	0.64	0.51
Water control	0.39	0.20	0.28	0.16	0.28

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

TABLE A.3.- REDUCTION OF MESOPHILIC AEROBIC BACTERIA (log_{10} CFU/g) ON ALFALFA SPROUTS PREVIOUSLY INOCULATED *ESCHERICHIA COLI* O157:H7 USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Clorox 1,000 ppm	3.57		4.24	4.78	5.49
Water control*			1.08	1.02	0.79
Clorox 2,000 ppm	3.67		5.02		5.77
Water control			1.08	1.02	0.79
Clorox 10,000 ppm	3.94	3.90	4.29	4.67	5.73
Water control	0.81	0.70	0.75	0.77	0.76
Clorox10,000 ppm+Cu ion	3.86	3.89	4.61	5.18	5.25
Water control	0.81	0.70	0.75	0.77	0.76
Vegi-Clean 5%	1.31	1.76	1.73	2.08	2.70
Water control	0.43		0.80	0.60	0.38
Vegi-Clean 5%+copper ion	1.40	1.76	1.80	1.88	2.36
Water control	0.43		0.80	0.60	0.38

* All water control data in this table have to be compared to the treatment results reported on the row placed above them

TABLE A.4.- REDUCTION OF MESOPHILIC AEROBIC BACTERIA (log_{10} CFU/g) ON ALFALFA SPROUTS PREVIOUSLY INOCULATED WITH *SALMONELLA* TYPHIMURIUM DT104 USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Sonication alone	0.51		0.78		0.87
Water control *	0.61	0.41	0.78	0.70	1.07
Clorox 200 ppm	0.82	1.36	1.60	2.27	2.20
Water control	0.61	0.41	0.78	0.70	1.07
Clorox 20,000 ppm	3.80	3.38	2.90	3.10	5.34
Water control	0.38	0.57	0.47	0.43	0.42
Clorox 200 ppm + sonication	1.22	1.34	2.38	2.85	2.73
Water control	0.61	0.41	0.78	0.70	1.07
Tsunami 80 ppm	0.94	0.85	0.89	0.92	1.15
Water control	0.61	0.41	0.78	0.70	1.07
Tsunami 800 ppm	2.08	2.23	2.78	3.04	3.18
Water control	0.38	0.57	0.47	0.43	0.42
Tsunami 80 ppm + sonication	0.85	1.15	2.31	1.46	1.51
Water control	0.61	0.41	0.78	0.70	1.07
Vegi-Clean 1%	0.82	1.25	1.50	1.39	1.67
Water control	0.61	0.41	0.78	0.70	1.07
Vegi-Clean 2%	1.15	1.30	1.43	1.39	1.67
Water control	0.38	0.57	0.47	0.43	0.42
Veg.-Clean 1% + sonication	0.70	1.30	1.51	1.57	2.58
Water control	0.61	0.41	0.78	0.70	1.07
Copper ion 1 ppm	0.73	0.33	0.48	0.42	0.82
Water control	0.61	0.41	0.78	0.70	1.07
Copper ion + sonication		0.22	0.34	0.45	0.77
Water control	0.61	0.41	0.78	0.70	1.07

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

TABLE A.5.- REDUCTION OF MESOPHILIC AEROBIC BACTERIA (log_{10} CFU/g) ON ALFALFA SPROUTS PREVIOUSLY INOCULATED WITH *LISTERIA MONOCYTOGENES* USING VARIOUS ANTIMICROBIAL TREATMENTS.

TREATMENT	TIME				
	30 sec	1 min	3 min	5 min	10 min
Clorox 20,000 ppm	2.46	3.47	3.59	4.59	5.53
Water control*	0.64	0.45	0.49	0.53	0.40
Vegi-Clean 2%	0.97	1.18	1.60	1.85	1.90
Water control	0.64	0.45	0.49	0.53	0.40

* All water control data in this table have to be compared to the treatment results reported on the row placed above them.

APPENDIX B

EFFICACY OF A FATTY ACID-BASED SANITIZER TO INACTIVATE MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS ARTIFICIALLY CONTAMINATED *ESCHERICHIA COLI* O157:H7, *SALMONELLA* TYPHIMURIUM DT104 AND *LISTERIA MONOCYTOGENES.*

TABLE B.1.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS ARTIFICIALLY CONTAMINATED WITH *E. COLI* O157:H7 USING 5X, 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Concentration	Exposure Time (min)	After Treatment	Reduction
0x (Water control)	5	7.05 ± 0.10^{a}	0.12 ± 0.10^{a}
	10	6.99 ± 0.05^{a}	0.18 ± 0.05^{a}
5x	5	4.65 ± 0.04^{b}	2.52 ± 0.04^{b}
	10	4.98 ± 0.01^{b}	2.19 ± 0.01^{b}
10x	5	$< 1.30 \pm 0.00^{c}$	$> 5.88 \pm 0.00^{c}$
	10	$< 1.76 \pm 0.47^{c}$	$> 5.42 \pm 0.47^{c}$
15x	5	$< 1.30 \pm 0.00^{c}$	$> 5.88 \pm 0.00^{c}$
	10	$< 1.30 \pm 0.00^{c}$	$> 5.88 \pm 0.00^{c}$

Inoculated seeds average: 7.18 ± 0.04^{a} log CFU/g
Means ± standard deviation (n = 3)
Means with different letters are significantly different ($p < 0.05$)

TABLE B.2.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS ARTIFICIALLY CONTAMINATED WITH *E. COLI* O157:H7 USING 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Concentration	Exposure Time (min)	After Treatment	Reduction
0x (Water control)	1	6.91 ± 0.05^{a}	0.27 ± 0.05^{a}
	3	6.83 ± 0.11^{a}	0.34 ± 0.11^{a}
	5	6.90 ± 0.08^{a}	0.27 ± 0.08^{a}
	10	6.97 ± 0.08^{a}	0.20 ± 0.08^{a}
10x	1	$< 1.16 \pm 0.46^{b}$	$>6.02 \pm 0.46^{b}$
	3	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$
	5	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$
	10	$< 1.16 \pm 0.46^{b}$	$>6.02 \pm 0.46^{b}$
15x	1	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$
	3	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$
	5	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$
	10	$< 0.70 \pm 0.00^{b}$	$>6.48 \pm 0.00^{b}$

Inoculated seeds average: 7.18 ± 0.08^{a} log CFU/g
Means ± standard deviation (n = 3)
Means with different letters are significantly different ($p < 0.05$)

TABLE B.3.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS ARTIFICIALLY CONTAMINATED WITH *SALMONELLA* TYPHIMURIUM DT 104 USING 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Concentration	Exposure Time (min)	After Treatment	Reduction
0x	1	7.45 ± 0.04^{a}	0.08 ± 0.04^{a}
(Water control)	3	7.41 ± 0.07^{a}	0.12 ± 0.07^{a}
	5	7.49 ± 0.09^{a}	0.04 ± 0.09^{a}
10x	1	4.91 ± 0.26^{b}	2.62 ± 0.26^{b}
	3	2.95 ± 0.33^{c}	4.58 ± 0.33^{c}
	5	2.18 ± 0.04^{d}	5.36 ± 0.04^{d}
15x	1	$< 0.70 \pm 0.00^{e}$	$>6.83 \pm 0.00^{e}$
	3	$< 0.70 \pm 0.00^{e}$	$>6.83 \pm 0.00^{e}$
	5	$< 0.70 \pm 0.00^{e}$	$>6.83 \pm 0.00^{e}$

Inoculated seeds average: 7.53 ± 0.02^{a} log CFU/g
Means ± standard deviation (n = 3)
Means with different letters are significantly different ($p < 0.05$)

TABLE B.4.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS ARTIFICIALLY CONTAMINATED WITH *LISTERIA MONOCYTOGENES* USING 10X AND 15X CONCENTRATIONS OF A FATTY ACID-BASED SANITIZER.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Concentration	Exposure Time (min)	After Treatment	Reduction
0x	1	6.91 ± 0.02^{a}	0.65 ± 0.02^{a}
(Water control)	3	6.94 ± 0.09^{a}	0.61 ± 0.09^{a}
	5	7.00 ± 0.03^{a}	0.56 ± 0.03^{a}
10x	1	5.54 ± 0.37^{b}	2.01 ± 0.37^{b}
	3	4.57 ± 0.42^{b}	2.99 ± 0.42^{b}
	5	4.32 ± 0.36^{b}	3.23 ± 0.36^{b}
15x	1	$< 0.70 \pm 0.00^{c}$	$> 6.86 \pm 0.00^{c}$
	3	$< 0.70 \pm 0.00^{c}$	$> 6.86 \pm 0.00^{c}$
	5	$< 0.70 \pm 0.00^{c}$	$> 6.86 \pm 0.00^{c}$

Inoculated seeds average: 7.56 ± 0.09^{a} log CFU/g
Means ± standard deviation (n = 3)
Means with different letters are significantly different ($p < 0.05$)

TABLE B.5.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS PREVIOUSLY INOCULATED WITH *S.* TYPHIMURIUM DT 104 USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment (log CFU/g) (S)	Reduction (log CFU/g)
Water control	3	7.57 ± 0.28^{abc}	0.75 ± 0.28
	5	7.52 ± 0.09^{abc}	0.80 ± 0.09
A.- (ASEP 1+2)	3	0.70 ± 0.00^{h}	$> 7.62 \pm 0.00$
	5	0.70 ± 0.00^{h}	$> 7.62 \pm 0.00$
B.- (ASEP 1)	3	6.52 ± 0.41^{bcd}	1.80 ± 0.41
	5	6.07 ± 0.19^{cde}	2.25 ± 0.19
C.- (ASEP 2)	3	4.49 ± 1.05^{ef}	3.83 ± 1.05
	5	5.32 ± 0.38^{def}	3.00 ± 0.38
D.- (E658 + LA)	3	2.51 ± 1.57^{g}	5.81 ± 1.57
	5	2.36 ± 1.51^{gh}	5.96 ± 1.51
E.- (E658 + GM)	3	7.01 ± 0.67^{abcd}	1.31 ± 0.67
	5	6.51 ± 0.41^{bdc}	1.81 ± 0.41
F.- (GM + LA)	3	7.41 ± 0.08^{abc}	0.91 ± 0.08
	5	7.42 ± 0.08^{abc}	0.90 ± 0.08
G.- (E658)	3	7.89 ± 0.13^{abc}	0.93 ± 0.13
	5	7.94 ± 0.06^{abc}	0.38 ± 0.06
H.- (LA)	3	7.34 ± 0.23^{abc}	0.98 ± 0.23
	5	7.28 ± 0.11^{abc}	1.04 ± 0.11
I.- (GM)	3	7.89 ± 0.13^{ab}	0.43 ± 0.13
	5	7.94 ± 0.06^{ab}	0.38 ± 0.06

Inoculated seeds average: 8.32 ± 0.23^{a} log CFU/g

Means ± standard deviation (n = 3).

Means with different letters are significantly different ($p < 0.05$)

TABLE B.6.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS PREVIOUSLY INOCULATED WITH *E. COLI* O157:H7 USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment	Reduction
Water control	3	7.77 ± 0.36^{ab}	0.64 ± 0.36
	5	7.57 ± 0.24^{ab}	0.84 ± 0.24
A.- (ASEP 1+2)	3	0.70 ± 0.00^{f}	$> 7.71 \pm 0.00$
	5	0.70 ± 0.00^{f}	$> 7.71 \pm 0.00$
B.- (ASEP 1)	3	6.80 ± 0.07^{bc}	1.61 ± 0.07
	5	6.28 ± 0.54^{bcd}	2.13 ± 0.54
C.- (ASEP 2)	3	5.88 ± 0.28^{cd}	2.53 ± 0.28
	5	3.61 ± 0.81^{e}	4.80 ± 0.81
D.- (E658 + LA)	3	5.50 ± 0.25^{cd}	2.91 ± 0.25
	5	5.05 ± 1.44^{de}	3.36 ± 1.44

Inoculated seeds average: 8.41 ± 0.12^{a} log CFU/g
Means ± standard deviation (n = 3).
Means with different letters are significantly different ($p < 0.05$)

TABLE B.7.- INACTIVATION OF MESOPHILIC AEROBIC BACTERIA ON ALFALFA SEEDS PREVIOUSLY INOCULATED WITH *LISTERIA MONOCYTOGENES* USING PEROXYACID (PA), CAPRIC/CAPRYLIC ACID (EMERY 658), LACTIC ACID (LA), GLYCEROL MONOLAURATE (GM) ALONE OR IN COMBINATION.

		Mesophilic Aerobic Bacteria (log CFU/g)	
Sanitizer Treatment	Exposure Time (min)	After Treatment	Reduction
Water control	3	7.17 ± 0.26^{a}	0.31 ± 0.26
	5	7.18 ± 0.09^{a}	0.30 ± 0.09
A- (Asep 1+2)	3	0.70 ± 0.00^{c}	$>6.79 \pm 0.00$
	5	0.70 ± 0.00^{c}	$>6.79 \pm 0.00$
C- (ASEP 2)	3	4.39 ± 0.85^{b}	3.09 ± 0.85
	5	3.76 ± 0.22^{b}	3.72 ± 0.22
D- (E658 + LA)	3	3.95 ± 0.55^{b}	3.53 ± 0.55
	5	4.34 ± 0.65^{b}	3.15 ± 0.65

Inoculated seeds average: 7.49 ± 0.05^{a}
Means ± standard deviation (n = 3).
Means with different letters are significantly different ($p < 0.05$)

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