To the Graduate Council:

I am submitting herewith a dissertation written by Robert C. Williams entitled "Effect of Ozone, Treatment Temperature, and Antimicrobials on Inactivation of *Escherichia coli* O157:H7 and *Salmonella* spp. in Apple Cider and Orange Juice." I have examined the final copy of this dissertation for form and content and recommend that it be accepted in partial fulfillment of the requirements for the degree of Doctor of Philosophy, with a major in Food Science and Technology.

David A. Golden, Major Professor

We have read this dissertation
and recommend its acceptance:

F. Ann Draughon

Alan G. Mathew

John R. Mount

Accepted for the Council:

Vice Provost and Dean of Graduate Studies

EFFECT OF OZONE, TREATMENT TEMPERATURE, AND ANTIMICROBIALS ON INACTIVATION OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* SPP. IN APPLE CIDER AND ORANGE JUICE

A Dissertation
Presented for the
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Degree
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Robert Charles Williams
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DEDICATION

This dissertation is dedicated to my wife

Christa M. Williams

whose love and support made this work possible.

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ABSTRACT

The studies presented in this dissertation were conducted to determine the effect of ozone, treatment temperature, and antimicrobials on the inactivation of *Escherichia coli* O157:H7 and *Salmonella* spp. in apple cider and orange juice. In the first experiment, inactivation of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with ozone was evaluated. A five-strain mixture of *E. coli* O157:H7 or *Salmonella* was inoculated (7 log cfu/ml) into apple cider and orange juice. Ozone (0.9 g ozone/h) was pumped into juices maintained at 4˚C, ambient temperature (~20˚C), and 50˚C for up to 30 min (*Salmonella*/50˚C), 75 min (*E. coli*/50˚C), 180 min (apple cider/4˚C) or 240 min (orange juice/4˚C and ambient). Samples were withdrawn, diluted in 0.1% peptone water, neutralized with 1.0 N NaOH, and surface plated onto recovery media. Recovery of *E. coli* O157:H7 was compared on tryptic soy (TSA), sorbitol MacConkey (SMAC), hemorrhagic coli (HC), and modified eosin methylene blue (MEMB) agars; recovery of *Salmonella* was compared on TSA, bismuth sulfite (BSA), and XLT4 agars. After treatment at 50˚C, *E. coli* O157:H7 populations were reduced to the limit of detection (1.0 log cfu/ml) in 45, 30, 45, and 30 min in apple cider, and 75, 60, 60, and 45 min in orange juice, as determined on TSA, HC, MEMB, and SMAC, respectively. In apple cider at 50˚C, *Salmonella* was reduced by 4.8 log cfu/ml (TSA) and to the limit of detection in 15 min as determined on BSA and XLT4, respectively. In orange juice at 50˚C, *Salmonella* populations were reduced to the limit of detection in 15, 10 and 5 min as determined on TSA, BSA, and XLT4, respectively. *E. coli* O157:H7 in apple cider at 4˚C was reduced by 4.8 log cfu/ml (TSA) and to the limit of detection in 180 min as determined on HC, MEMB, and SMAC. *E. coli* O157:H7 populations in

orange juice at 4˚C decreased by 5.4, 5.7, 5.5, and 5.3 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *Salmonella* in apple cider at 4˚C was reduced in apple cider by 4.5 log cfu/ml (TSA) and to the limit of detection in 180 and 150 min as determined on BSA, and XLT4, respectively. *Salmonella* populations in orange juice at 4˚C decreased by 4.2, 4.4, and 4.9 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. *E. coli* O157:H7 populations in apple cider at ambient temperature decreased by 3.0, 4.5, 4.0, and 3.4 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *E. coli* O157:H7 populations in orange juice at ambient temperature decreased by 2.3, 3.0, 3.6, and 2.5 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *Salmonella* populations in apple cider at ambient temperature decreased by 3.3, 4.8, and 5.5 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. *Salmonella* populations in orange juice at ambient temperature decreased by 4.2, 4.3, and 4.8 log cfu/ml as determined on TSA, BSA, and XLT4, respectively.

The second experiment was composed of two studies and was conducted to determine the inactivation of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with ozone in combination with antimicrobials. A five-strain mixture of nalidixic acid-resistant *E. coli* O157:H7 or *Salmonella* was inoculated (7 log cfu/ml) into apple cider and orange juice. Ozone (O_3; 0.9 g ozone/h) was pumped into juices containing either dimethyl dicarbonate (DMDC; 250 ppm [Study 1]; 250 and 500 ppm [Study 2]) or hydrogen peroxide (HP; 300 ppm [Study 1]; 300 and 600 ppm [Study 2]) at 4˚C for up to 90 min (Study 1) or 60 min followed by 24-h refrigerated (4˚C) storage (Study 2). Samples were withdrawn, diluted in 0.1M phosphate buffer, neutralized with

1.0 N NaOH, and surface plated onto tryptic soy agar + 50 ppm nalidixic acid (TSAN). Study 1: No combination of treatments resulted in a 5 log cfu/ml reduction of either pathogen. Overall, O_3/DMDC (250 ppm) was more effective than O_3/HP (300 ppm) for reducing populations of *E. coli* O157:H7 and *Salmonella* ($P < 0.05$). Greater inactivation occurred in apple cider than in orange juice ($P < 0.05$) during O_3 treatment, but inactivation in control (air + antimicrobials) juices did not differ ($P > 0.05$). O_3 treatment in combination with antimicrobials was more effective than controls (i.e., antimicrobials + air) ($P < 0.05$). Overall, effectiveness of O_3/DMDC depended on juice, as greater inactivation of pathogens occurred in O_3/DMDC treated apple cider than O_3/DMDC treated orange juice ($P < 0.05$). However, inactivation of pathogens by O_3/HP did not differ by juice ($P > 0.05$). During O_3/DMDC treatment in apple cider, reductions in *Salmonella* populations were greater than reductions in *E. coli* O157:H7 populations ($P < 0.05$). However, under the same treatment in orange juice, there were no differences between the two pathogens ($P > 0.05$). Overall, O_3/HP caused greater inactivation of *E. coli* O157:H7 than *Salmonella* ($P < 0.05$). Study 2: All combinations of antimicrobial plus ozone treatments, followed by refrigerated storage, caused greater than a 5-log cfu/ml reduction of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice, except O_3/DMDC (250 ppm) treatment in orange juice reduced *E. coli* O157:H7 populations by less than 5 log cfu/ml. For all combinations tested, inactivation of *E. coli* O157:H7 and *Salmonella* was greater in apple cider than in orange juice ($P < 0.05$). Inactivation of *E. coli* O157:H7 in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only [note: O_3/HP (600 ppm) > O_3/DMDC (250 ppm)] ($P < 0.05$). Inactivation of *E. coli*

O157:H7 in orange juice followed the order: O_3/DMDC (500 ppm) = O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/DMDC (250 ppm) >O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in orange juice followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only ($P < 0.05$). Ozone treatment of apple cider and orange juice at 4°C, in combination with mild heating (50°C), or in combination with antimicrobials (dimethyl dicarbonate, hydrogen peroxide) may provide an alternative to thermal pasteurization for reduction of *E. coli* O157:H7 and *Salmonella* spp. in apple cider and orange juice.

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PART I

INTRODUCTION

On January 19, 2001 the United States Food and Drug Administration (FDA) published its final rule for "Hazard Analysis Critical Control Point (HACCP); procedures for safe and sanitary processing and importing of juice" (FDA, 2001). As a result, fruit juice processors engaged in interstate or intrastate commerce must implement a HACCP program that includes procedures that provide a minimum 5-log reduction in populations of the "pertinent" pathogen in the juice being processed. Only retail establishments that limit their juice business to direct consumer sales (e.g.; farmer's markets, restaurants) are exempt from the HACCP regulation.

The effective date for compliance to this final rule is January 22, 2002. However, small businesses (fewer than 500 employees) and very small businesses (fewer than 100 employees and fewer than 100,000 units of juice sold) have until 1 and 2 years past the effective date, respectively, to be in compliance. Juices that have not been processed to reduce or eliminate pathogens, including those packaged and sold by retail-only establishments, must carry the following statement:

> "WARNING: This product has not been pasteurized and, therefore, may contain harmful bacteria which can cause serious illness in children, the elderly, and persons with weakened immune systems" (FDA, 2001).

This HACCP regulation is in response to several outbreaks of foodborne illness, specifically *Escherichia coli* O157:H7, *Salmonella* spp. and *Cryptosporidium* spp. infections, associated with consumption of unpasteurized apple juice/cider and unpasteurized orange juice, that occurred primarily during the 1990's (Besser et al., 1993;

Buxton et al., 1999; CDC, 1999; Cody et al., 1999; Cook et al., 1998; Millard et al., 1994; Singh et al., 1995). However, outbreaks of foodborne illness associated with juice have been reported since the 1970's (Anderson, 2001).

At least eight outbreaks of foodborne illness associated with consumption of unpasteurized apple juice/cider, affecting approximately 629 persons, have been reported since 1974 (Anderson, 2001). In five of these, *E. coli* O157:H7 was implicated, *Cryptosporidium parvum* in two, and *Salmonella* Typhimurium in one. Since 1989, at least six outbreaks of foodborne illness associated with the consumption of unpasteurized or improperly pasteurized orange juice have occurred, affecting approximately 731 persons. The following microorganisms were implicated in these outbreaks: *S.* Enteriditis, *S.* Muenchen, *S.* Anatum, *S. typhi*, *S.* Hartford, *S.* Gaminara, *S.* Rubislaw, and *Bacillus cereus* (Anderson, 2001).

JUSTIFICATION

Production and consumption of fresh (unpasteurized) apple cider is common in many regions of the United States. Fresh apple cider is primarily consumed during the Fall harvest season, but frozen storage of unpasteurized cider results in year-round availability in many parts of the country. In the United States, fresh apple cider is generally regarded as the unclarified, freshly pressed juice of apples, characterized by suspended particles and darker color than clarified apple juice (Semanchek and Golden, 1996). Additionally, apple cider is most often unpasteurized whereas clarified apple juice is normally pasteurized (Zhao et al., 1993).

Unpasteurized juices (especially fresh apple cider) are products desired by

consumers because of their fresh taste and full aroma. However, due to recent outbreaks and poor production practices of many small processors, it is likely that pathogenic bacterial contamination of unpasteurized juices will continue. Currently, the only treatment widely available for enhancing the safety of apple cider is pasteurization, but many people perceive adverse effects of pasteurization on flavor and aroma of fresh juices. Furthermore, many apple cider producers are small processors that cannot implement pasteurization due to high capital investment and production expenses (Kozempel et al., 1998), and respondents to a U.S. Apple Association survey overwhelmingly reported that they had no plans to implement pasteurization (Daly, 1997). Fresh juice producers are currently searching for alternative processes that allow production of a safe product and compliance with FDA regulations while maintaining characteristics of fresh, unpasteurized juices. The use of ozone and antimicrobial agents offer hope of meeting these demands.

Ozone is the triatomic allotrope of oxygen, and it is characterized by a high oxidation potential and bactericidal and viricidal properties (Burleson, 1975; Horvath et al., 1985; Kim et al., 1999). Antimicrobial activity of ozone is attributable to its diffusion capability through biological membranes and its high oxidation potential (Hunt and Marinas, 1997). Byun et al. (1998), reported that ozone reduces populations of *E. coli* O157:H7 in phosphate buffer. Da Silva et al. (1998) reported that gaseous ozone treatment of fresh fish decreased bacteria associated with spoilage. Restaino et al. (1995) found that ozone treatment kills *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* Typhimurium in water containing organic matter.

Ozone has been evaluated for its efficacy in preserving a variety of food products

including, milk, gelatin, albumin, casein, and meat products (Kim et al., 1999). However, the efficacy of ozone for inactivating bacterial pathogens in fresh juices has not been studied.

Historically, antimicrobial treatment of fresh apple cider and orange juice has been for the prevention of spoilage. Two common antimicrobials, sodium benzoate and potassium sorbate extend shelf-life by inhibiting yeasts and molds (Downing, 1989). Fisher and Golden (1998) reported that dimethyl dicarbonate (DMDC), sodium bisulfite, sodium benzoate, and sodium bisulfite + sodium benzoate were effective for inactivating *E. coli* O157:H7 in apple cider. Regardless of temperature, DMDC was more effective at reducing populations of *E. coli* O157:H7 in apple cider than other antimicrobials tested. Wright et al. (2000) found that acetic acid treatment followed by hydrogen peroxide treatment was effective for reducing levels of *E. coli* O157:H7 on the surface of intact apples. However, the efficacy of antimicrobial treatments in combination with ozone for the reduction of bacterial pathogens in fresh juices has not been reported.

OBJECTIVES

The objectives of this research were:

1. to determine the efficacy of ozone treatment (0.9 g/h) at 4, ~20, and 50°C for inactivating *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without preservatives.

2. to determine the survival of *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without

preservatives treated with ozone (4°C, 0.9 g/h) in combination with dimethyl dicarbonate (250 ppm) and hydrogen peroxide (300 ppm).

3. to determine the survival of *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without preservatives treated with ozone (4°C, 0.9 g/h) in combination with dimethyl dicarbonate (250 ppm, 500 ppm) and hydrogen peroxide (300 ppm, 600 ppm) and stored at 4°C.

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PART II

LITERATURE REVIEW

The FDA defines juice as the aqueous liquid expressed or extracted from one or more fruits or vegetables, purees of the edible portions of one or more fruits or vegetables, or any concentrates of such liquid or puree (FDA, 2001a). The scope of new HACCP regulations for juices is limited to products sold as juice or used as an ingredient in beverages (FDA, 2001a). Although it is recognized that non-beverage products may contain juice as an ingredient, non-beverage products containing juice are not regulated by the juice HACCP rule. This rule is intended to regulate products that have contributed to or are believed to be likely vehicles of foodborne illness outbreaks.

FRUIT JUICES

Apple Cider-

Currently, no legal definition for apple cider exists in the United States. The terms sweet, fresh, and country have been applied to what Semanchek and Golden (1996) define as the unclarified, freshly pressed juice of apples, characterized by suspended particles and darker color than clarified apple juice. Downing (1989) quoted the R. L. Labelle definition of apple cider as a simple old-fashioned product, normally oxidized in color and flavor and still containing some, if not most, of the suspended solids that make the product opaque. Additionally, fresh apple cider is most often unpasteurized whereas apple juice is typically pasteurized (Zhao et al., 1993). In the United States, sweet cider and hard cider are used to differentiate between non-fermented and fermented apple cider, respectively (Downing, 1989). It is the fresh or sweet-type, unpasteurized apple cider that has been implicated in most of the recent outbreaks of juice-borne *E. coli* O157:H7 infections and cryptosporidiosis.

The process of cider making begins with harvesting cider apples at appropriate maturity, with attention to avoiding unripe (poorly flavored, high acidity, high astringency) and overripe (difficult to press) apples (Proulx and Nichols, 1980; Bump, 1989). Improved storage methods allow for apples to be used for cider long after harvesting, but many small-volume producers limit cider-making to the harvest season (Bump, 1989). Harvested cider apples may be washed to remove dirt, twigs, leaves, pesticide residues, and insects (Proulx and Nichols, 1980) and inspected for foreign materials and decay (Bump, 1989). Rotten or moldy apples are discarded (Proulx and Nichols, 1980). Bins of apples may be unloaded onto a leafing screen in a container of water, then directed toward a conveyor system by circulating pumps (Bump, 1989). Apples are then inspected again to remove decayed fruit and conveyed to a grinder (Bump, 1989). Apples are ground in stainless steel hammer or grating mills to a fine mash that is gradually pressed to extract the juice (Bump, 1989).

Several types of pressing equipment are available including hydraulic, screw, basket, belt, and pneumatic presses, and pressing systems may either be batch or continuous (Bump, 1989). Rack and frame was, for many years, the most commonly used press. In this system, apple mash is pumped into nylon cloth mounted in a frame on a rack. The top of the cloth is folded over enclosing the mash and forming a "cheese." Racks containing apple "cheese" are stacked and a hydraulic press is used to gradually apply pressure to the racks, and liquid from the apple mash is collected and packaged (Bump, 1989). At this point the liquid is characteristic of fresh or sweet (unpasteurized) cider.

Surveys performed by the U.S. Apple Association in 1996 revealed that many

apple cider producers (approximately 450 respondents) produce only seasonally (94%) and less than 5,000 gallons per year (46%). Only 7% of surveyed cider processors reported production of greater than 50,000 gallons per year (Daly, 1997). Annual cider income was less than $40,000 for 71% of responding producers, and 42% restricted trade to direct sales. A large proportion of cider producers (53%) reported using a mixture of tree-picked apples and "drops" (fallen apples harvested from the ground) for cider production, and 2% reported using drops exclusively (Daly, 1997).

The use of drops has been cited as a likely source of *E. coli* O157:H7 in contaminated cider. Apples that drop to the ground are more likely to come in contact with contaminated soil and animal feces which are known sources for *E. coli* O157:H7. Recommendations have been to not allow livestock (especially cattle) to graze in orchards and to avoid using drops. However, cider producers (especially in New England) have reported that the use of drops is required to meet the demand for product (Daly, 1997).

Investigations of reported outbreaks have suggested the use of drops as a source of *E. coli* O157:H7 contamination due to deposition of the microorganism from soil or manure onto the surface of the fruit (Besser et al., 1993; Millard et al., 1994). However, *E. coli* O157:H7 internalization in intact apples has also been reported (Burnett et al., 2000; Buchanan et al., 1999), and extensive washing procedures of intact apples did not prevent juice contamination in at least one major outbreak (Cody et al., 1999). Pathogen infiltration is believed to be due to the open-blossom morphology of apples or introduction through wounded tissue. *E. coli* O157:H7 inoculated into wounded apple tissue reportedly reaches the core of the apple within one day and grows in fruit held at

temperatures above refrigeration (Janes et al., 2000a; Janes et al., 2000b).

Sanitation during production of apple cider was also found to be inadequate with regard to even basic good food manufacturing practices. Only 75% of respondents in the U.S. Apple survey reported washing and brushing apples prior to pressing, 5% reported brushing alone, and 3% reported no brushing or washing at all prior to pressing (Daly, 1997).

Orange Juice-

Oranges intended for juice manufacture are typically hand-picked, placed in bins, loaded onto trucks and transported to processing facilities (Townsend, 2000). Mechanical means of harvest, although not widely used, include devices that shake trees causing oranges to fall into a catch frame (Townsend, 2000). Picked oranges are washed, graded, and sorted by size prior to juice extraction. After extraction, the juice is diverted to a finisher (screen) where pulp, seeds, and peels are removed (Townsend, 2000). At this point, the juice (approximately 11.8° Brix) is pasteurized and bottled (not from concentrate), concentrated to approximately 65° Brix, frozen, and shipped to juice packaging companies (frozen concentrated orange juice), or bottled as unpasteurized orange juice.

Citrus fruits used for juicing do not normally contact the ground. Furthermore, due to the morphology of intact citrus fruits, internalization of pathogenic bacteria into these fruits is not believed to be a hazard to public health (FDA, 2001a). Poor manufacturing practices, including ineffective pest control, have been most often cited as factors contributing to *Salmonella* contamination of orange juice (Cook et al., 1998;

Singh et al., 1995).

ESCHERICHIA COLI O157:H7

Escherichia coli O157:H7 has been recognized as a foodborne pathogen since 1982 when it was associated with two outbreaks of hemorrhagic colitis (Buchanan and Doyle, 1997). Illness caused by *E. coli* O157:H7 is characterized by diarrhea, cramps, and short-lived fever that develops within one to three days, and may be followed by development of hemorrhagic colitis, characterized by bloody diarrhea, dehydration, and severe abdominal pain that begins about two days after infection and lasts for about ten days (Buchanan and Doyle, 1997). The most severe illness caused by *E. coli* O157:H7 infection is hemolytic uremic syndrome (HUS) that is characterized by red blood cell destruction, low platelet counts, lack of urine production, renal failure, and occasionally death (Buchanan and Doyle, 1997). HUS, the primary cause of death among *E. coli* O157:H7 victims, typically develops seven days after infection and may last for nine or more days (Buchanan and Doyle, 1997).

Outbreaks of *E. coli* O157:H7 infection have most often implicated ground beef, and cattle have been identified as an important reservoir for this pathogen (Doyle et al., 1997; Buchanan and Doyle, 1997). However, a number of *E. coli* O157:H7 infections have been associated with consumption of acidic foods including apple cider, salami, cantaloupe, and mayonnaise-containing salad dressing (Buchanan and Doyle, 1997).

Survival in Acidic Environments-

E. coli O157:H7 possesses unusual resistance to acidic environments. It has been

shown to survive for several days to several weeks in acidic foods including, ground apples (Fisher and Golden, 1998a), apple cider (Lakins et al, 2000; Zhao et al. 1993), sausages (Clavero and Beuchat, 1996), and mayonnaise (Zhao and Doyle, 1994). Survival of *E. coli* O157:H7 in acidic environments is temperature dependent. Several investigators have reported that *E. coli* O157:H7 survives better in acidic foods at refrigeration temperatures (i.e., 4 to 10°C) than at higher temperatures (e.g., 25°C) (Conner and Kotrola, 1995; Miller and Kaspar, 1994; Semanchek and Golden, 1996). Conner and Kotrola (1995) reported that the presence of organic acids in culture broth actually enhanced survival of *E. coli* O157:H7 at 4°C as compared to unacidified controls. Leyer et al. (1995) reported that acid adaptation of *E. coli* O157:H7 by culturing the organism at pH 5.0 prior to testing, enhanced its subsequent survival in salami and apple cider. Additionally, once induced, mechanisms of acid resistance in *E. coli* O157:H7 remain active for long periods (>28 days) at 4°C (Lin et al., 1996). Therefore, the practice of refrigerating apple cider for preservation may enhance the ability of *E. coli* O157:H7 to survive storage.

SALMONELLA SPP.

Salmonella is one of the leading causes of foodborne illness in the U.S. and foodborne salmonellosis has increased worldwide in the last few decades (D'Aoust, 1997). Salmonellosis is caused by the ingestion of food containing virulent *Salmonella.* The illness is characterized by nausea, vomiting, abdominal pain, headache, chills, and diarrhea with onset typically 12 to 14 hours after consumption of contaminated food, and a duration of 2 to 3 days (Jay, 1992). However, chronic conditions such as Reiter's

syndrome, ankylosing spondylitis, and aseptic reactive arthritis occasionally follow *Salmonella* infection (D'Aoust, 1997).

The most common vehicles of *Salmonella* outbreaks have been meat and meat products, poultry, and eggs (Jay, 1992). However, outbreaks in milk, ice cream, chocolate, paté and acidic foods including, mayonnaise, cantaloupe, and orange juice have occurred (D'Aoust, 1997).

Survival in Acidic Environments-

Roering et al. (1999) reported that *Salmonella* Typhimurium survives for up to seven days in refrigerated (approximately 4°C) apple cider (pH 3.3 to 3.5). In orange juice, salmonellae survived for 27 and 73 days at pH 3.5 and 4.4, respectively (Parish et al., 1997). Storage of fruit juices at refrigeration temperatures may enhance the survival of *Salmonella*.

RECENT JUICE-ASSOCIATED OUTBREAKS

Two outbreaks, one occurring on the West Coast of the United States and the other on the East Coast, illustrate the cause for concern about the safety of unpasteurized apple cider/juice. During October and November 1991, in Fall River, Massachusetts and the surrounding area, a cluster of 23 cases of *E. coli* O157:H7 infection were identified Besser et al., 1993). These cases were strongly associated with consumption of unpasteurized apple cider produced at a single, local mill. Patients reported the following symptoms: diarrhea (96%), abdominal pain (87%), bloody diarrhea (70%), vomiting (35%), and fever (17%). At least four children were diagnosed with hemolytic uremic

syndrome (HUS), but no deaths were reported. *E. coli* O157:H7 was not isolated from environmental samples from the cider mill (first collected two months after the outbreak), cider purchased from the mill, cider (fermented) from a patient's refrigerator, or stool specimens from cattle in a field beside the mill. However, poor manufacturing practices, including the use of drops for 90% of apples pressed and failure to wash or otherwise sanitize apples were suggested as contributing factors to the outbreak (Besser et al., 1993).

In contrast, apple cider processing practices employed by the California company, Odwalla Inc., were considered state of the art. However, in 1996, several cases of *E. coli* O157:H7 infection occurring in Washington state were epidemiologically linked to consumption of Odwalla brand products containing unpasteurized apple juice (Cody et al., 1999). Ultimately, 70 cases of *E. coli* O157:H7 infection (65 primary, 2 secondary, 3 indeterminate) occurring in California (26 cases), Colorado (5 cases), Washington (29 cases), and British Columbia (10 cases) were identified. Inspections of the Odwalla processing plant, where the apple juice was processed, provided no likely route of contamination of the product. Investigators concluded that apples were contaminated with *E. coli* O157:H7 prior to entrance into the processing plant. However, Odwalla policy dictating that suppliers provide only tree picked apples was regarded as unenforceable, and juice contamination occurred despite sorting, washing and brushing procedures (Cody et al., 1999).

In October 1993, 160 cases of *Cryptosporidium* infection occurred among attendees of a school agricultural fair in central Maine (Millard et al., 1994). Illness was associated with consumption of fresh apple cider pressed at the fair. Apples used for

making the implicated apple cider were collected by high school students from uncultivated trees located on the edge of a pasture where cows had been recently grazing. Students shook trees and collected apples from the ground, and the apples were sprayed with municipal water prior to pressing. Of the 160 primary cases, 84% reported diarrhea and 82% reported vomiting. Prior to this outbreak, *Cryptosporidium* infections had only been associated with person-to-person and waterborne transmission. The outbreak in Maine provided the first well-documented evidence that *Cryptosporidium* infections may be transmitted via food (Millard et al., 1994).

In June 1999, the Washington State Health Department and the Oregon Health Division began investigating *Salmonella* Muenchen infections that were occurring in each state (Buxton et al., 1999). *S.* Muenchen infections (accounting for approximately 1.6% of total reported *Salmonella* isolates nationwide) in both states were epidemiologically linked to consumption of unpasteurized orange juice produced by the Sun Orchards Company. By July 1999, 79 patients were identified reporting diarrhea (94%), fever (75%), and bloody diarrhea (43%) (CDC, 1999a). Sun Orchards had a HACCP plan in place during the time that the outbreak occurred, but control failures that contributed to the contamination of the orange juice were not reported (Buxton et al., 1999).

During May and June of 1995, an outbreak of *Salmonella* Hartford infection occurred at a theme park in Orlando, Florida. Sixty-two persons suffering from salmonellosis were identified and, ultimately, thirty-two persons were enrolled in a case study (Cook et al., 1998). Patients in the case study reported diarrhea (97%), fever (97%), headache (73%), bloody stool (71%), and vomiting (66%) with a mean duration

of seven days. *Salmonella* Hartford (representing 0.2% of reported *Salmonella* isolates nationwide) infection was associated with consumption of unpasteurized orange juice produced primarily for the theme park by a small, local processor. Environmental studies of the processing plant revealed several deficiencies including: a poorly sealed processing room, cracks and holes in walls and ceilings, bird and rodent droppings in processing areas, and the observation of frogs in the vicinity of processing equipment. Orchards that supplied oranges to this processor harvested by hand-picking fruits which were then dropped to the ground, gathered into large bins, and shipped to the processing facility. Although all oranges sampled from each orchard (n = 95) were negative for salmonellae, a frog captured just outside of the processing facility was positive for *S.* Hartford, indicating that ineffective pest control may have been a contributing factor for the contamination of the juice.

In 1992, an outbreak of enterotoxigenic *E. coli* infection, associated with consumption of orange juice purchased from roadside vendors, occurred in Faridpur, India. Although no detailed information was provided, juice contamination was reportedly related to unhygienic practices of a vendor (Singh et al., 1995).

FDA REGULATIONS FOR JUICE

As a result of foodborne outbreaks associated with juices, the FDA issued juice HACCP regulations. A primary standard of this regulation is a 5-log reduction in populations of the "pertinent" pathogen in the juice being processed. The 5-log reduction performance standard was advised by the Fresh Produce Working Group of the National Advisory Committee on Microbiological Criteria for Foods (NACMCF). The NACMCF

committee added a 100-fold (2-log) safety margin to levels of *E. coli* that they believed may typically occur in untreated juice (about 3 log cfu/ml) and postulated a "worst case scenario" in which bovine fecal contamination of produce may occur (FDA, 2001a). Additionally, a 5-log pathogen reduction performance standard has a historical basis in that it is used for *Salmonella* inactivation for in-shell egg pasteurization and for inactivation of *E. coli* O157:H7 in fermented sausage (FDA, 2001a). Application of a 5-log reduction begins where the treatment has direct contact with any and all pathogens that may be present (FDA, 2001a). For almost all types of juice, only direct treatment of expressed juice will satisfy this requirement, with the single exception that surface treatments of intact citrus fruits may be used to satisfy part or all of the performance standard.

A pertinent pathogen is defined as the most resistant microorganism of public health significance that is likely to occur in the juice (Anderson, 2001). Microorganisms that are considered pertinent pathogens include, *Escherichia coli* O157:H7 in apple juice/cider and *Salmonella* spp. in orange juice. Resistance refers to the ability of the microorganism to withstand processing and storage conditions to which a particular juice product will be subjected. The determination of the pertinent pathogen for any particular juice product is the responsibility of each juice processor. The pertinent pathogen is based on historical association of the pathogen with a specific type of juice product and the resistance of that microorganism to the processing practices used.

Inactivation kinetics of pathogenic microorganisms and ultimately resistance, may vary widely according to the type of processing technique used. For example, historical indicator microorganisms (i.e, *Coxiella burnetti* in milk, *Clostridium botulinum*

in low-acid canned foods) represent the "pertinent" pathogen in those products when thermally processed (IFT, 2000). However, use of alternative processes to treat such products may require targeting a different microorganisms for destruction than would be targeted for thermal pasteurization. Therefore, the ability of a microorganism to resist thermal treatment does not necessarily indicate its ability to resist other processes.

In addition to *E. coli* O157:H7 and *Salmonella* spp., *Cryptosporidium parvum* and *Listeria monocytogenes* have been considered as pertinent pathogens for juices. Outbreaks of *E. coli* O157:H7 infections, salmonellosis, and cryptosporidiosis associated with the consumption of unpasteurized juices have been reported. No outbreaks of foodborne illness associated with juice, nor any juice recalls, have been associated with *L. monocytogenes*. However, the FDA believes that, because of the ubiquitous nature of this microorganism and its relatively high resistance to heat, it should be considered as a potential pertinent pathogen in juices (FDA, 2001a).

Some comments made to the FDA in response to proposed HACCP regulations for juices argued that mandatory pasteurization was the only reasonable way to solve safety issues related to juices. Others commentors argued that mandatory pasteurization is not acceptable because nutritional value is lost from heat treatment, some consumers prefer unpasteurized juice, pasteurized juice may become contaminated after treatment and still put consumers at risk, and the apple cider and fresh juice industry would be destroyed (FDA, 2001a). FDA agreed with the latter comment and additionally argued that physical and chemical hazards that may occur in juices would not be corrected by pasteurization alone. As a result, alternative processing techniques have become of interest in the hope that these methods will allow processors to meet the 5-log reduction

performance standard while allowing retention of some or all of the organoleptic properties associated with fresh juices.

ALTERNATIVE PROCESSING

The term "alternative process" may be defined as a process or treatment that differs significantly from a traditional or generally accepted process used for a particular product or type of products. Historically, thermal pasteurization has been the process of choice for extending shelf-life and improving microbiological safety of juices. Therefore, in juice processing, "alternative processes" refers to alternatives to thermal pasteurization. Alternative processing techniques that may be applicable to juice processing include: ozone, ultraviolet light, microwave and radio frequency, ohmic and inductive heating, high pressure processing, pulsed electric fields, high voltage arc discharge, pulsed light, oscillating magnetic fields, ultrasound, X-rays, and various antimicrobial treatments.

As previously stated, application of a 5-log reduction begins where the treatment has direct contact with any and all pathogens that may be present (FDA, 2001a). Although, *E. coli* O157:H7 internalization in intact apples has been reported (Burnett et al., 2000; Buchanan et al., 1999), internalization of pathogenic bacteria is not believed to be a significant public health risk in intact citrus fruits (FDA, 2001a). Therefore, the FDA requires that the 5-log standard be met by treatments applied directly to the juice, with the exception that citrus juice processors may include treatments of fruit surfaces (FDA, 2001a). Only intact citrus fruits may be treated with surface decontamination steps to meet the 5-log reduction performance standard. Therefore, any process that is not

directly applied to the expressed juice is not acceptable for demonstration of a 5-log reduction, with the single exception of citrus fruits.

Traditional Processing: Pasteurization-

Fresh (unpasteurized) apple cider represents a minority of the total juices consumed in the U.S. The National Food Processors Association reports that 98% (>2 billion gallons/year) of fruit juices consumed in the U.S. are pasteurized, while the remaining approx. 38 million gallons are not pasteurized (Johnston, 2000). Juices are pasteurized to destroy vegetative pathogenic and spoilage microorganisms and inactivate enzymes. The pH of fruit juices is typically below 4.5, and although pathogenic bacteria do not grow in most fruit juices, they may die very slowly (Wilbey, 1999) and pose a health hazard throughout the shelf-life of the product.

Pathogenic microorganisms are typically not as resistant to heating in juices as spoilage microorganisms, and pasteurization of fruit juices is designed to eliminate the more resistant yeasts and lactic acid bacteria (Wilbey, 1999). Enzymes in juices (e.g., pectinase in orange juice, polyphenol oxidase in apple juice) are typically more resistant to heat inactivation than microorganisms. Although pasteurization at 70˚C for 60 sec. or 85˚C for 30 sec. in citrus juices is sufficient to prevent microbial spoilage, heating to 90˚C for 10 sec. or 85˚C for 4 min. is required to inactivate pectinase (Wilbey, 1999).

Dark color is characteristic of fresh apple cider, but prevention of dark color in apple juice requires pasteurization at 89˚C for 90 sec. to inhibit polyphenol oxidase activity (Wilbey, 1999). No legal standards for pasteurization of apple cider currently exist in the U.S. However, pasteurization of apple cider at 160˚F (~71˚C) for 10 sec. is

recommended to inactivate pathogenic and spoilage microorganisms (McLellan, 1998).

Some consumers prefer fresh unpasteurized apple cider because of perceived negative impact of heating on the organoleptic quality. In fact, heating apple cider to pasteurization temperatures has been shown to reduce aroma (Karwowska et al., 1973). Additionally, the cost to pasteurize juice products is prohibitive for many small juice processors. Kozempel et al. (1998) estimated capital costs for a medium size apple cider pasteurization facility (cider production of about 15 million gal/yr) to be $185,000 and annual production costs to be $93,000 ($0.006/gal). Many apple cider processors do not produce enough apple cider to make the capital investment required for pasteurization equipment financially feasible. Therefore, alternative processing techniques may provide a means for improving juice safety while maintaining product quality and economic feasibility.

Ozone-

Ozone is the triatomic allotrope of oxygen, and it is characterized by a high oxidation potential and bactericidal and viricidal properties (Burleson, 1975; Horvath et al., 1985; Kim et al., 1999). Ozone, discovered by Schönbein in 1840, is often recognized for its strong odor that is detectable by humans at low concentrations (Horvath et al., 1985). "Ozone" is a derivative of the Greek word "ozein," which means "to smell" (Lamarre, 1997; Horvath et al., 1985). The oxidation potential of ozone (- 2.07 V) is higher than hypochlorous acid (- 1.49 V) or chlorine (- 1.36 V), and it decomposes quickly in aqueous solution containing organic matter (Kim et al., 1999). Ozone decomposes into hydroperoxyl, hydroxyl, and superoxide radicals with varying degrees

of stability and reactivity (Kim et al., 1999). Suggested mechanisms responsible for ozone mediated inactivation of microorganisms are direct reaction with molecular ozone and indirect reaction with ozone initiated radicals (Hunt and Marinas, 1997: Kim et al. 1999). The microbiocidal activity of ozone is attributable to its diffusion capability through biological membranes and it high oxidation potential (Hunt and Marinas, 1997).

Due to its reactivity and difficulty with storage, ozone is usually generated as needed from ultraviolet (UV) lamps (185 nm) or corona discharge, with corona discharge being superior for volume production of ozone (Kim et al., 1999). A relatively new approach to ozone production involves electrolysis of water resulting in much higher concentrations of gaseous ozone (10-18% by weight) than are attainable with UV lamps (0.03 ppm) or corona discharge (4% by weight) (Kim et al., 1999). Ozone production is greater when pure oxygen is used, but when using air, dry air is preferred to prevent corrosion of equipment caused by nitric acid (Kim et al., 1999).

Ohlmuller discovered the bactericidal effect of ozone in 1890, and by the early 1900's, ozone was being used on a limited basis to disinfect drinking water in France, Germany, Switzerland and the U. S. (Horvath et al., 1985). However, early ozone generators were not reliable due to deficiencies in design and materials, and chlorine was chosen instead for the treatment of drinking water.

Ozone has been evaluated for its efficacy in preservation of milk, gelatin, albumin, casein, and meat products, and it has been used for the artificial aging of wine, sanitation of brewing facilities, and odor control (Kim et al., 1999). Currently, widespread use of ozone is limited to disinfection of bottled water for which ozone is approved as a GRAS substance by the FDA (Kim et al., 1999). However, the 2001 FDA

ruling providing for the safe use of ozone in gaseous and aqueous phases as an antimicrobial agent on food, makes possible the use of ozone in a variety of food products (FDA, 2001b)

Research to determine the efficacy of ozone for destruction of foodborne pathogens has focused on basic studies concerning the effect of ozone treatment of bacterial cell suspensions in culture and applied research on the effect of ozone treatment of food products. Yang and Chen (1979a) reported that the efficacy of ozone for inactivation of microoganisms suspended in ground poultry meat rinsate was better under the following conditions: longer contact times, lower temperatures (2°C vs. 25°C), lower pH (pH 3 vs. pH 5), moderate NaCl concentrations (1% and 2.5% vs. 5.0%), and low organic load (0% egg albumin vs. 0.5 or 1.0% egg albumin). They concluded that increased bactericidal effects of ozone are related to its increased solubility and stability in these conditions. Byun et al. (1998) assessed the effect of ozone on *E. coli* O157:H7 by applying gaseous ozone to inoculated TSA plates and sparging gaseous ozone through inoculated phosphate buffer. They reported that D-values for *E. coli* O157:H7 were longer in TSA than in phosphate buffer. This may indicate that the effectiveness of ozone is better in substrates that contain less organic load or when introduced directly into aqueous suspensions. In shrimp-meat extract, ozone was more soluble at 5°C than 25°C, but less soluble than in distilled water or water containing 2% NaCl, and ozone decomposition rates at 5 and 25°C were the same (Chen et al., 1992). Da Silva et al. (1998) reported that gaseous ozone treatment of fresh fish (0.25 ppm, 60 min. initially followed by 30 min. exposure daily) decreased spoilage flora on fish and improved sensory scores as compared to controls.

Outbreaks of foodborne illness associated with consumption of juice have primarily implicated Gram negative rod-shaped bacteria (i.e., *E. coli* O157:H7 and *Salmonella*). Yang and Chen (1979b) found that ozone inactivated Gram negative rod-shaped bacteria better than Gram positive rods or Gram positive cocci in suspensions of poultry meat microflora. Restaino et al. (1995) found that ozone treatment (0.149 - 0.188 ppm) kills foodborne microorganisms including, *Pseudomonas aeruginosa*, *Zygosaccharomyces bailii*, *Enterococcus faecalis*, *Escherichia coli*, *Listeria monocytogenes*, *Bacillus cereus*, *Salmonella* Typhimurium, *Yersinia enterocolitica*, and *Staphylococcus aureus* in deionized water, deionized water + 20 ppm soluble starch, and deionized water + 20 ppm bovine serum albumin at approximately 20°C. They also reported that inactivation rates were lower in deionized water containing bovine serum albumin than in deionized water alone.

In addition to its effects on vegetative cells, ozone is an effective sporicidal agent. Foegeding (1985) reported that ozone concentrations of 1.5 ppm reduced *Bacillus cereus* T spores by 1 to 3 log cfu/ml in ozone demand-free water (pH 3), and that decreased pH resulted in increased sensitivity to ozone. In the same study, *Clostridium botulinum*, *Clostridium perfringens*, and *Bacillus stearothermophilus* spores were also inactivated by ozone. Further, Foegeding (1985) reported that spores with their coats removed were more susceptible to the lethal effects of ozone, indicating that the spore coat is the primary protective barrier against ozone. Additionally, ozone treatment (0.188 ppm) was found to be ineffective against conidia of *Aspergillus niger* in deionized water over 5 minutes of treatment (Restaino et al., 1995). Generally, ozone is more effective against vegetative bacterial cells than against bacterial spores (Kim et al., 1999).

Due to their low pH, spoilage of fruit juices is often caused by yeasts and lactic acid bacteria. Molds are also known to grow at the surface of fruit juices causing spoilage. Naito and Sawair (2000) found that dissolved ozone concentrations of 0.5 to 5.0 ppm decreased populations of lactic acid bacteria in water (Naito and Sawair, 2000). They reported that, although sensitivity to ozone depended on the type of lactic acid bacteria tested, inactivation by ozone was linearly related to ozone concentration with 5.0 ppm being more destructive than 0.5 ppm.

In addition to its antibacterial activity, ozone is an effective fungicide. Gaseous ozone (at concentrations of 0.6 to 1.5 ppm and 2.5 to 3.0 ppm) has been shown to prevent mold growth on eggs and beef, respectively, under refrigerated storage and 90% relative humidity (Kim et al., 1999). Liew and Prange (1994) reported that gaseous ozone was effective for reducing growth of *Sclerotinia sclerotiorum* and *Botrytis cinerea* in stored carrots, but carrot damage was observed with increasing concentrations of residual ozone (up to 22 ppm after 60 days).

Studies on the effects of ozone on shelf-life extension of foods have primarily focused on the prevention of spoilage microflora (i.e., lactic acid bacteria, yeasts, and molds). However, studies have reported the effect of ozone treatment on the quality of intact fruit during storage. Barth et al. (1995), in a study on the effect of ozone on thornless blackberries, reported that, in addition to inhibition of fungal growth, maintaining ozone concentrations of 0.1 and 0.3 ppm in storage rooms resulted in the extension of market life, including better retention of color than controls. In contrast, carrot damage, including electrolyte leakage, surface pitting, bleaching and reduced intensity of carrot color occurred during ozone treatment, and the defects increased as

ozone concentration increased (up to 22 ppm) (Liew and Prange, 1994). Perez et al. (1999) reported that ozone treatment of strawberries at 0.35 ppm for 3 days at 2°C followed by storage for 4 days at 20°C (to simulate retail conditions) was ineffective for preventing fungal growth on strawberries and caused a 40% reduction of strawberry aroma volatiles. However, they reported that ozone treated strawberries have three times as much vitamin C as control strawberries at the end of refrigerated storage.

In the U.S., ozone is approved for use as an antimicrobial in foods, for the treatment of bottled water, and as a sanitizer. Historically, wide-spread use of ozone in the United States has been for disinfection of bottled water for which ozone is approved for use, with regulatory status as generally recognized as safe (GRAS; Kim et al., 1999). In 1997, a panel of experts, organized by research and development enterprises on behalf of the Electric Power Research Institute (EPRI), affirmed ozone as GRAS for use as a food processing disinfectant or sanitizer in the United States (Kim et al., 1999). The FDA had no objection to this affirmation and, as a result, food processors in the United States may use ozone as a food disinfectant or sanitizer when used in accordance with good manufacturing practices (Graham, 1997). In 2000, EPRI filed a food additive petition proposing that the food additive regulations be amended to provide for the safe use of ozone in gaseous and aqueous phases as an antimicrobial agent for the treatment, storage, and processing of foods (FDA, 2000a). This petition was approved on June 26, 2001, and ozone is now approved for the direct treatment of foods (FDA 2001b).

Antimicrobial Treatments-

Historically, antimicrobial treatment of fresh apple cider and orange juice has

been for the prevention of spoilage. Two common antimicrobials, sodium benzoate (benzoic acid) and potassium sorbate (sorbic acid), are added to apple cider at levels of 0.03 to1% and 0.025 to 0.01%, respectively, to extend shelf-life by inhibiting yeasts and molds (Downing, 1989). Benzoic acid is most effective at pH below 4.5 because its antimicrobial activity is optimal in the undissociated form (Chipley, 1993). Although the antimicrobial activity spectrum of benzoates includes bacteria, it is generally not effective against spoilage bacteria at levels commonly used (Downing, 1989). At concentrations higher than those normally used, benzoic acid imparts an undesirable flavor to products that is especially detectable in fruit beverages (Downing, 1989). Therefore, benzoic acid is not generally recommended for the prevention of spoilage caused by bacteria (Chipley, 1993).

Sorbic acid is most effective as an antimicrobial at or below its pK_a of 4.76, but it is effective at pH up to 6.5 (Sofos and Busta, 1993). Sorbic acid inhibits yeasts and molds, and its antibacterial activity is selective (Sofos and Busta, 1993). Li *et al.* (1989) used a combination of acidification with HCl (pH 2.5) and 0.03% potassium sorbate for the preservation of unpasteurized orange juice. During 10 weeks of storage at 10°C, the treated juice maintained acceptable quality. Unlike benzoic acid, sorbic acid is not as likely to impart undesirable flavors to products at levels commonly used (Sofos and Busta, 1993; Downing, 1989). Regardless of whether benzoic acid, sorbic acid, or a combination of both is used, maximum effectiveness is achieved when levels of spoilage microbes are initially low in the product (Downing, 1989). In the U. S., sodium benzoate and potassium sorbate are GRAS and sanctioned in a number of products with standards of identity (Chipley, 1993; Sofos and Busta, 1993).

In addition to use in the prevention of spoilage, researchers have investigated the use of preservatives for the inactivation of foodborne pathogens in juice products. Zhao et al. (1993) reported that potassium sorbate (0.1%) alone was ineffective for reducing survival of *E. coli* O157:H7 in apple cider at 8 or 25°C. Sodium benzoate (0.1%), however, reduced the duration of detectable populations of *E. coli* O157:H7 in apple cider from 15-20 days to 2-10 days at 8°C and from 2-3 days to less than 1-2 days at 25°C, respectively (Zhao et al., 1993). A combination of sodium benzoate (0.1%) and potassium sorbate (0.1%) resulted in greater inactivation than sodium benzoate alone (Zhao et al., 1993). Uljas and Ingham (1999) reported that a combination of 0.1% sorbic acid, followed by 12-h storage at 25°C, then followed by freeze-thawing (48 h at -20°C; 4 h at 4°C) reduced populations of *E. coli* O157:H7 by 5 log cfu/ml in apple cider. Venkitanarayanan et al. (1999) found that in peptone water supplemented with either 1.5% lactic acid plus 0.1% hydrogen peroxide or 1.5% lactic acid plus 0.005% glycerol monolaurate, *E. coli* O157:H7 was reduced by at least 5 log cfu/ml within 20 min.

Addition of 3% citric acid to orange juice caused a 2 log cfu/ml reduction in *Listeria monocytogenes* populations within 48 h at 4°C (Phelps et al., 2000). Fisher and Golden (1998b) reported that 0.025% dimethyl dicarbonate (DMDC), 0.0046% sodium bisulfite (NaS), 0.45% sodium benzoate (SB), and sodium bisulfite + sodium benzoate (NaS/SB) were effective for inactivating *E. coli* O157:H7 in apple cider at 4, 10, and 25°C. Generally, effectiveness for inactivating *E. coli* O157:H7 followed the order DMDC > NaS/SB > SB > NaS. Inactivation of *E. coli* O157:H7 was better at 4 and 10°C than 25°C, and regardless of temperature, DMDC was more effective for reducing populations of *E. coli* O157:H7 in apple cider than other antimicrobials tested.

Many investigations have focused on the use of diethyl dicarbonate (DEDC) for the reduction of yeasts (primarily in wine), molds, and bacteria, and, although fewer investigations been performed using DMDC, it has been shown to be equally effective or superior to DEDC as an antimicrobial agent (Ough, 1993a). DMDC is currently allowed by the FDA for use as a yeast inhibitor in wine, ready-to-drink teas, non-juice containing beverages with added electrolytes, and carbonated juice and certain non-juice containing beverages in amounts of 200 to 250 ppm depending on the product (FDA, 2000b). However, DMDC is not currently approved for use in juices.

Sulfites were once considered GRAS in the U.S., but health concerns related to the sensitivity of some asthmatics to relatively low amounts of sulfur dioxide prompted the FDA to rescind GRAS status (Ough, 1993b). Sulfites, including sodium bisulfite, are regulated for the amount of sulfur dioxide allowed in a treated food product. Sulfites are not allowed for use on raw fruits and vegetables, and regulated levels of sulfur dioxide in other products range from a maximum allowable level of 20 mg/kg in dry soup mix to 2000 mg/kg in dried fruit (Ough, 1993b). Allowable levels of sulfur dioxide are 300 mg/kg and 1000 mg/kg in single-strength and concentrated fruit juices, respectively (Ough, 1993b).

In addition to direct treatment of juices, the use of anitmicrobial treatments for the surface disinfection of intact fruits has also been investigated. Wright et al. (2000) found that 200 ppm sodium hypochlorite, phosphoric acid fruit wash (commercial), 5% acetic acid, 5% acetic acid followed by 3% hydrogen peroxide, and a peroxyacetic acid sanitizer (commercial) were all effective for reducing levels of *E. coli* O157:H7 on the surface of intact apples. The most effective treatments were 5% acetic acid and the

peroxyacetic acid sanitizer, causing reductions of 3.1 and 2.6 log cfu/cm^2, respectively. However, Wisniewsky et al., (2000) found that when used at manufacturer recommended levels, peroxyacetic acid, chlorine dioxide, and chlorine-phosphate buffer did not reduce populations of *E. coli* O157:H7 by 5 logs. Concentrations of 2.1 to 14 times the recommended levels were needed to achieve a 5 log reduction using the most effective sanitizer tested, peroxyacetic acid. Treatments of 100 and 200 ppm chlorine dioxide, 200 ppm acid anionic sanitizer, 80 ppm peroxyacetic acid, 2% trisodium phosphate, and deionized water only reduced *E. coli* O157:H7 levels on oranges by 1.8 to 3.1 logs.

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PART III

PRELIMINARY STUDIES

DEVELOPMENT OF AN OZONE GAS INTRODUCTION SYSTEM

Prior to initiation of studies described in this dissertation, studies on the application of ozone directly into orange juice and unclarified apple cider had not been reported. Therefore, preliminary investigations on the efficacy of ozone treatment for the reduction of foodborne pathogens in fresh juices were required to develop equipment and methods for the direct introduction of ozone into juices. Ozone generators used for these studies were obtained from Golden Buffalo (Orange, CA) and consist of ultraviolet (UV) lamps (1 or 2 per generator) inside capped PVC tubes. Electrical wiring for lamps and PVC tubing for air flow were routed through drilled holes in the caps of each tube. The generators work by pumping ambient air at 2.4 L/min through the capped PVC tubes containing UV lamps. Ozone is generated by UV light energy on molecular oxygen and conveyed outward via 0.25 inch PVC tubing. Ozone is delivered from an outlet tube as an ozone/air mixture. As a result, outgoing ozone/air mixtures must be bubbled directly through juice in order for ozone to be dissolved into the product and effectuate destruction of foodborne microorganisms. In order to produce maximum ozone concentration in given quantity of juice, maximization of ozone/air surface area in contact with the juice and contact time are needed for any given temperature. To this end, several equipment set-ups were tested.

Originally, a one liter graduated cylinder containing one liter of apple cider was tested. An ozone generator outlet tube was fitted with a stainless steel inlet filter (typically used for HPLC applications) and lowered inside a graduated cylinder containing juice. Ozone gas passing through the inlet filter produced very fine bubbles, as desired, but resulted in excessive foaming of both apple cider and orange juice. Both

juices foamed and escaped the cylinder within seconds of establishing gas flow. In an attempt to control foaming, a submersible pump was connected to PVC tubing which, at its terminus, was connected to the drain stem of a Beuchner funnel. When the pump was turned on it circulated the juice in the cylinder and sprayed juice through small holes in the top of the funnel which were directed towards the juice in the cylinder producing a "shower-head" effect. The idea was that the mechanical force of raining juice would destabilize the foam and keep it within controllable limits. This method only allowed control of foaming for a few seconds, and was deemed ineffective. Introduction of ozone into one liter of juice in a 2-L Erlenmeyer flask also did not allow acceptable control of foaming.

Finally, it was discovered that introducing ozone gas into one liter of juice in a four liter Erlenmeyer flask prevented foam overflow of apple cider due to high surface area to volume ratio and high headspace of the flask. However, orange juice foam overflow occurred within 30 minutes of beginning treatment. Additionally, it was also observed that orange juice foam is more stable at 4°C than ambient temperatures (20°C) or 50°C. Antifoaming agents such as vegetable oil were tried, but the addition of Antifoam-289 (Acros; Fair Lawn, NJ) to orange juice in 0.5 ml quantities at 30 minute intervals was required for acceptable control of foaming at 4°C, but an initial 0.5 ml dose in orange juice at ambient temperature and 50°C was typically satisfactory for the duration of testing.

It was observed that, due to the limited overall size of the inlet filter, and the large surface area of the bottom of the 4-L flask, ozone gas bubbles were not being effectively distributed throughout the juice being treated, even while stirring at medium speed.

Therefore, an ozone dispersal ring was developed by perforating PVC tubing along its outside, horizontal circumference and shaping into a ring to fit the inside, bottom circumference of the 4-L flask. Perforations were made along the outside, horizontal circumference of the dispersal ring with a 20 gauge needle and the tubing ring was attached to a T-connector on the end of the ozone generator outlet tubing. This arrangement forced the ozone/air mixture to bubble out of the tubing along its entire circumference and, in combination with stirring at medium speed on a stirrer/hotplate, this allowed more thorough and even distribution of ozone gas in the juice. However, it was found that perforations in PVC tubing tended to seal, possibly due to sanitizing treatments (10% bleach; 1+ hours), and prevent gas flow. To overcome this problem, two 10-cm segments of PVC tubing were removed and perforated Teflon tubing segments were used in their place. Teflon tubing is far more rigid than PVC tubing, and, as a result, building the entire dispersal tubing out of Teflon tubing was not possible. Additionally, it was found that perforating the Teflon tubing with a 27 ½ gauge needle provided the most functional bubble size. Even after repeated sanitizing treatments, perforations in Teflon tubing remained open.

Further studies investigated the effect of combining two ozone outputs to result in one outlet and pressurizing the juice containing flask to two p.s.i. However, both adjustments resulted in no observable effect.

OZONE CONCENTRATION DETERMINATIONS

Methods for ozone determination including the indigo colorimetric, iodometric, and UV absorption require test materials that are free of other colored or UV absorbing

materials. Many of these methods were developed for use in water, for which they work quite well, but due to coloration and suspended particles in juices, these methods are not acceptable for determining ozone concentration in apple cider and orange juice. Currently, no standard method exists for direct determination of ozone in treated juices. Therefore, preliminary studies were conducted to assess the ability of oxidation/reduction (redox) potential (mV) measurements, using a pH meter (Accumet AB-15; Fisher Scientific; Fair Lawn, NJ) set to display mV, to estimate the concentration of ozone in treated juices. Acidified water (pH 3.3; HCl) was treated with ozone for 4 h at 4°C. At one hour intervals, mV measurements were taken and a sample was withdrawn and analyzed using an ozone test kit (Vacu-Vials; Chemetrics, Calcerton, VA). Measurements decreased during treatment from 191 mV (0 h) to 177 mV (4 h), while ozone concentration increased from 0.02 ppm (0 h) to 0.88 ppm (4 h). This experiment was carried out at ambient temperature and at 50°C. At ambient temperature, mV readings of acidified water (pH 3.4) decreased from 180 to 173 at 0 and 4 h, respectively, while ozone concentration increased from 0.02 to 0.66 ppm during the same period. At 50°C in acidified water (pH 3.3), mV readings decreased from 165 to 157 at 0 and 4 h, respectively, while ozone concentration increased from 0.03 to 0.19 ppm during the same period. No reliable relationship between mV readings and ozone concentration was found and after 4 h ozone treatment of apple cider at 4°C, readings changed only from 170 mV (0 h) to 172 mV (4 h). The use of mV readings in apple cider to approximate ozone concentration was found to be unfeasible, and as a result ozone treatment levels are reported as the ozone output rated by the manufacturer.

OZONE IN COMBINATION WITH ANTIMICROBIAL AGENTS

Studies were conducted to determine the effect of wicking hydrogen peroxide (HP) into juices during ozone treatment on the inactivation of foodborne bacteria. A wicking apparatus, developed by Golden Buffalo, consisted of a T-shaped PVC connector of approximately one inch tube diameter, fitted with 1/4 inch reducers on both of the top ends. This apparatus was connected "in-stream" of the air line (i.e., between the pump and the ozone generator), and a wick that was pulled through a rubber stopper, was screwed into the bottom of the T-shaped connector, so that the upper terminus of the wick would be directly in the air stream. The bottom 2/3 of the wick was placed into a 250 ml Erlenmeyer flask containing either 3 or 30% HP. HP is wicked into the air stream prior to entrance into the ozone generator and, inside the generator, HP is split by U.V. light into highly active OH radicals. However, wicking 3% HP into juice inoculated with *E. coli* O157:H7 resulted in only a 0.4 log cfu/ml greater reduction than ozone alone. Wicking 30% HP into apple cider inoculated with *E. coli* O157:H7 was much poorer, with no apparent reductions over ozone alone.

Addition of 3 and 1.5% hydrogen peroxide directly into apple cider inoculated with *E. coli* O157:H7 resulted in rapid decreases in populations of *E. coli* O157:H7 in apple cider at 4˚C. Although effective, 1.5 and 3.0% HP in juice is much higher than acceptable limits.

Dimethyl dicarbonate (DMDC; 250 ppm) causes appreciable reductions in *E. coli* O157:H7 in apple cider during ozone treatment at 4˚C. However, neither combinations of DMDC and sodium benzoate (0.045%) nor DMDC and sodium bisulfite (0.0046%) caused an appreciable reduction.

Preliminary studies indicated that wicking HP into juice, using the protocol described, was ineffective for inactivation of *E. coli* O157:H7 in apple cider during ozone treatment at 4°C. HP added at concentrations of 1.5 and 3.0% in combination with ozone treatment was very effective, but above acceptable limits. Finally, combinations of DMDC and sodium benzoate and sodium bisulfite were also ineffective for reducing populations of *E. coli* O157:H7 in apple cider.

PART IV

EFFECT OF OZONE AND TREATMENT TEMPERATURE ON SURVIVAL OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* SPP. IN APPLE CIDER AND ORANGE JUICE

ABSTRACT

Inactivation of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with ozone was evaluated. A five-strain mixture of *E. coli* O157:H7 or *Salmonella* was inoculated (7 log cfu/ml) into apple cider and orange juice. Ozone (0.9 g ozone/h) was pumped into juices maintained at 4°C, ambient temperature (~20°C), and 50°C for up to 30 min (*Salmonella*/50°C), 75 min (*E. coli*/50°C), 180 min (apple cider/4°C) or 240 min (orange juice/4°C and ambient). Samples were withdrawn, diluted in 0.1% peptone water, neutralized with 1.0 N NaOH, and surface plated onto recovery media. Recovery of *E. coli* O157:H7 was compared on tryptic soy (TSA), sorbitol MacConkey (SMAC), hemorrhagic coli (HC), and modified eosin methylene blue (MEMB) agars; recovery of *Salmonella* was compared on TSA, bismuth sulfite (BSA), and XLT4 agars. After treatment at 50°C, *E. coli* O157:H7 populations were reduced to the limit of detection (1.0 log cfu/ml) in 45, 30, 45, and 30 min in apple cider, and 75, 60, 60, and 45 min in orange juice, as determined on TSA, HC, MEMB, and SMAC, respectively. In apple cider at 50°C, *Salmonella* was reduced by 4.8 log cfu/ml (TSA) and to the limit of detection in 15 min as determined on BSA and XLT4, respectively. In orange juice at 50°C, *Salmonella* populations were reduced to the limit of detection in 15, 10 and 5 min as determined on TSA, BSA, and XLT4, respectively. *E. coli* O157:H7 in apple cider at 4°C was reduced by 4.8 log cfu/ml (TSA) and to the limit of detection in 180 min as determined on HC, MEMB, and SMAC. *E. coli* O157:H7 populations in orange juice at 4°C decreased by 5.4, 5.7, 5.5, and 5.3 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *Salmonella* in apple cider at 4°C was reduced in apple cider by 4.5 log cfu/ml (TSA) and to the limit of detection in 180 and 150 min as

determined on BSA, and XLT4, respectively. *Salmonella* populations in orange juice at 4°C decreased by 4.2, 4.4, and 4.9 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. *E. coli* O157:H7 populations in apple cider at ambient temperature decreased by 3.0, 4.5, 4.0, and 3.4 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *E. coli* O157:H7 populations in orange juice at ambient temperature decreased by 2.3, 3.0, 3.6, and 2.5 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *Salmonella* populations in apple cider at ambient temperature decreased by 3.3, 4.8, and 5.5 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. *Salmonella* populations in orange juice at ambient temperature decreased by 4.2, 4.3, and 4.8 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. Ozone treatment of apple cider and orange juice at 4°C or in combination with mild heating (50°C) may potentially provide an alternative to thermal pasteurization for reduction of *E. coli* O157:H7 and *Salmonella* spp. in apple cider and orange juice.

INTRODUCTION

Outbreaks of foodborne illness associated with juice have been reported since the 1970s (Anderson, 2001). However, during the 1990s, several foodborne illness outbreaks caused by *Escherichia coli* O157:H7 and *Salmonella* spp. were associated with consumption of unpasteurized apple and orange juices (Besser et al., 1993; Buxton et al., 1999; CDC, 1999; Cody et al., 1999; Cook et al., 1998; Millard et al., 1994; Singh et al., 1995). Concerns over the safety of unpasteurized juices has led the FDA to issue hazard analysis critical control point (HACCP) regulations for safe and sanitary processing of juice (FDA, 2001). Under these regulations, fruit juice processors engaged in interstate

or intrastate commerce must implement a HACCP program that includes a process or processes that provide a minimum 5-log reduction in populations of the "pertinent" pathogen in the juice being processed (FDA, 2001).

Some juice producers in the U.S. have considered thermal pasteurization as a means of accomplishing a minimum 5-log reduction in pathogens. However, many juice producers, particularly small seasonal operators, are unable to utilize thermal pasteurization for economic reasons or are opposed to thermal pasteurization because of perceived adverse effects on product quality and acceptability. Technological alternatives to traditional thermal pasteurization, such as ozone treatment, are now being investigated.

Ozone is the triatomic allotrope of oxygen and it is characterized by a high oxidation potential and bactericidal and viricidal properties (Burleson, 1975; Horvath et al., 1985; Kim et al., 1999). Antimicrobial activity of ozone is attributable to its diffusion capability through biological membranes and its high oxidation potential (Hunt and Marinas, 1997). Byun et al. (1998) reported that ozone reduces populations of *E. coli* O157:H7 in buffer. Da Silva et al. (1998) reported that gaseous ozone treatment of fresh fish decreased bacteria associated with spoilage. Restaino et al. (1995) found that ozone treatment kills *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* Typhimurium in water containing organic matter.

Ozone has been evaluated for its efficacy for preserving a variety of food products including, milk, gelatin, albumin, casein and meat products (Kim et al., 1999). However, application of ozone directly to fruit juices for the reduction of pathogens has not been evaluated. The objective of this study was to determine the efficacy of ozone treatment (0.9 g/ h), applied directly to unpasteurized apple cider and orange juice, for

the reduction of *E. coli* O157:H7 and *Salmonella* at 4˚C, ambient temperature, and under mild heating (50˚C).

MATERIALS AND METHODS

Strains and preparation of inoculum-

Five strains of *E. coli* O157:H7 (E0019, beef; 994, salami; H1730, lettuce-associated outbreak; F4546, alfalfa sprout-associated outbreak; cider, apple cider), as well as, *S.* Agona (alfalfa-associated outbreak), *S.* Baildon (lettuce/tomato-associated outbreak), *S.* Gaminara (orange juice), *S.* Michigan (cantaloupe-associated outbreak) and *S.* Montevideo (tomato-associated outbreak), were used in this study. All cultures, with the exception of the *E. coli* O157:H7 cider isolate, were obtained from Dr. Larry Beuchat, University of Georgia.

Cultures of each strain were grown separately in tryptic soy broth (Difco; Detroit, MI) at 35˚C and transferred at 24-h intervals. Broth cultures of each strain of either *E. coli* O157:H7 or *Salmonella* were combined to obtain a mixed culture containing equal proportions of the five strains. Equal portions of the individual strains were mixed and centrifuged (11,000 x *g*, 10 min; Biofuge 17-R; Heraeus Sepatech; Germany), the spent culture medium was decanted, and the cell pellet was resuspended in 0.1% peptone water (PW) (Bacto Peptone; Difco; Becton-Dickinson; Sparks, MD) prior to inoculation of juices.

Inoculation of fruit juices-

Frozen, unpasteurized apple cider (pH ~3.8), purchased from a local processor

and refrigerated, pasteurized orange juice (pH ~3.8), obtained from a local supermarket, were used. Juices were thawed under refrigeration, if required (apple cider only), and held at 4°C prior to experimentation. One liter of juice was transferred to a sterile 4-L Erlenmeyer flask, warmed to the target temperature by direct heat on a stirrer/hotplate (Corning PC-620) if required (ambient and 50°C), and the flask was placed into an ice-waterbath or heated waterbath equipped with stirrers to maintain juice at 4 or 50°C, respectively. No waterbath was used for ambient temperature studies. Juices were inoculated with 10 ml of the mixed *E. coli* O157:H7 or *Salmonella* culture and mixed for 2 min speed prior to addition of antimicrobial agents.

Ozone application-

An Activated Oxygen Generator (Golden Buffalo; CA) designed to produce 0.9 g ozone/h at a flow rate of 2.4 L/min was used. Ozonated air was pumped directly into the juice through a delivery tube (0.25 inch i.d.; Nalgene 180 PVC; Nalge Nunc Int. Corp; Rochester, NY) and sparged through a stainless steel inlet filter (porosity: 10 μm; 0.5 x 1.12"; Upchurch Scientific; Oak Harbor, WA) [4°C] or perforated tubing ring fit to the bottom inside circumference of the 4L flask [ambient and 50°C] (0.25 inch i.d.; Nalgene 180 PVC and 890 Teflon FEP; Nalge Nunc Int. Corp; Rochester, NY). During application of ozone, the contents of the flask were stirred on medium speed using a stirrer to ensure dispersal of ozone. All studies were conducted under an exhaust hood.

Bacteriological Analysis-

Samples (5 ml) were withdrawn at 5-min intervals for up to 30 min

(*Salmonella*/50˚C), 15-min intervals for up to 75 min (*E. coli*/50˚C), or 30-min intervals for up to 180 min (apple cider/4˚C) or 240 min (orange juice/4˚C; ambient studies). Samples were neutralized with 1 N NaOH (Fisher Scientific; Fair Lawn, NJ) and serially diluted in 0.1% PW, and surface plated (0.1 ml) onto tryptic soy agar (TSA; Difco; Becton-Dickinson; Sparks, MD), sorbitol MacConkey agar (SMAC; Oxoid; Basingstoke, England), hemorrhagic coli agar (HC), and modified eosin methylene blue (MEMB) agar to compare recovery of *E. coli* O157:H7; recovery of *Salmonella* was compared on TSA, bismuth sulfite (BSA), and XLT4 agars. All media were incubated for 48 h at 35˚C prior to analysis. MEMB was prepared as described by Clavero and Beuchat (1995), HC as described in the FDA Bacteriological Analytical Manual, and all others according to manufacturer specifications.

E. coli O157:H7 isolates from inoculated apple cider were analyzed for indole, methyl-red, Voges-Proskauer, and citrate (IMViC) reactions and presumptive *E. coli* isolates were confirmed using a latex agglutination test for the O157 antigen (drySPOT™ *E. coli* O157; Oxoid; Basingstoke, England). *Salmonella* isolates from apple cider were analyzed for reactions on triple sugar iron agar (TSI; Difco; Becton-Dickinson; Sparks, MD) and lysine iron agar (LIA; Difco; Becton-Dickinson; Sparks, MD) slants, and presumptive *Salmonella* isolates were confirmed using the Salmonella O antiserum (Poly A-I & Vi; Difco; Detroit, MI) agglutination test.

Statistical analysis-

All experiments were performed in triplicate. Recovery of *E. coli* O157:H7 and *Salmonella* by direct plating was statistically analyzed using the mixed procedure (PROC

MIXED) of SAS version 8.1 (SAS Institute, Cary, NC). The experimental design was a randomized block design, nested treatment arrangement, repeated measures with sampling, blocked on rep. Means were separated using LSMeans; significant differences are defined at $P<0.05$.

RESULTS

Populations of background microflora (aerobic mesophiles) in unpasteurized apple cider were generally in the range of 5.5 to 6.0 log cfu/ml. However, populations of background microflora in pasteurized orange juice were generally below 2.0 log cfu/ml. Additionally, neither *E. coli* O157:H7 nor *Salmonella* spp. were detected in either unpasteurized apple cider or orange juice used in this study.

Initial populations of *E. coli* O157:H7 and *Salmonella* spp. in inoculated juices were approximately 7.0 log cfu/ml and the limit of detection was 1.0 log cfu/ml based on the plating scheme used. Therefore, populations that reached the detection limit represent a minimum 6.0 log cfu/ml reduction.

Generally, inactivation of *E. coli* O157:H7 and *Salmonella* spp. during treatment with ozone (0.9 g/h) followed the order 50°C > 4°C > ambient temperature in apple cider and orange juice for the time periods tested ($P<0.05$), with the exception that in orange juice, reductions of *Salmonella* at 4°C and ambient temperatures did not differ ($P>0.05$). At any temperature tested (i.e., 4°C, ambient, 50°C) juice type (i.e., apple cider, orange juice) did not influence survival of *E. coli* O157:H7or *Salmonella* ($P > 0.05$). Regardless of treatment temperature, *E. coli* O157:H7 in apple cider and orange juice was recovered best on TSA ($P<0.05$) while recovery on HC, MEMB, or SMAC did not differ ($P>0.05$),

with the exception that recovery on orange juice at 50°C followed the order TSA > MEMB > HC > SMAC ($P < 0.05$). Regardless of temperature, *Salmonella* in apple cider was recovered in the following order TSA > BSA > XLT4 ($P<0.05$). In orange juice at 4 and 20°C, *Salmonella* recovery followed the order TSA > BSA = XLT4 ($P < 0.05$), while at 50°C recovery followed the order TSA > BSA > XLT4 ($P < 0.05$).

No appreciable reductions in populations of *E. coli* O157:H7 or *Salmonella* spp. in apple cider or orange juice during control (air) treatments at 4 or 20°C were observed. However, after control treatment for 75 min, populations of *E. coli* O157:H7 in apple cider at 50°C decreased by 2.0, 4.5, 3.0, and 5.9 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively. *E. coli* O157:H7 populations in orange juice at 50°C decreased by 3.1, 5.7, 5.7, and 6.0 log cfu/ml as determined on TSA, HC, MEMB, and SMAC, respectively, after control treatment for 75 min. After control treatment for 30 min, *Salmonella* populations in apple cider at 50°C were reduced by 2.8, 3.5, and 6.0 log cfu/ml as determined on TSA, BSA, and XLT4, respectively. *Salmonella* populations in orange juice at 50°C decreased by 5.3, 5.5, and 6.0 log cfu/ml as determined on TSA, BSA, and XLT4, respectively, after control treatment for 15 min.

After treatment with ozone, *E. coli* O157:H7 populations in apple cider at 4°C decreased by 4.8 log cfu/ml (TSA) and to the limit of detection in 180 min as determined on HC, MEMB, and SMAC (Fig. 1). *E. coli* O157:H7 in orange juice at 4°C decreased by 5.4, 5.7, 5.5, and 5.3 log cfu/ml in 240 min as determined on TSA, HC, MEMB, and SMAC, respectively (Fig. 2). *Salmonella* populations in apple cider at 4°C decreased by 4.5 log cfu/ml (TSA) in 180 min, and to the limit of detection in 180 min and 150 min as determined on BSA, and XLT4, respectively (Fig. 3). *Salmonella* populations in orange

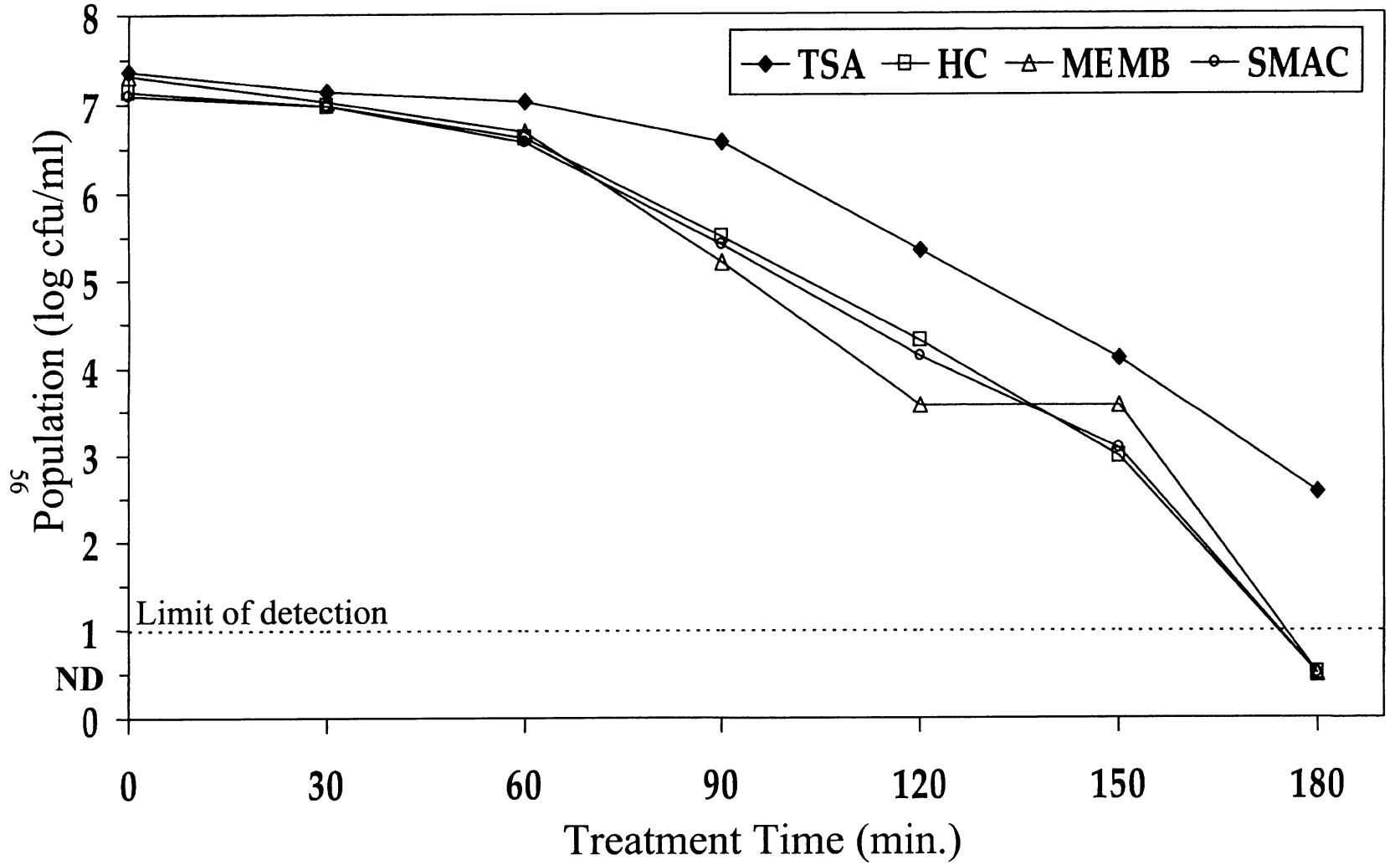

Figure 1. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at 4°C. ND = not detected.

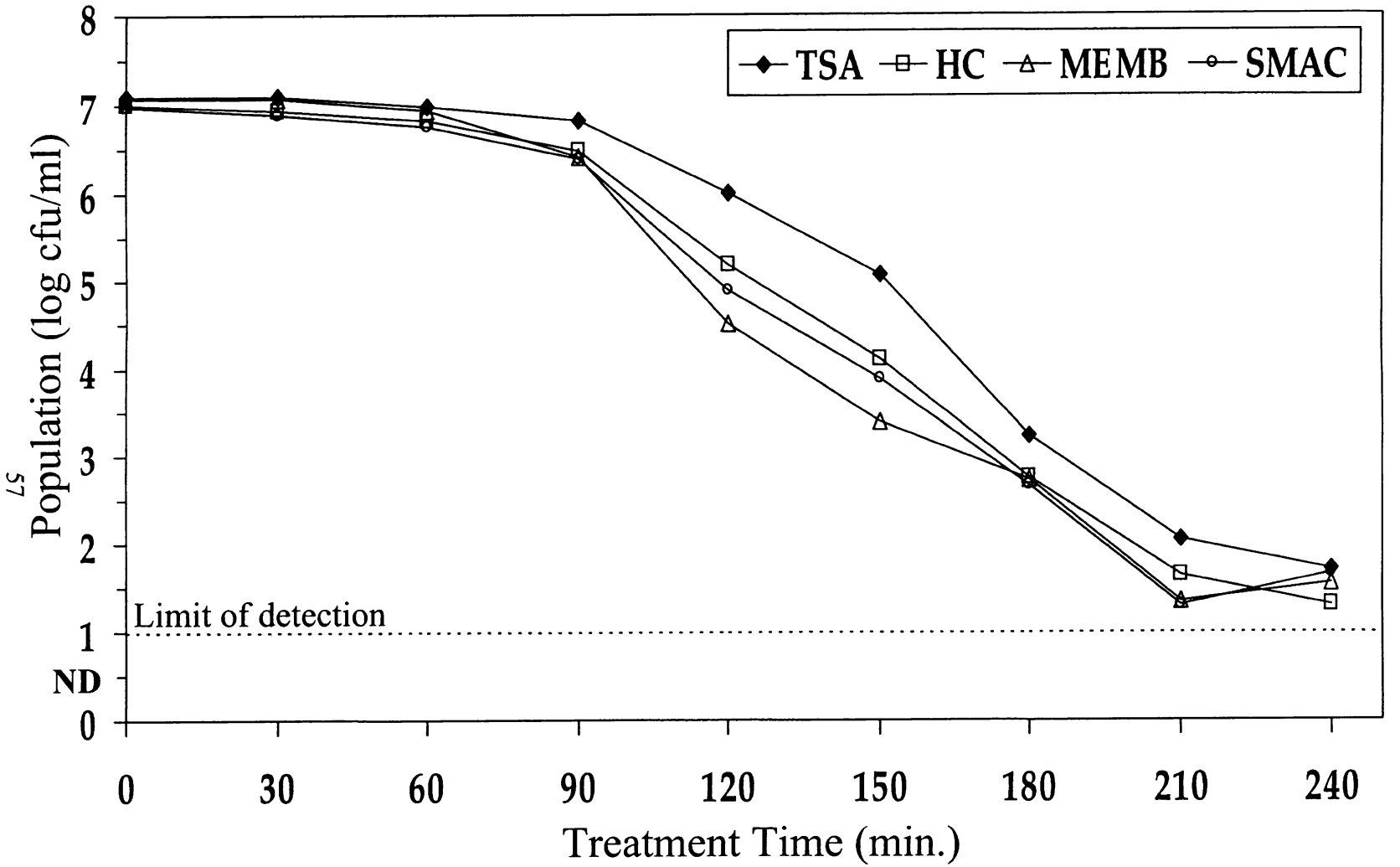

Figure 2. Inactivation of *E. coli* O157:H7 in orange juice during ozone treatment (0.9 g ozone/h) at 4°C. ND = not detected.

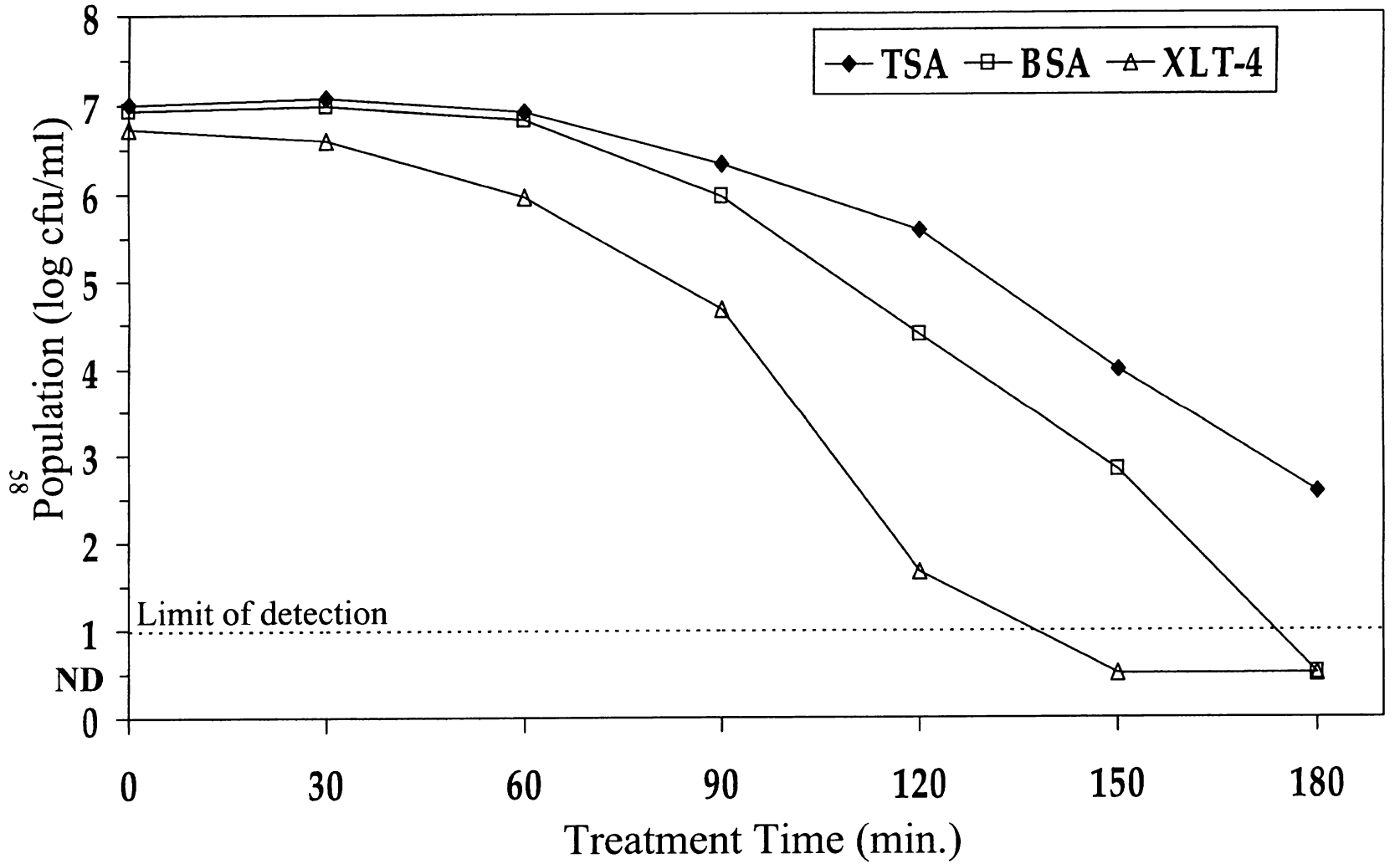

Figure 3. Inactivation of *Salmonella* spp. in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at 4°C. ND = not detected.

juice at 4°C decreased by 4.2, 4.4, and 4.9 log cfu/ml in 240 min as determined on TSA, BSA, and XLT4, respectively (Fig. 4).

E. coli O157:H7 populations in apple cider at ambient temperature (approx. 20°C) decreased by 3.0, 4.5, 4.0, and 3.4 log cfu/ml in 240 min as determined on TSA, HC, MEMB, and SMAC, respectively (Fig. 5). *E. coli* O157:H7 populations in orange juice at ambient temperature decreased by 2.3, 3.0, 3.6, and 2.5 log cfu/ml in 240 min as determined on TSA, HC, MEMB, and SMAC, respectively (Fig. 6). *Salmonella* populations in apple cider at ambient temperature decreased by 3.3, 4.8 and 5.5 log cfu/ml in 240 min as determined on TSA, BSA, and XLT4, respectively (Fig. 7). *Salmonella* populations in orange juice at ambient temperature decreased by 4.2, 4.3, and 4.8 log cfu/ml in 240 min as determined on TSA, BSA, and XLT4, respectively (Fig. 8).

In apple cider at 50°C, *E. coli* O157:H7 populations were reduced to the limit of detection in 45, 30, 45, and 30 min as determined on TSA, HC, MEMB, and SMAC, respectively (Fig. 9). *E. coli* O157:H7 populations in orange juice at 50°C decreased to the limit of detection in 75, 60, 60, and 45 min as determined on TSA, HC, MEMB, and SMAC, respectively (Fig. 10). In apple cider at 50°C, *Salmonella* was reduced by 4.75 log cfu/ml (TSA) in 30 min and to the limit of detection in 15 min as determined on BSA, and XLT4 (Fig 11). In orange juice at 50°C, *Salmonella* populations were reduced to the limit of detection in 15, 10, and 5 min as determined on TSA, BSA, and XLT4, respectively (Fig 12).

DISCUSSION

Direct application of ozone was effective for reducing *E. coli* O157:H7 and

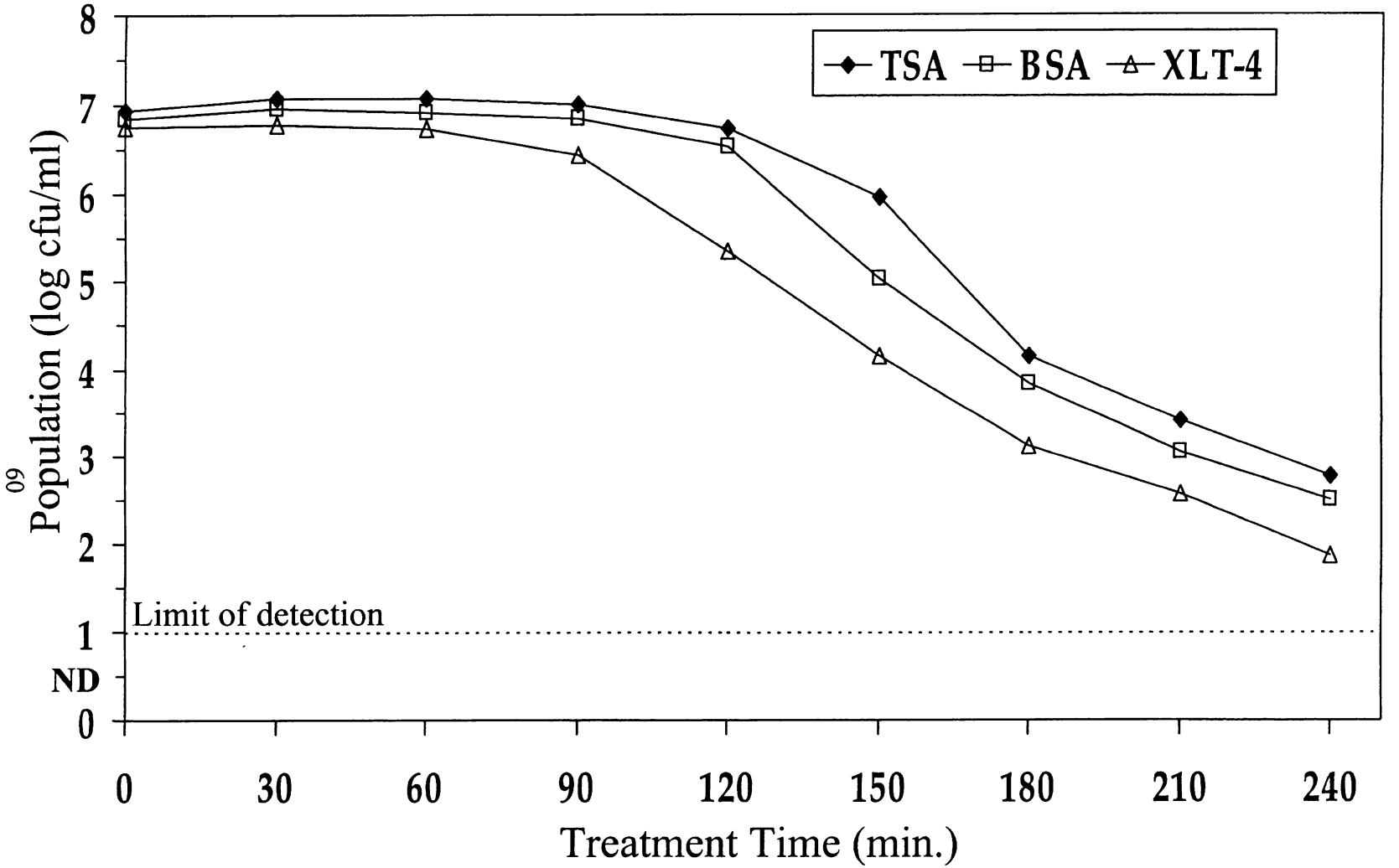

Figure 4. Inactivation of *Salmonella* spp. in orange juice during ozone treatment (0.9 g ozone/h) at 4°C. ND = not detected.

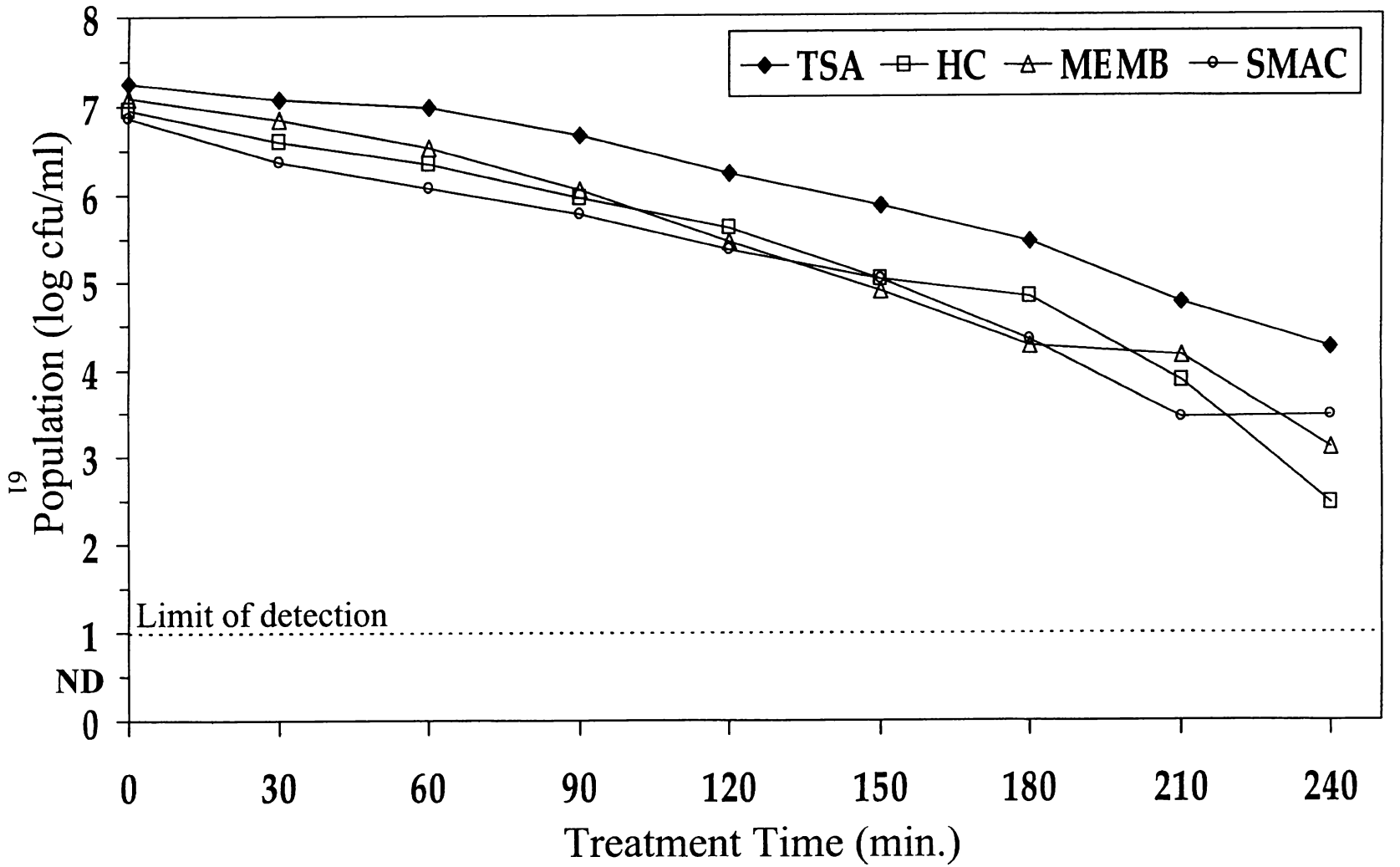

Figure 5. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at ambient temperature (~20°C). ND = not detected.

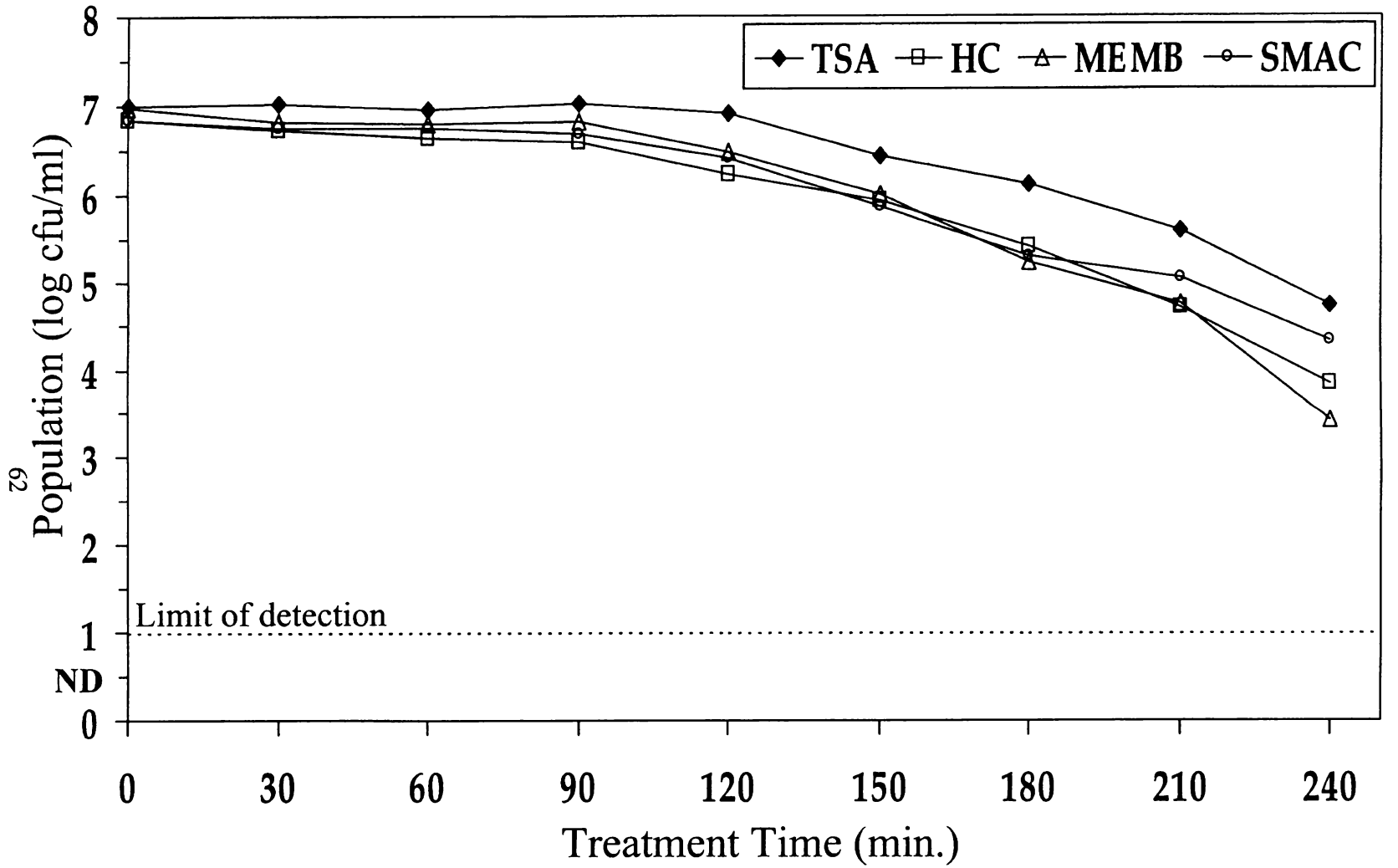

Figure 6. Inactivation of *E. coli* O157:H7 in orange juice during ozone treatment (0.9 g ozone/h) at ambient temperature (~20°C). ND = not detected.

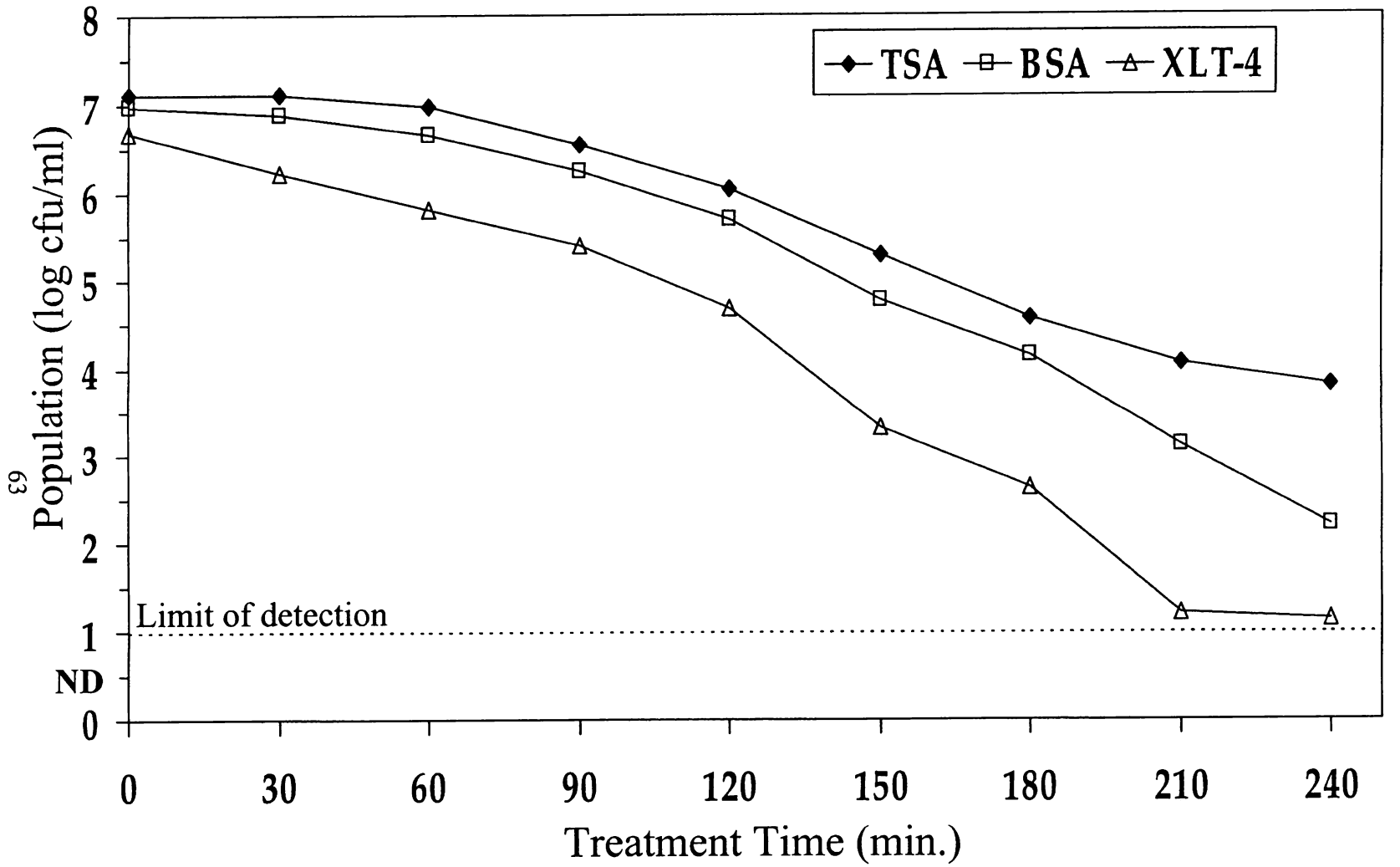

Figure 7. Inactivation of *Salmonella* spp. in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at ambient temperatures (~20°C). ND = not detected.

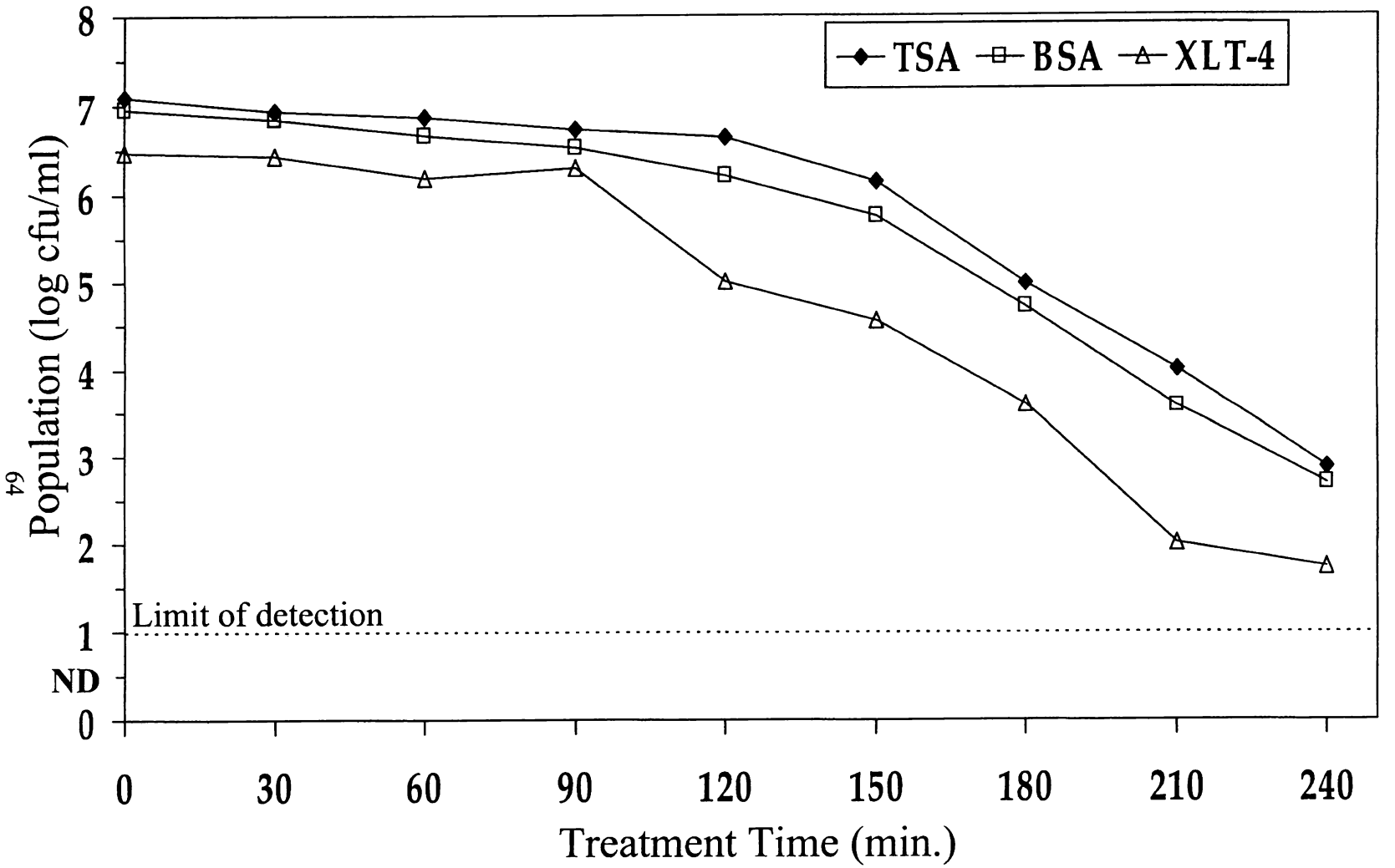

Figure 8. Inactivation of *Salmonella* spp. in orange juice during ozone treatment (0.9 g ozone/h) at ambient temperature (~20°C). ND = not detected.

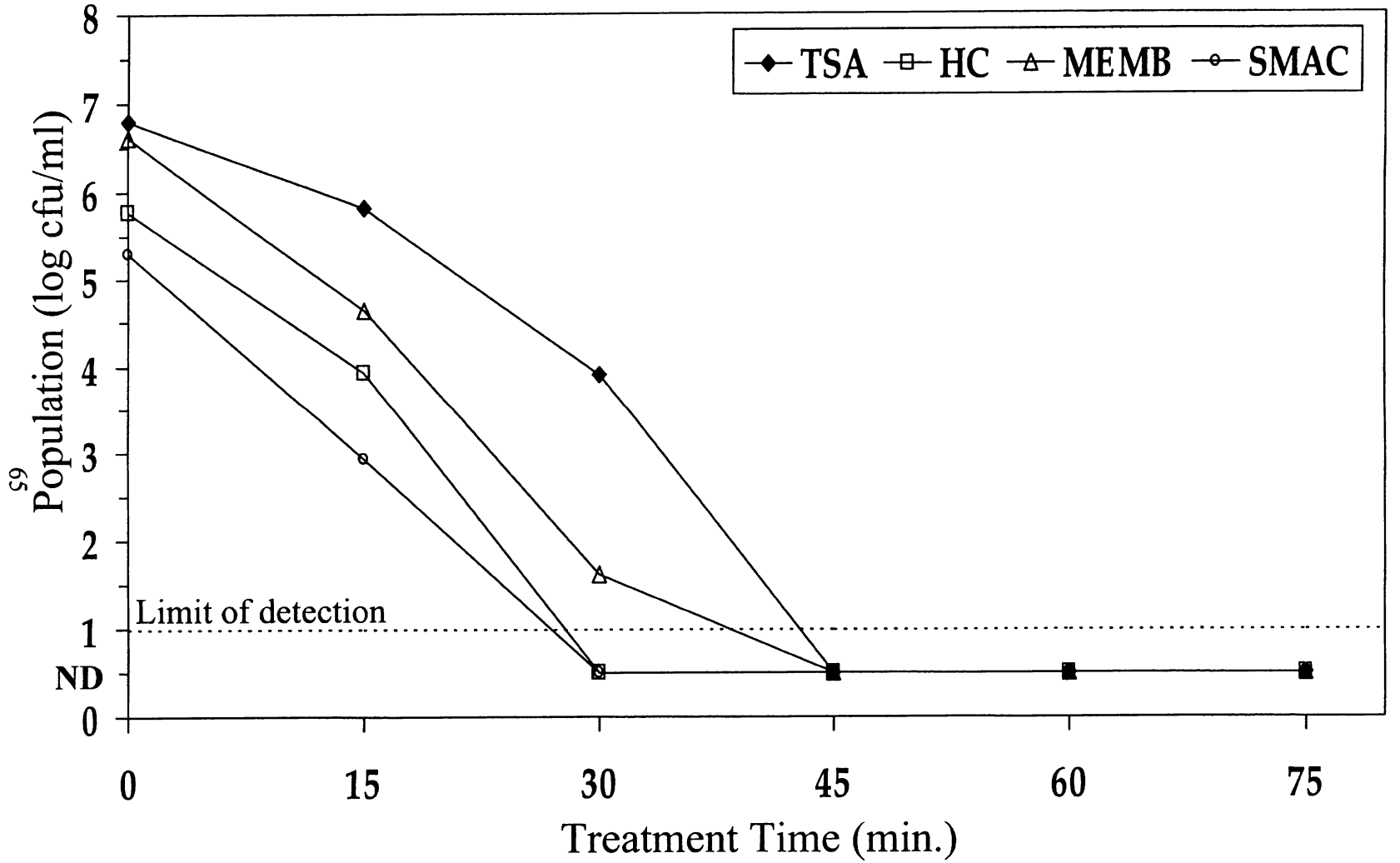

Figure 9. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at 50°C. ND = not detected.

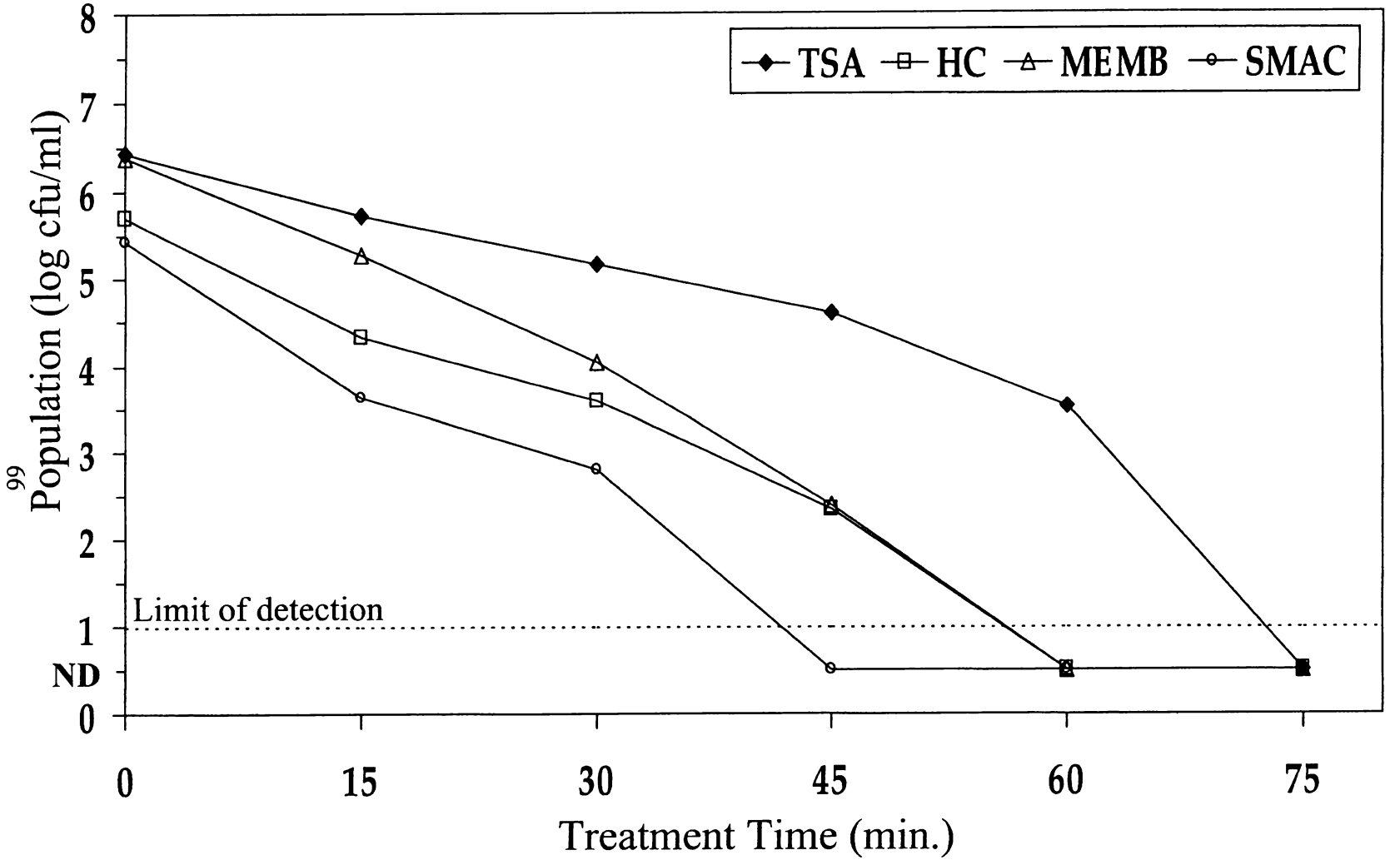

Figure 10. Inactivation of *E. coli* O157:H7 in orange juice during ozone treatment (0.9 g ozone/h) at 50°C. ND = not detected.

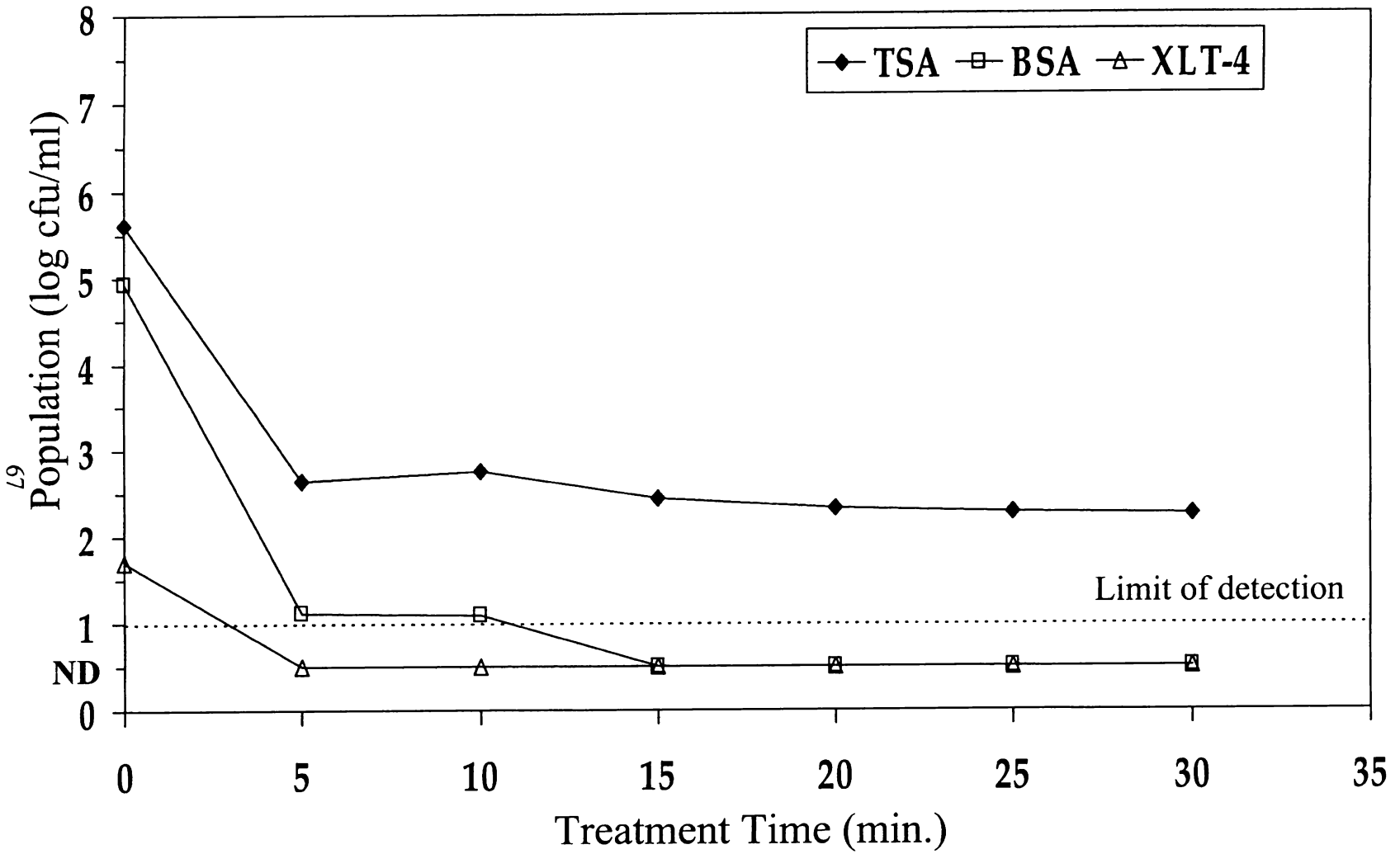

Figure 11. Inactivation of *Salmonella* spp. in unpasteurized apple cider during ozone treatment (0.9 g ozone/h) at 50°C. ND = not detectable.

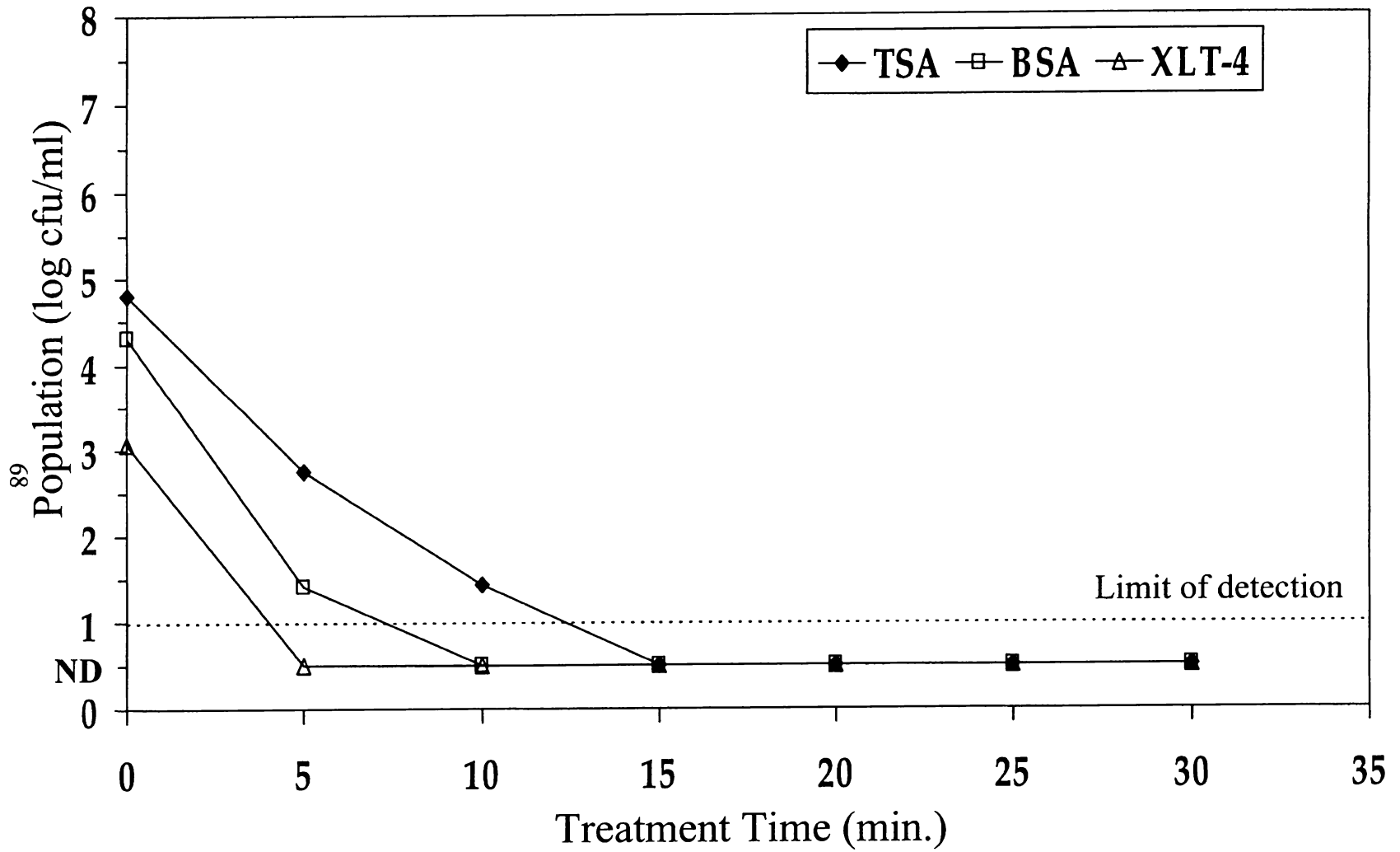

Figure 12. Inactivation of *Salmonella* spp. in orange juice during ozone treatment (0.9 g ozone/h) at 50°C. ND = not detectable.

Salmonella populations in apple cider and orange juice. However, the magnitude of reductions was influenced by the temperature and duration of ozone treatment. During ozone treatment at 4°C, populations of *E. coli* were reduced by 4.8 (apple cider) and 5.4 log cfu/ml (orange juice) in 180 and 240 min, respectively, as determined on TSA. Therefore, under the protocols used in this study, processing times of 3 to 4 hours at 4°C were necessary to approach the 5-log reduction standard required by FDA regulations. Mild heating (50°C) in combination with ozone treatment reduced treatment time to achieve greater than 5-log reductions of *E. coli* O157:H7 to 45 and 75 min in apple cider and orange juice, respectively.

Organic load present during treatment is known to decrease the effectiveness of ozone for the inactivation of microorganisms. Restaino et al. (1995) found that the type of organic material affects ozone effectiveness more than the amount of organic material present. They reported that 20 ppm bovine serum albumin added to deionized water reduced the effectiveness of ozone for inactivating microorganisms, whereas 20 ppm soluble starch had no effect on ozone activity. Labbe et al. (2001) found that 3% sucrose in phosphate buffer markedly affected inactivation of two strains of yeasts and one type of bacterium isolated from maple sap, and that ozone treatment (1 mg ozone/ml) of maple sap, which contains 2 to 3% sucrose, was only moderately effective (< 3 log reduction) for reducing aerobic mesophilic populations during a 40-min treatment time. Apple cider and orange juice contain organic compounds including sugars, pectic substances, and ascorbic acid (OJ) that may react with ozone causing delayed inactivation of microorganisms as compared to inactivation in pure water.

The mechanism for inactivation of microorganisms by ozone includes contact

with ozone molecules or contact with ozone decomposition molecules (radicals). Decreasing pH and temperature are associated with increasing stability of the ozone molecule (Kim et al., 1999). Increasing pH and temperature are associated with a reduction in the stability of ozone and subsequent greater development of ozone induced radicals. In the present study, inactivation of *E. coli* and *Salmonella* during ozone treatment at 4°C was generally better than at 20°C, and ozone treatment at 50°C was better than treatment at either 4°C or ambient temperature. Our preliminary studies showed that during ozone treatment of acidified water (HCl; pH 3.0 to 3.4) ozone concentrations of 0.88 and 0.66 ppm were obtained at 4°C and 23°C, respectively, after four hours. Treatment of acidified water for 75 min at 50°C resulted in an ozone concentration of 0.19 ppm. Differences in reductions *E. coli* O157:H7 and *Salmonella* in this study may have been due to higher concentrations of ozone at 4°C and higher reactivity of ozone at 50°C, in addition to the effects of elevated temperature.

Differences in recovery of *E. coli* O157:H7 and *Salmonella* on non-selective (i.e., TSA) versus selective (i.e., HC, MEMB, SMAC [*E. coli*] and BSA, XLT4 [*Salmonella*]) media indicate that both of these pathogens were sublethally injured during ozone application, regardless of treatment temperature. This illustrates the importance of media selection when determining effectiveness of ozone treatment in juices as part of a process validation. Several investigators have reported the ability of heat and acid to cause injury in *E. coli* O157:H7 and *Salmonella* (Dickson and Siragusa, 1994; Duffy et al., 1999; Mackey and Derrick, 1982; Silk and Donnelly, 1997; Semancheck and Golden, 1998). However, bacterial injury caused by ozone treatment of foods has not been widely reported and further investigation of ozone induced injury of microorganisms in foods is

needed.

This study demonstrates that ozone is a potential alternative to traditional thermal pasteurization to control *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice. However, ozone treatment-related processing problems such as juice foaming, off-gasing of ozone, and difficulties associated with determining ozone concentration in juice must be solved before practical application of ozone for the treatment of fruit juice can be utilized.

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PART V

EFFECT OF OZONE IN COMBINATION WITH DIMETHYL DICARBONATE OR HYDROGEN PEROXIDE ON SURVIVAL OF *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA* SPP. IN APPLE CIDER AND ORANGE JUICE

ABSTRACT

Inactivation of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice treated with ozone in combination with antimicrobials was evaluated. A five-strain mixture of nalidixic acid-resistant *E. coli* O157:H7 or *Salmonella* was inoculated (7 log cfu/ml) into apple cider and orange juice. Ozone (O_3; 0.9 g ozone/h) was pumped into juices containing either dimethyl dicarbonate (DMDC; 250 ppm [Study 1]; 250 and 500 ppm [Study 2]) or hydrogen peroxide (HP; 300 ppm [Study 1]; 300 and 600 ppm [Study 2]) at 4°C for up to 90 min (Study 1) or 60 min followed by 24-h refrigerated (4°C) storage (Study 2). Samples were withdrawn, neutralized with 1.0 N NaOH, diluted in 0.1M phosphate buffer, and surface plated onto tryptic soy agar + 50 ppm nalidixic acid (TSAN). Study 1: No combination of treatments resulted in a 5 log cfu/ml reduction of either pathogen. Overall, O_3/DMDC (250 ppm) was more effective than O_3/HP (300 ppm) for reducing populations of *E. coli* O157:H7 and *Salmonella* ($P < 0.05$). Greater inactivation occurred in apple cider than in orange juice ($P < 0.05$) during O_3 treatment, but inactivation in control (air + antimicrobials) juices did not differ ($P > 0.05$). O_3 treatment in combination with antimicrobials was more effective than controls (i.e., antimicrobials + air) ($P < 0.05$). Overall, effectiveness of O_3/DMDC depended on juice, as greater inactivation of pathogens occurred in O_3/DMDC treated apple cider than O_3/DMDC treated orange juice ($P < 0.05$). However, inactivation of pathogens by O_3/HP did not differ by juice ($P > 0.05$). During O_3/DMDC treatment in apple cider, reductions in *Salmonella* populations were greater than reductions in *E. coli* O157:H7 populations ($P < 0.05$). However, under the same treatment in orange juice, there were no differences between the two pathogens ($P > 0.05$). Overall, O_3/HP caused greater inactivation of *E.*

coli O157:H7 than *Salmonella* ($P < 0.05$). Study 2: All combinations of antimicrobial plus ozone treatments, followed by refrigerated storage, caused greater than a 5-log cfu/ml reduction of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice, except O_3/DMDC (250 ppm) treatment in orange juice reduced *E. coli* O157:H7 populations by less than 5 log cfu/ml. For all combinations tested, inactivation of *E. coli* O157:H7 and *Salmonella* was greater in apple cider than in orange juice ($P < 0.05$). Inactivation of *E. coli* O157:H7 in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only [note: O_3/HP (600 ppm) > O_3/DMDC (250 ppm)] ($P < 0.05$). Inactivation of *E. coli* O157:H7 in orange juice followed the order: O_3/DMDC (500 ppm) = O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/DMDC (250 ppm) >O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in orange juice followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only ($P < 0.05$). This study indicates that ozone treatment in combination with dimethyl dicarbonate or hydrogen peroxide followed by refrigerated storage may potentially provide an alternative to thermal pasteurization to meet the 5-log reduction standard in apple cider and orange juice.

INTRODUCTION

Historically, antimicrobial treatment of apple cider and orange juice has been for the prevention of spoilage. The most common antimicrobials used in fresh juices, sodium benzoate and potassium sorbate, extend shelf-life by inhibiting yeasts and molds

(Downing, 1989). Recent concerns over the safety of fresh fruit juices has led to an interest in alternative treatments, including antimicrobials, for the inactivation of foodborne pathogens in juices.

Several outbreaks of foodborne illness caused by *Escherichia coli* O157:H7 and *Salmonella* spp. that occurred during the 1990s were associated with consumption of unpasteurized apple and orange juices (Besser et al., 1993; Buxton et al., 1999; CDC, 1999; Cody et al., 1999; Cook et al., 1998; Millard et al., 1994; Singh et al., 1995). These outbreaks led the FDA to issue hazard analysis critical control point (HACCP) regulations for safe and sanitary processing of juice (FDA, 2001). A primary performance standard required by this new HACCP regulation is a minimum 5-log reduction in populations of the "pertinent" pathogen in the juice being processed (FDA, 2001).

Juice producers in the U.S. have considered pasteurization as a means of accomplishing a minimum 5-log reduction in pathogens. However, many juice producers, particularly small seasonal operations, are unable to utilize pasteurization for economic reasons or are opposed to pasteurization because of perceived adverse effects on product quality and acceptability. Technologies alternative to traditional pasteurization, such as ozone and antimicrobial treatments, are now being investigated.

Ozone is a triatomic allotrope of oxygen and it is characterized by a high oxidation potential and bactericidal and viricidal properties (Burleson, 1975; Horvath et al., 1985; Kim et al., 1999). Ozone has been shown to reduce populations of *E. coli* O157:H7 in phopsphate buffer (Byun et al.,1998). Ozone treatment is reportedly effective at reducing spoilage bacteria on fish (Da Silva et al., 1998). Restaino et al. (1995) found that ozone

treatment kills microorganisms including, *Escherichia coli*, *Listeria monocytogenes*, and *Salmonella* Typhimurium, in water containing organic matter.

Fisher and Golden (1998a) reported that dimethyl dicarbonate, sodium bisulfite, sodium benzoate, and sodium bisulfite + sodium benzoate were effective for inactivating *E. coli* O157:H7 in apple cider. Regardless of temperature, DMDC was more effective for reducing populations of *E. coli* O157:H7 in apple cider than other antimicrobials tested. Wright et al. (2000) found that acetic acid treatment followed by hydrogen peroxide treatment was effective for reducing populations of *E. coli* O157:H7 on the surface of intact apples.

Ozone has been evaluated for its efficacy in preserving a variety of food products (Kim et al., 1999). However, the efficacy of antimicrobial treatments in combination with ozone for the reduction of bacterial pathogens in fresh juices has not been reported. The objective of this study was to determine the efficacy of dimethyl dicarbonate and hydrogen peroxide in combination with ozone treatment (0.9 g/ h), applied directly to unpasteurized apple cider and orange juice, for the reduction of *E. coli* O157:H7 and *Salmonella* at 4°C.

MATERIALS AND METHODS

Strains and preparation of inoculum-

Five strains of *E. coli* O157:H7 (E0019, beef; 994, salami; H1730, lettuce-associated outbreak; F4546, alfalfa sprout-associated outbreak; cider, apple cider), as well as, *S.* Agona (alfalfa-associated outbreak), *S.* Baildon (lettuce/tomato-associated outbreak), *S.* Gaminara (orange juice), *S.* Michigan (cantaloupe-associated outbreak) and

S. Montevideo (tomato-associated outbreak), were used in this study. All cultures with the exception of the *E. coli* O157:H7 cider isolate were obtained from Dr. Larry Beuchat, University of Georgia.

Cultures of each strain, previously adapted to nalidixic acid, were grown separately in tryptic soy broth (TSB; Difco; Becton-Dickinson; Sparks, MD) containing 50 ppm nalidixic acid (Fisher Scientific; Fair Lawn, NJ) at 35°C and transferred at 24-h intervals. Broth cultures of each strain of either *E. coli* O157:H7 or *Salmonella* were combined to obtain a mixed culture containing equal proportions of the five strains. Equal portions of the individual strains were mixed and centrifuged (11,000 x *g*, 10 min) (Biofuge 17-R; Heraeus Sepatech; Germany), the spent culture medium was decanted, and the cell pellet was resuspended in 0.1% peptone water (PW) (Bacto Peptone; Difco; Becton-Dickinson; Sparks, MD) prior to inoculation of juices.

Inoculation of fruit juices-

Unpasteurized apple cider (pH ~3.8), purchased from a local processor and pasteurized orange juice (pH ~3.8), obtained from a local supermarket, were used. Juices were thawed under refrigeration, if required (apple cider only), and held at 4°C prior to experimentation. One liter of juice was transferred to a sterile 4-L Erlenmeyer flask and the flask was placed into an ice-waterbath atop a stir-plate to maintain the juice at 4°C. Juices were inoculated with 10 ml of the mixed *E. coli* O157:H7 or *Salmonella* culture and mixed for 2 min prior to addition of antimicrobial agents.

Antimicrobial agents-

Studies to determine the combined effect of ozone and antimicrobial agents utilized dimethyl dicarbonate (DMDC; 99.8%; Bayer Corp.; Pittsburgh, PA) and hydrogen peroxide (HP; 30% in water; Fisher Scientific; Fair Lawn, NJ) added directly to juices. Study 1 determined the effect of DMDC (250 ppm) or HP (300 ppm) on populations of the pathogens in the juices during 90 min of ozone treatment. Study 2 determined the effect of DMDC (250 and 500 ppm) or HP (300 and 600 ppm) on the populations of pathogens in the juices during a 60 min ozone treatment followed by 24 h of refrigerated (4°C) storage. Antimicrobial treated juices were mixed for one minute prior to introduction of ozone.

Ozone application-

An Activated Oxygen Generator (Golden Buffalo; CA) designed to produce 0.9 g ozone/h at a flow rate of 2.4 L/min was used. Ozone was pumped directly into the juice through a delivery tube (0.25 inch i.d.; Nalgene 180 PVC; Nalge Nunc Int. Corp; Rochester, NY) and sparged through a perforated tubing ring (0.25 inch i.d.; Nalgene 180 PVC and 890 Teflon FEP; Nalge Nunc Int. Corp; Rochester, NY) fitted to the bottom inside circumference of the 4-L flask. During application of ozone, the contents of the flask were stirred on medium speed using a stirrer/hotplate to ensure dispersal of ozone.

Bacteriological analysis-

Samples (5 ml) were withdrawn at 15-min intervals for up to 90 min (study 1) or 30-min intervals for 60 min ozone treatment followed one sampling after 24 h of refrigerated

(4˚C) storage (study 2). All samples were neutralized with 1 N NaOH (Fisher Scientific; Fair Lawn, NJ), serially diluted in 0.1 M phosphate buffer (PB; BBL/Becton-Dickinson; Sparks, MD), and surface plated onto tryptic soy agar (TSA; Difco; Becton-Dickinson; Sparks, MD) containing 50 ppm nalidixic acid (Fisher Scientific; Fair Lawn, NJ) [TSAN] which was then incubated for 48 h at 35˚C.

Statistical analysis-

Both experiments were performed in triplicate. Recovery of *E. coli* O157:H7 and *Salmonella* by direct plating was statistically analyzed using the mixed procedure (PROC MIXED) of SAS version 8.1 (SAS Institute, Cary, NC). The experimental design for both studies was a randomized block design, factorial treatment arrangement, repeated measures with sampling, blocked on rep. Means were separated using LSMeans; significant differences are defined at $P<0.05$.

RESULTS

Study 1

Overall, O_3/DMDC (250 ppm) was more effective than O_3/HP (300 ppm)for reducing populations of *E. coli* O157:H7 and *Salmonella* ($P <0.05$). Greater inactivation occurred in apple cider than orange juice ($P < 0.05$) during ozone treatment, but control (air + antimicrobials) juices did not differ ($P >0.05$). Ozone treatment in combination with antimicrobials was more effective than controls (i.e., antimicrobials + air) ($P <0.05$). Overall, effectiveness of O_3/DMDC depended on juice, as greater inactivation of pathogens occurred in O_3/DMDC treated apple cider than O_3/DMDC treated orange juice

(P< 0.05). However, inactivation of pathogens by O_3/HP did not differ by juice (P >0.05). During O_3/DMDC treatment in apple cider, reductions in *Salmonella* populations were greater than reductions in *E. coli* O157:H7 populations (P < 0.05). However, under the same treatment in orange juice, there were no differences between the two pathogens (P >0.05). Overall, O_3/HP caused greater inactivation of *E. coli* O157:H7 than *Salmonella* (P < 0.05).

After ozone treatment, *E. coli* O157:H7 populations decreased by 4.2 and 2.3 log cfu/ml in DMDC and HP treated apple cider, respectively (Fig 13). In orange juice, *E. coli* O157:H7 populations decreased by 3.5 and 2.5 log cfu/ml during O_3/DMDC or O_3/HP treatment, respectively (Fig 14). *Salmonella* populations decreased by 4.1 and 2.1 log cfu/ml in apple cider treated with O_3/DMDC and O_3/HP, respectively (Fig 15). After ozone treatment, *Salmonella* populations decreased 1.3 and 1.1 log cfu/ml in DMDC and HP treated orange juice, respectively (Fig 16).

Study 2

For all combinations tested, inactivation of *E. coli* O157:H7 and *Salmonella* was greater in apple cider than in orange juice (P <0.05). Overall, inactivation of *E. coli* O157:H7 followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3/DMDC (250 ppm) > O_3 only > air only (P < 0.05). Overall, inactivation of *Salmonella* followed the order: O_3/DMDC (500 ppm) > O_3/DMDC (250 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) > O_3 only > air only (P <0.05). Inactivation of *E. coli* O157:H7 in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only [note: O_3/HP (600 ppm) >

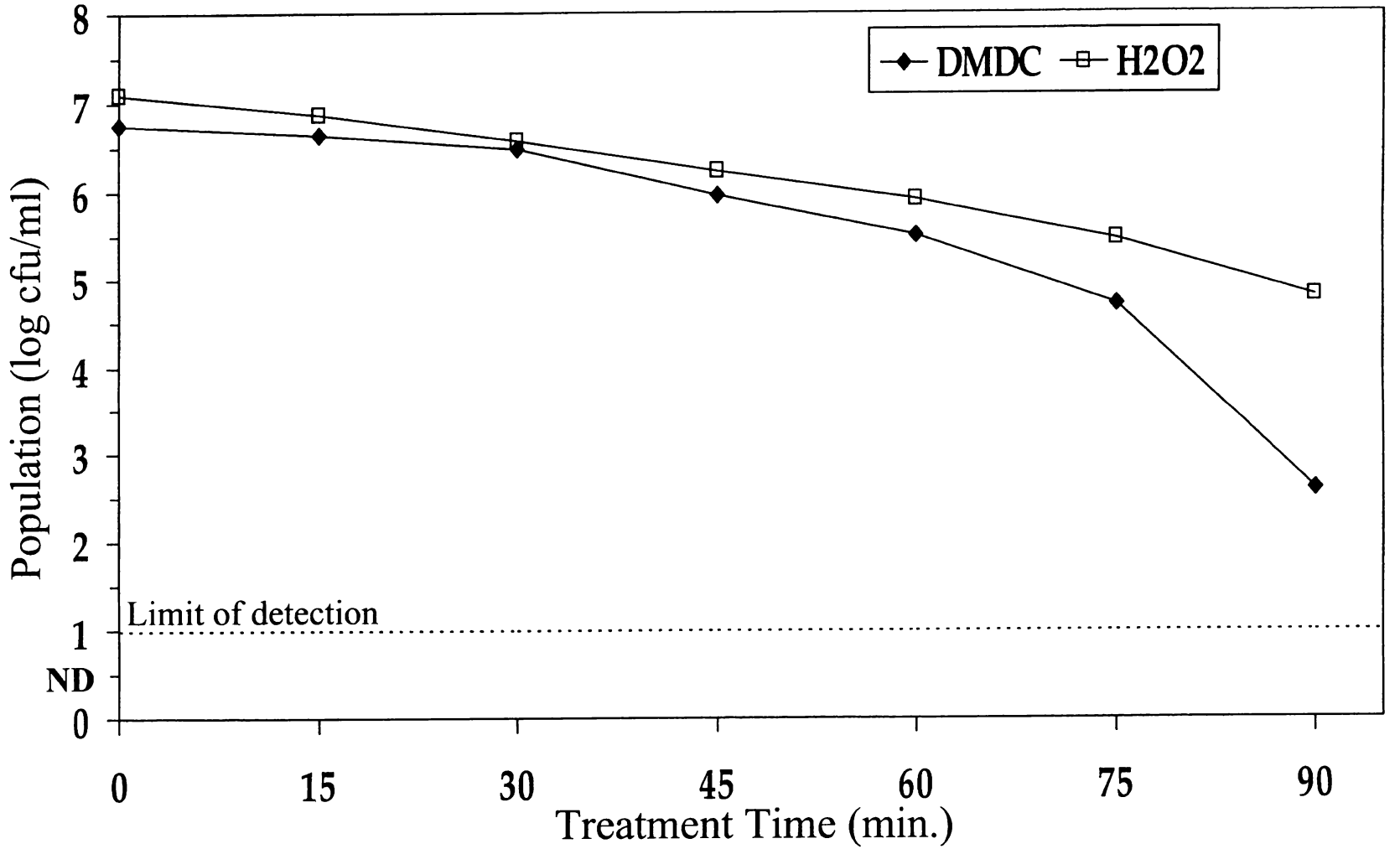

Figure 13. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during treatment with dimethyl dicarbonate (DMDC; 250 ppm) or hydrogen peroxide (H_2O_2; 300 ppm) in combination with ozone (0.9 g ozone/h) at 4°C. ND = not detectable.

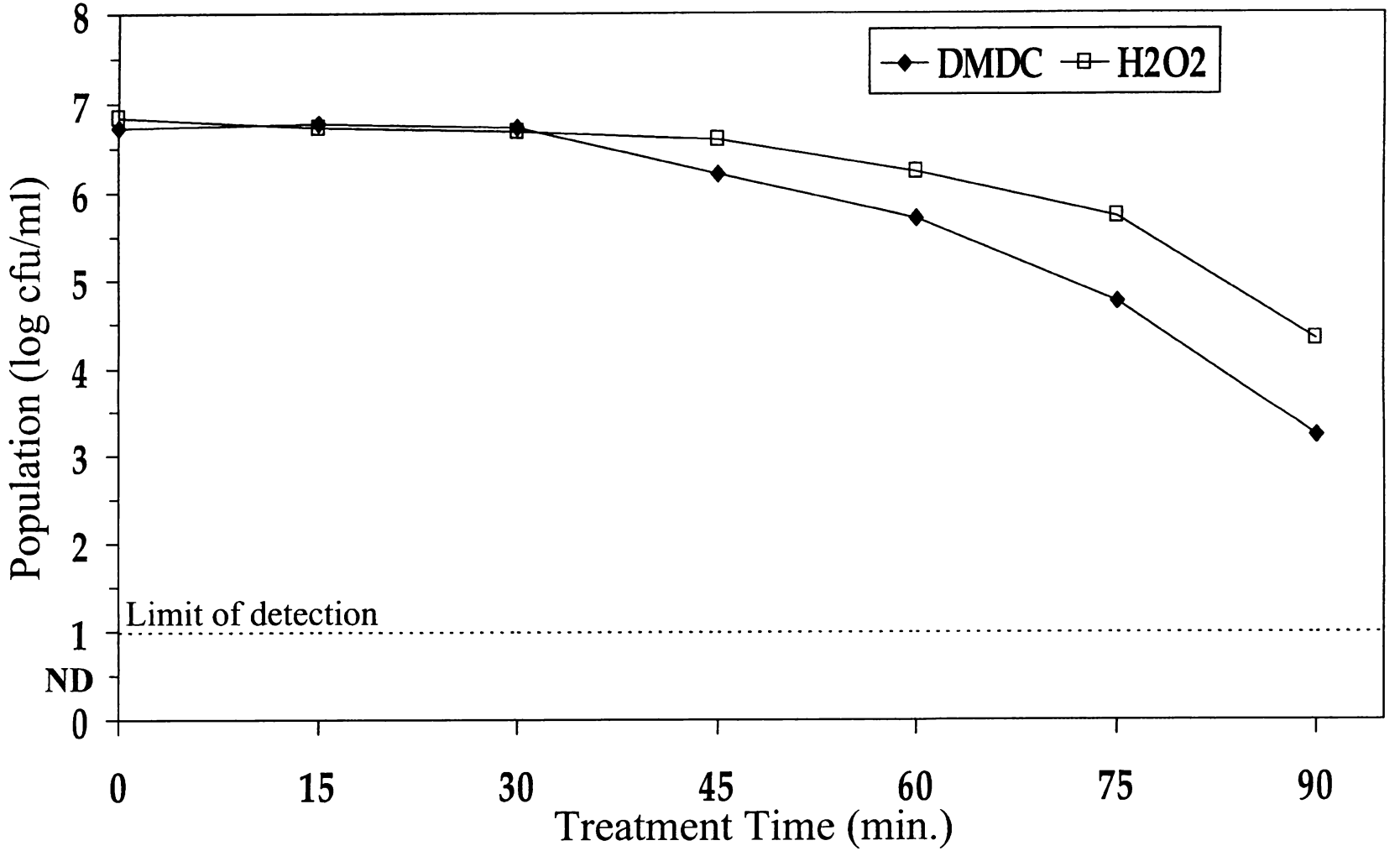

Figure 14. Inactivation of *E. coli* O157:H7 in orange juice during treatment with dimethyl dicarbonate (DMDC; 250 ppm) or hydrogen peroxide (H_2O_2; 300 ppm) in combination with ozone (0.9 g ozone/h) at 4°C. ND = not detectable.

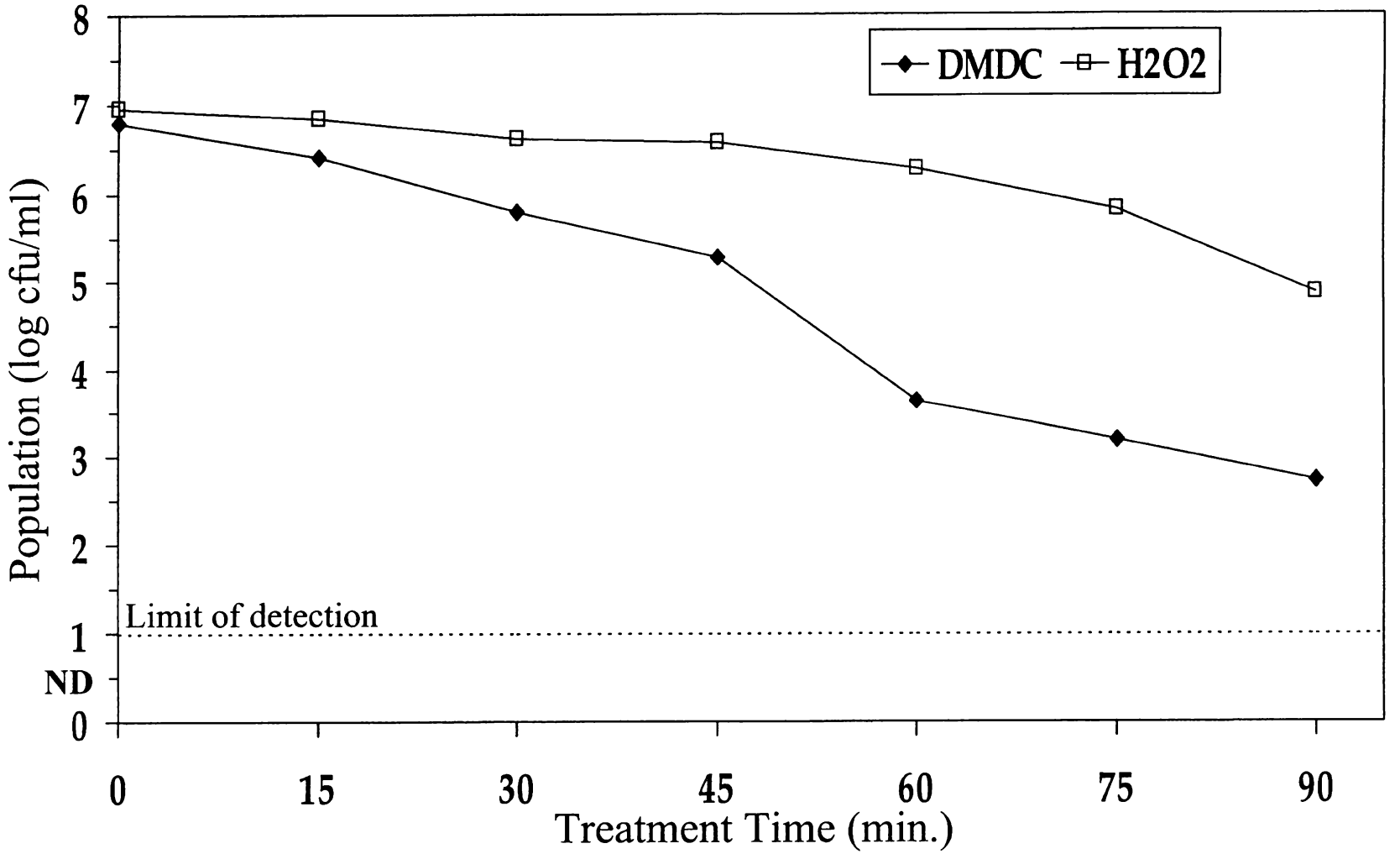

Figure 15. Inactivation of *Salmonella* spp. in unpasteurized apple cider during treatment with dimethyl dicarbonate (DMDC; 250 ppm) or hydrogen peroxide (H_2O_2; 300 ppm) in combination with ozone (0.9 g ozone/h) at 4°C. ND = not detectable.

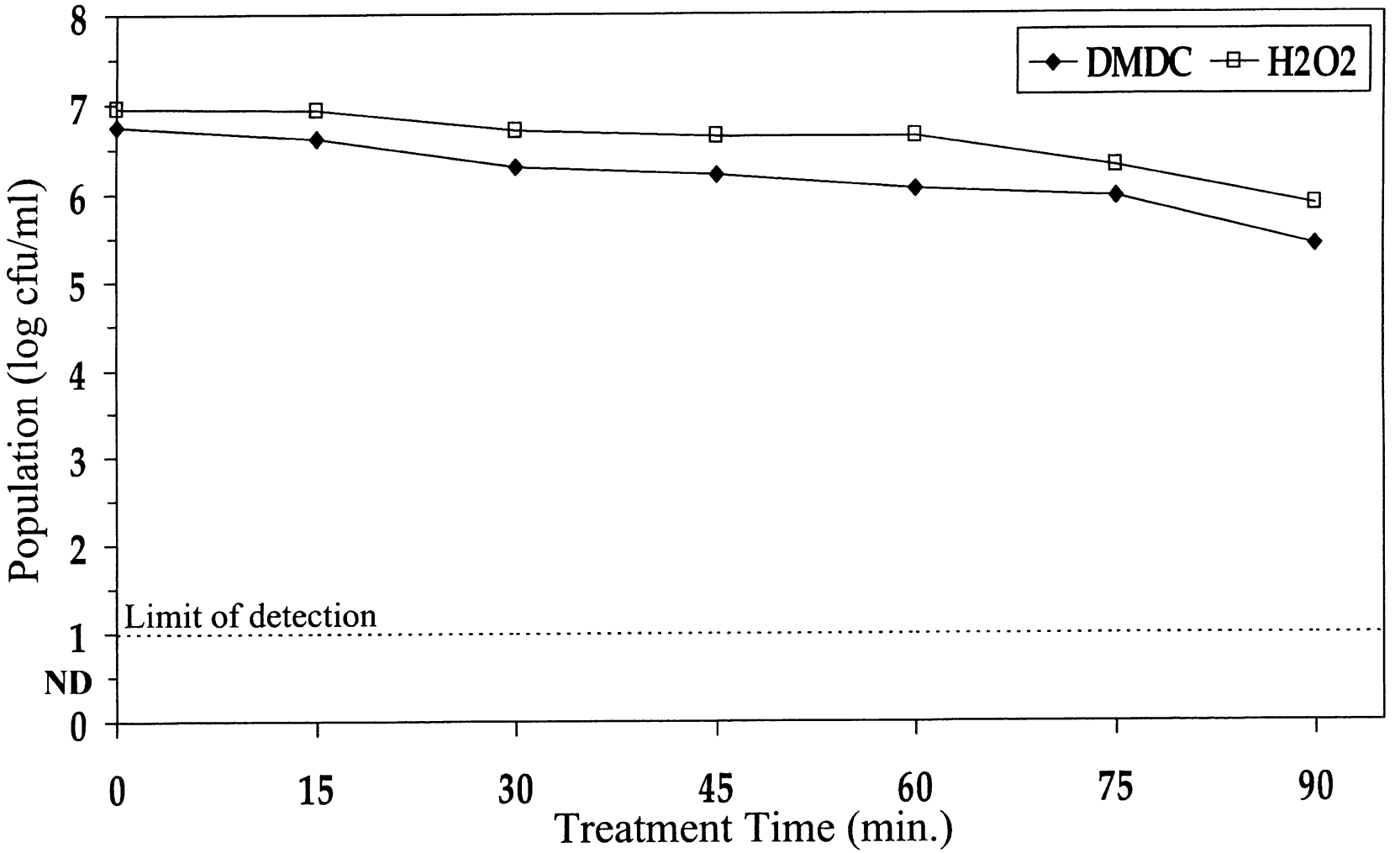

Figure 16. Inactivation of *Salmonella* spp. in orange juice during treatment with dimethyl dicarbonate (DMDC; 250 ppm) or hydrogen peroxide (H_2O_2; 300 ppm) in combination with ozone (0.9 g ozone/h) at 4°C. ND = not detectable.

O_3/DMDC (250 ppm)] ($P < 0.05$). Inactivation of *E. coli* O157:H7 in orange juice followed the order: O_3/DMDC (500 ppm) = O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in apple cider followed the order: O_3/DMDC (500 ppm) > O_3/DMDC (250 ppm) >O_3/HP (600 ppm) > O_3/HP (300 ppm) > O_3 only > air only ($P < 0.05$). Inactivation of *Salmonella* in orange juice followed the order: O_3/DMDC (500 ppm) > O_3/HP (600 ppm) = O_3/HP (300 ppm) = O_3/DMDC (250 ppm) > O_3 only > air only ($P < 0.05$).

E. coli O157:H7 populations in apple cider decreased by 5.5, 5.7, 5.8, 5.8, 0.9, and 0.1 log cfu/ml after treatment with O_3/DMDC (500 ppm), O_3/DMDC (250 ppm), O_3/HP (600 ppm), O_3/HP (300 ppm), O_3 only, and air only, respectively, after 24 h storage at 4°C (Figs. 17 and 18). *E. coli* O157:H7 populations in orange juice decreased by 5.5, 3.7, 5.6, 5.7, 0.4, and 0.01 log cfu/ml after treatment with O_3/DMDC (500 ppm), O_3/DMDC (250 ppm), O_3/HP (600 ppm), O_3/HP (300 ppm), O_3 only, and air only, respectively, after 24 h storage at 4°C (Figs. 19 and 20). *Salmonella* populations in apple cider decreased by 5.8, 5.8, 5.9, 5.7, 1.0, and 0.3 log cfu/ml after treatment with O_3/DMDC (500 ppm), O_3/DMDC (250 ppm), O_3/HP (600 ppm), O_3/HP (300 ppm), O_3 only, and air only, respectively, after 24 h storage at 4°C (Figs. 21 and 22). *Salmonella* populations in orange juice decreased by 5.7, 5.6, 5.7, 5.8, 1.8, and 0.0 log cfu/ml after treatment with O_3/DMDC (500 ppm), O_3/DMDC (250 ppm), O_3/HP (600 ppm), O_3/HP (300 ppm), O_3 only, and air only, respectively, after 24 h storage at 4°C (Figs. 23 and 24). Ozone treatment + DMDC (500 ppm) in apple cider, caused 5.5 and 5.8 log cfu/ml reductions in *E. coli* O157:H7 and *Salmonella*, respectively, during treatment for 60 min.

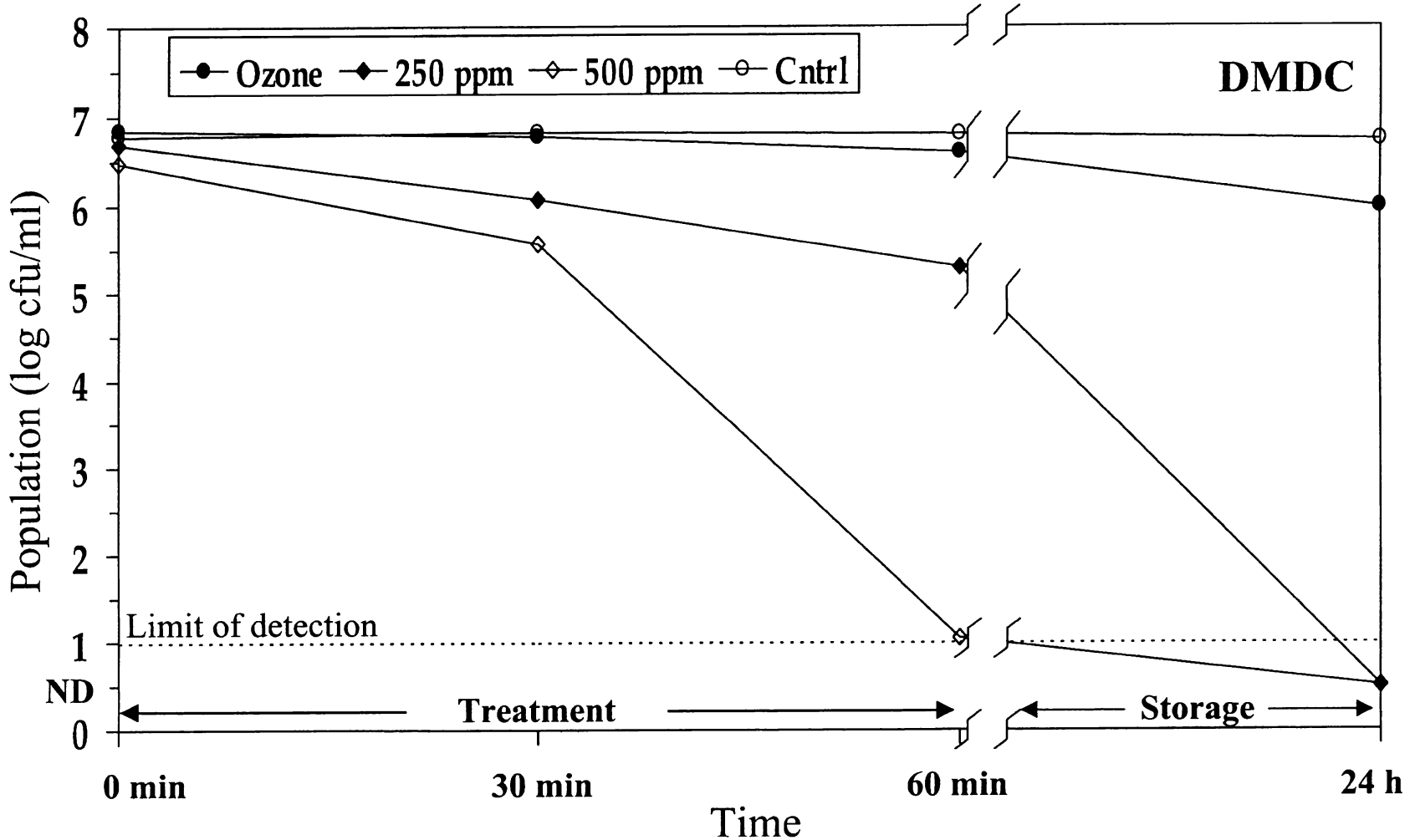

Figure 17. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during treatment with dimethyl dicarbonate (DMDC) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

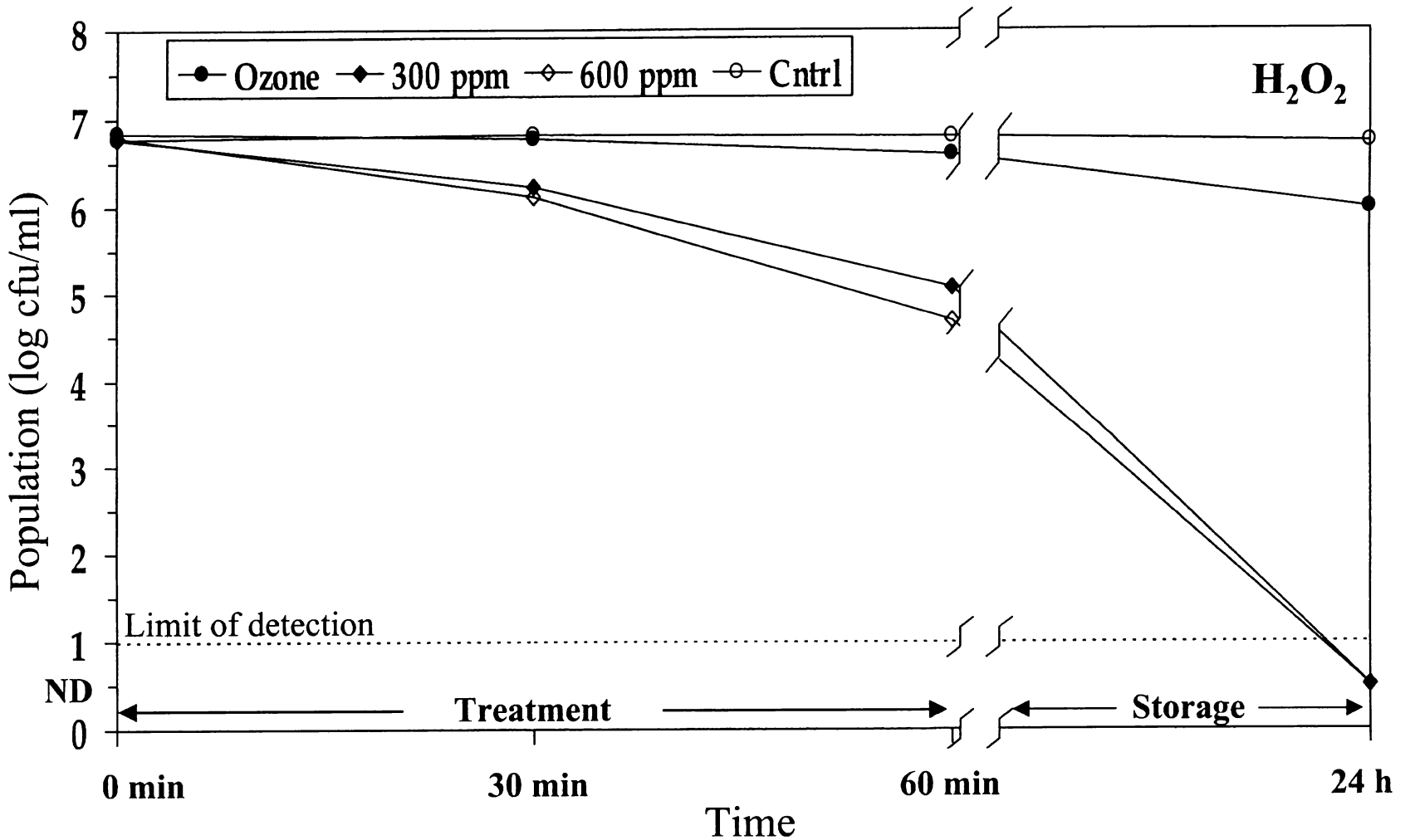

Figure 18. Inactivation of *E. coli* O157:H7 in unpasteurized apple cider during treatment with hydrogen peroxide (H_2O_2) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

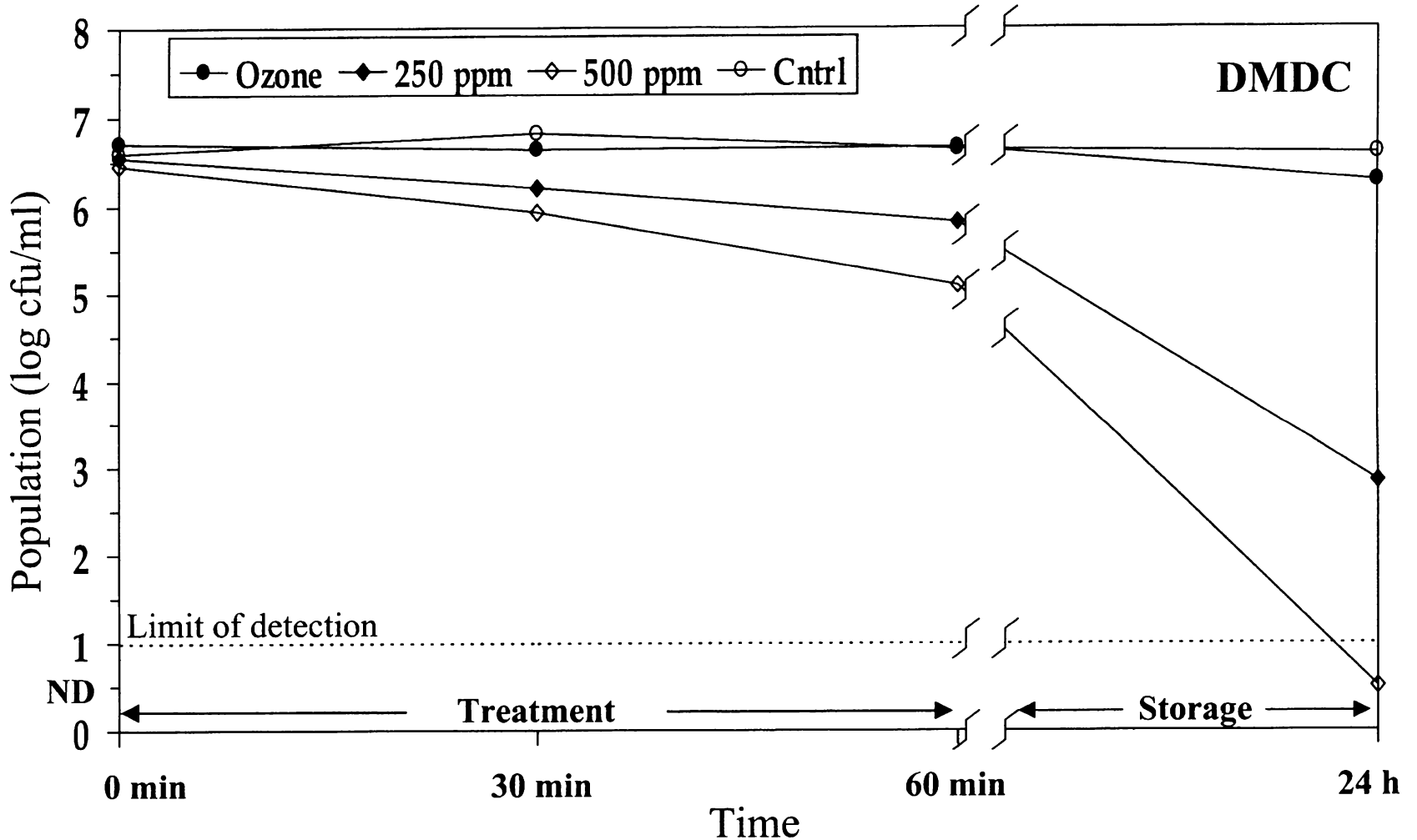

Figure 19. Inactivation of *E. coli* O157:H7 in orange juice during treatment with dimethyl dicarbonate (DMDC) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

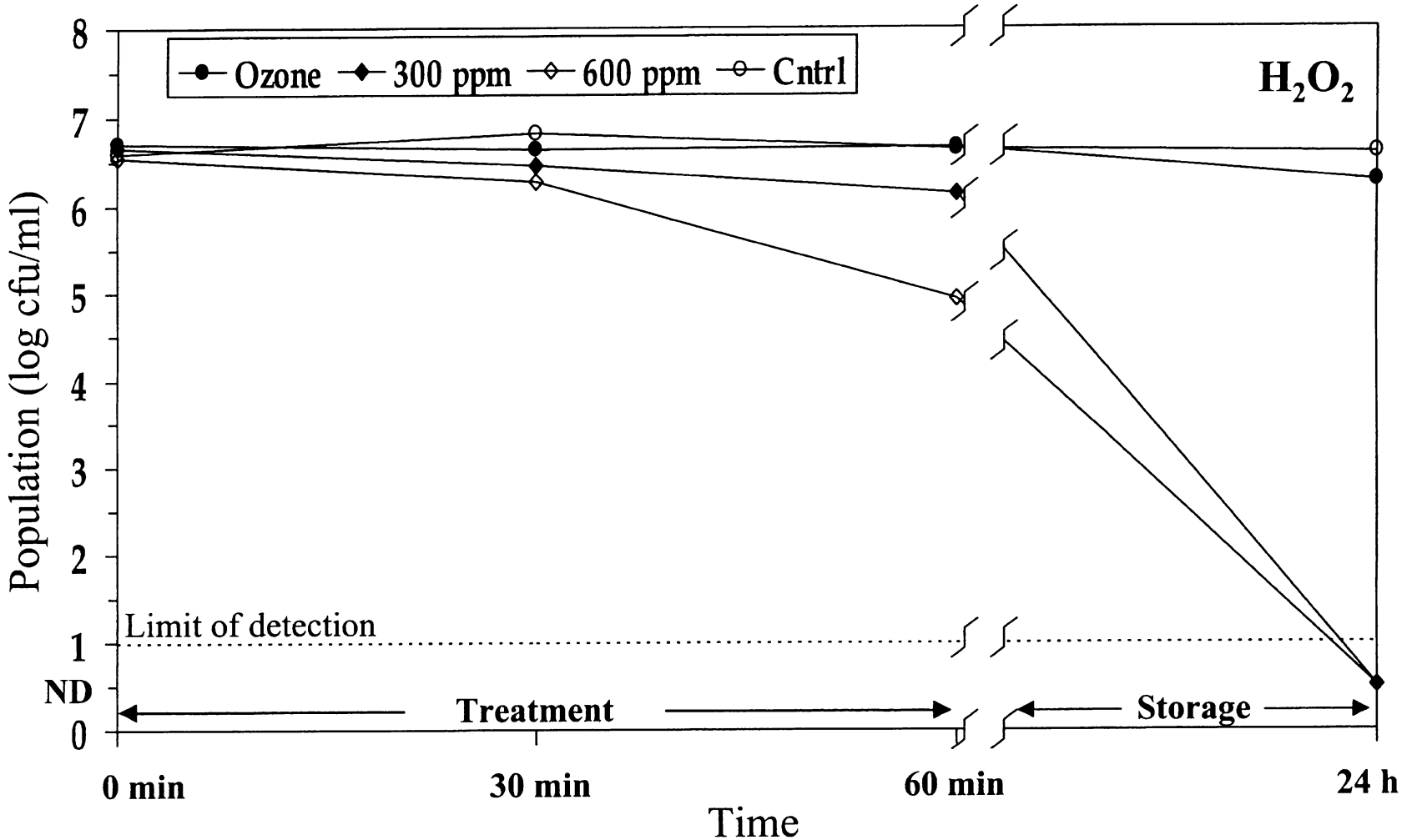

Figure 20. Inactivation of *E. coli* O157:H7. in orange juice during treatment with hydrogen peroxide (H_2O_2) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

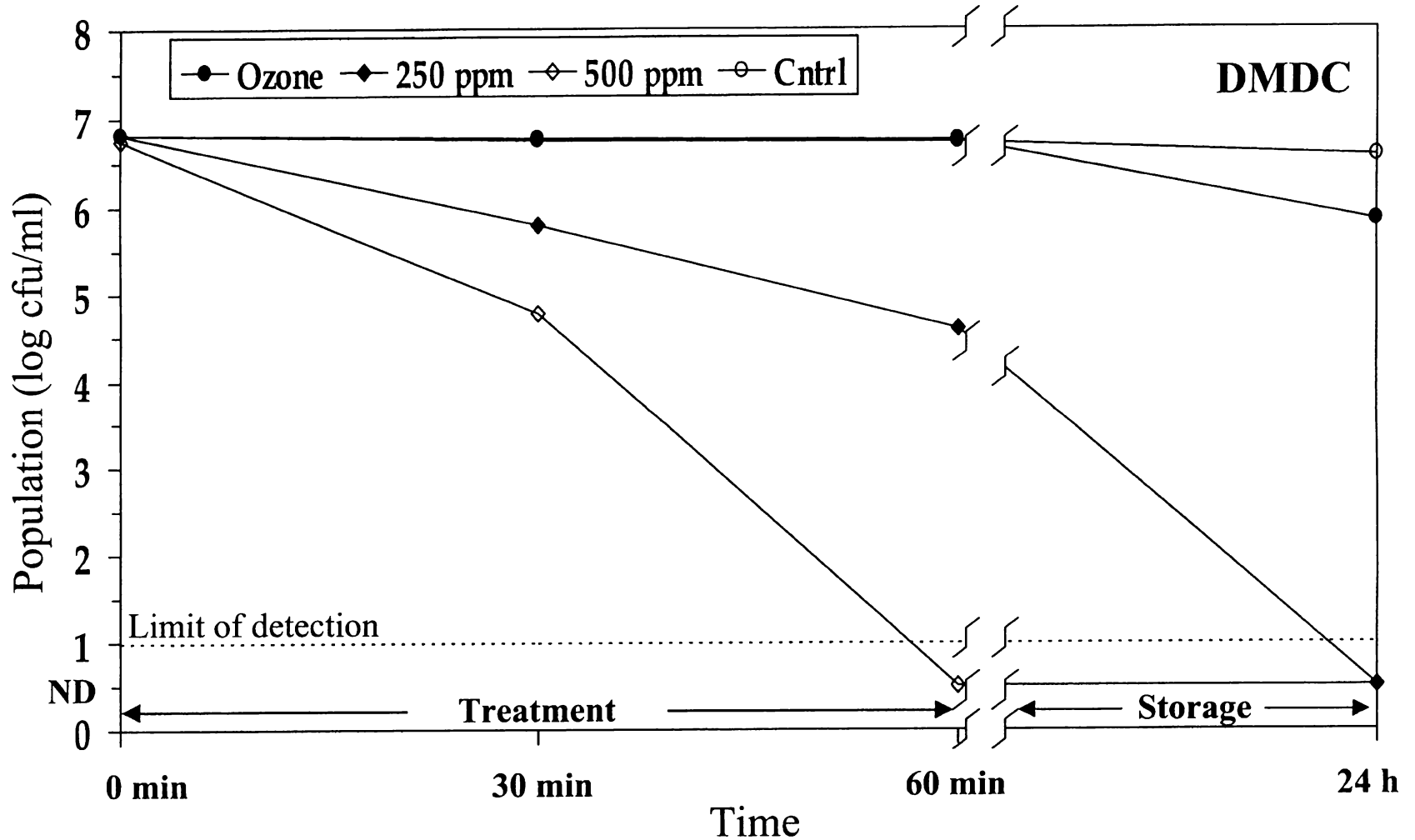

Figure 21. Inactivation of *Salmonella* spp. in unpasteurized apple cider during treatment with dimethyl dicarbonate (DMDC) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

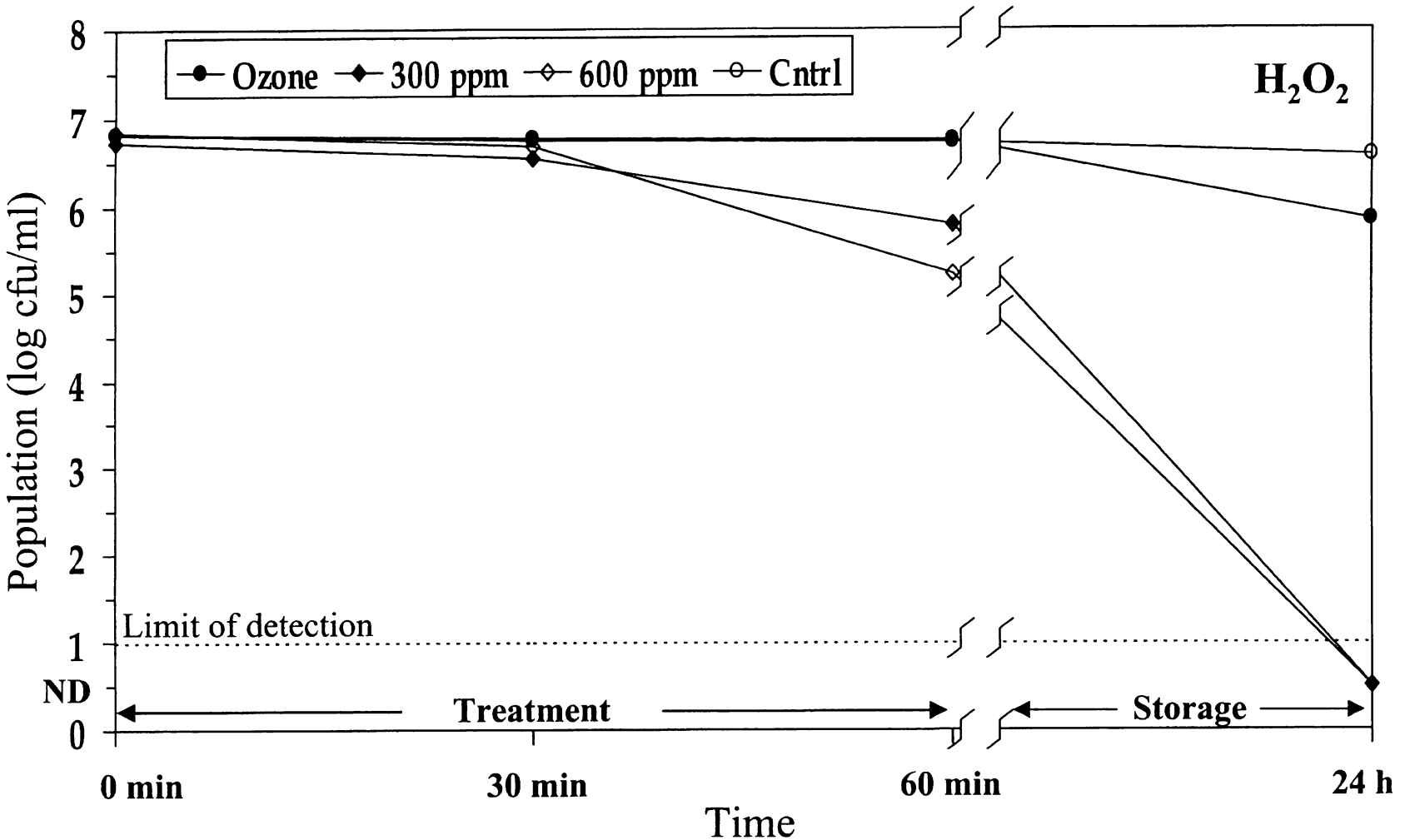

Figure 22. Inactivation of *Salmonella* spp. in unpasteurized apple cider during treatment with hydrogen peroxide (H_2O_2) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

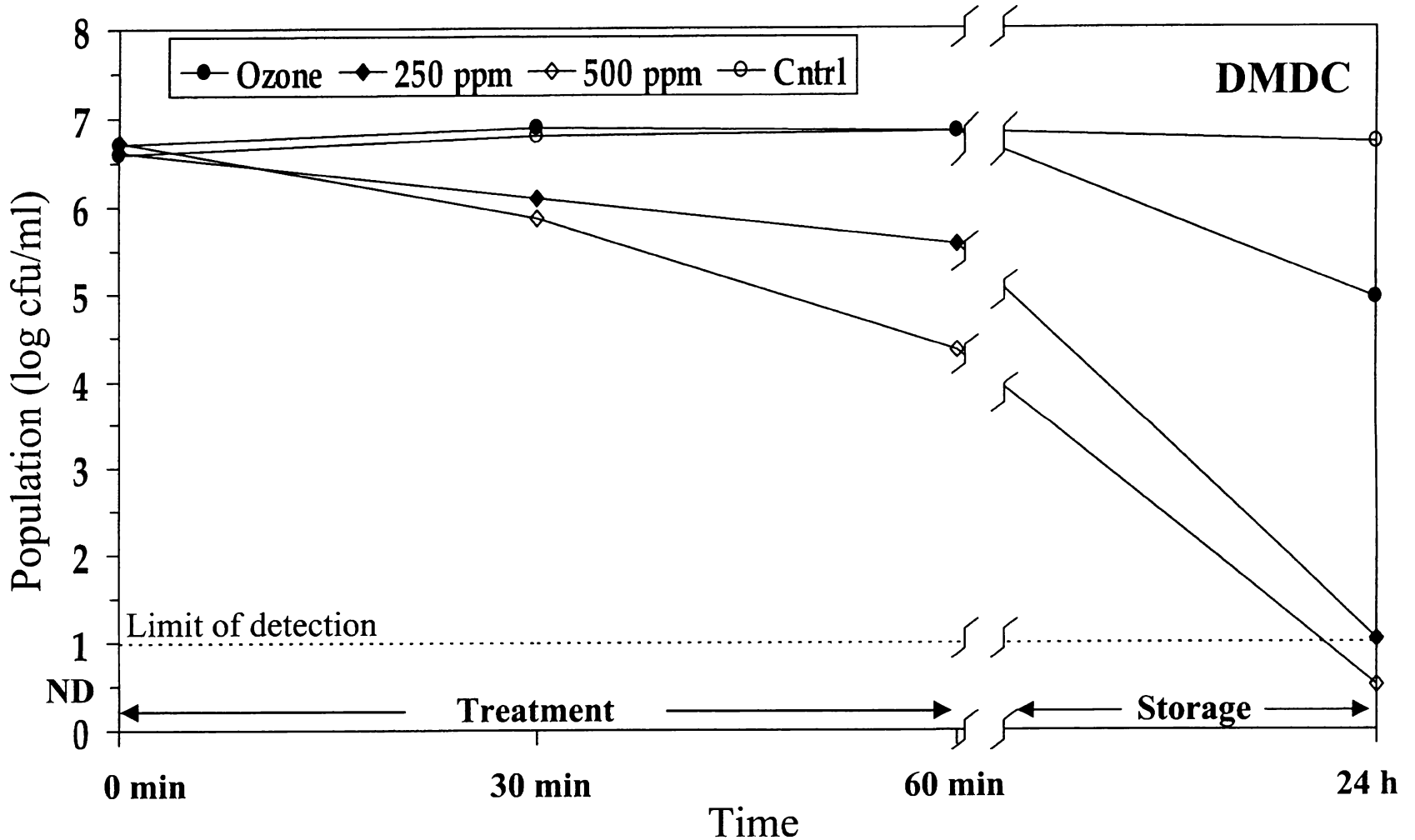

Figure 23. Inactivation of *Salmonella* spp. in orange juice during treatment with dimethyl dicarbonate (DMDC) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

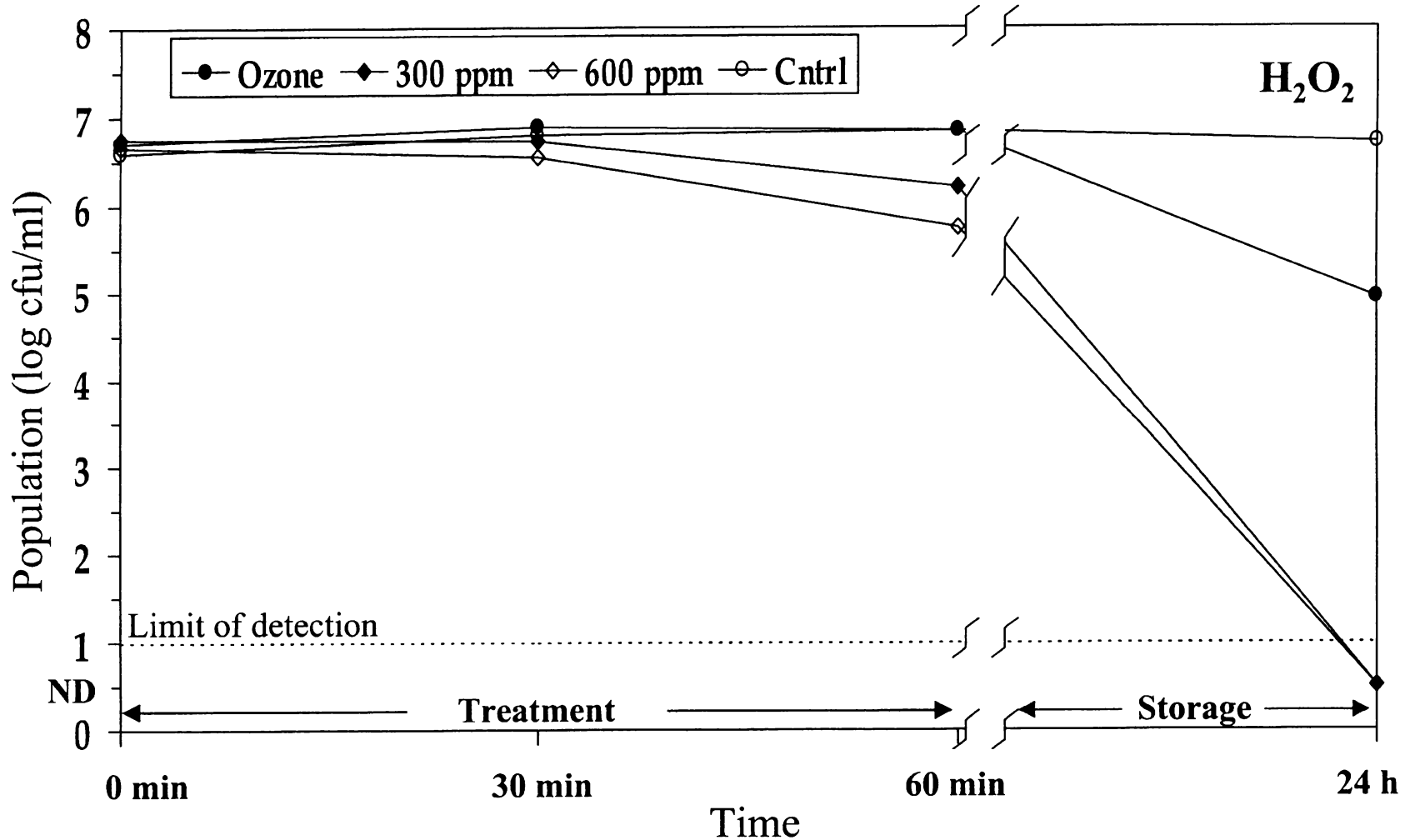

Figure 24. Inactivation of *Salmonella* spp. in orange juice during treatment with hydrogen peroxide (H_2O_2) in combination with ozone (0.9 g ozone/h) at 4°C followed by 24-h storage at 4°C. ND = not detectable.

DISCUSSION

No combination of treatments in study 1 resulted in a 5-log cfu/ml reduction of either *E. coli* O157:H7 or *Salmonella* in either apple cider or orange juice during 90 min of treatment. However, as demonstrated in study 2, all combinations of antimicrobials (O_3/DMDC 500 ppm, O_3/DMDC 250 ppm, O_3/HP 600 ppm, or O_3/HP 300 ppm) caused greater than 5-log reductions for *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice after 24 h storage at 4°C, with the exception that O_3/DMDC (250 ppm) in orange juice reduced populations of *E. coli* O157:H7 less than 5-log cfu/ml during ozone treatment or subsequent storage. These results indicate that the antimicrobial activity of DMDC and HP continues to cause reductions in pathogen populations during storage for 24 h at 4°C.

Greater reductions in populations of *E. coli* O157:H7 and *Salmonella* were observed in ozone and antimicrobial treated apple cider than orange juice for both studies, with the exception that in study 1 there was no difference between juices for the HP (300 ppm) plus ozone treatment. The orange juice used in this study was a "homestyle" type that contained added pulp. This orange juice contains more ascorbic acid and presumably more organic matter than apple cider. Organic matter is known to have a quenching effect on ozone (Restaino et al., 1995) and antimicrobials, resulting in reduced availability for interaction with microorganisms. For example, the antimicrobial activity of HP is primarily oxidative and may be diminished by ascorbic acid (a reducing agent), resulting in reduced effectiveness.

In study 1, O_3/DMDC (250 ppm) was more effective after 90 min treatment than HP (300 ppm), while in study 2, O_3/HP (300 ppm) was more effective than O_3/DMDC (250

ppm) for reducing *E. coli* O157:H7 and *Salmonella*. Study 1 samples were taken during 90 min treatment only, whereas study 2 samples were taken during treatment and after 24-h of refrigerated storage. Therefore, O_3/DMDC (250 ppm) appears to act rapidly to inactivate microorganisms, while HP requires longer contact time to inactivate microoganisms.

Unpreserved apple cider has been shown to allow survival of *E. coli* O157:H7 for 18 days (Fisher and Golden, 1998b) and 21 days (Miller and Kaspar, 1994) at 4°C and 31 days at 8°C (Zhao et al., 1993). Fisher and Golden (1998a) reported that 250 ppm dimethyl dicarbonate (DMDC) was effective for inactivating *E. coli* O157:H7 in apple cider at 4, 10, and 25°C. However, *E. coli* O157:H7 remained viable in DMDC treated apple cider for at least 3 days at 4°C. In the current study, combining ozone treatment (60 min) with DMDC (250 ppm) resulted in reduction of *E. coli* O157:H7 to undetectable levels within 24 h. Ozone treatment + DMDC (500 ppm) reduced populations of *E. coli* O157:H7 by greater than 5 log cfu/ml in apple cider within 60 min and did not require additional storage at 4°C.

This study demonstrates that combining ozone and antimicrobial treatment followed by refrigerated storage may potentially be used to achieve a 5-log cfu/ml reduction of *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice. However, survival of both pathogens is influenced by juice and antimicrobial characteristics.

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PART VI
SUMMARY

Several outbreaks of foodborne illness, specifically *Escherichia coli* O157:H7, *Salmonella* spp. and *Cryptosporidium* spp. infections, associated with consumption of unpasteurized apple juice/cider and unpasteurized orange juice, occurred during the 1990's (Besser et al., 1993; Buxton et al., 1999; CDC, 1999; Cody et al., 1999; Cook et al., 1998; Millard et al., 1994; Singh et al., 1995). In response, the United States Food and Drug Administration (FDA) issued hazard analysis and critical control point (HACCP) procedures for safe and sanitary processing and importing of juice (FDA, 2001). According to this regulation, juice processors engaged in interstate or intrastate commerce must implement a HACCP program that includes procedures that provide a minimum 5-log reduction in populations of the "pertinent" pathogen in the juice being processed. Pertinent pathogens include, *E. coli* O157:H7 in apple cider and *Salmonella* spp. in orange juice.

Unpasteurized juices (especially fresh apple cider) are products desired by consumers because of their fresh taste and full aroma. However, as highlighted by outbreaks and reports of poor production practices of many small processors, it is likely that pathogenic bacterial contamination of unpasteurized juices will continue. Currently, the only treatment widely available for enhancing the safety of apple cider is pasteurization, but many people perceive adverse effects of pasteurization on flavor and aroma of fresh juices. Furthermore, many apple cider producers are small processors that cannot implement pasteurization due to high capital investment and production expenses (Kozempel et al., 1998), and respondents to a 1996 U.S. Apple Association survey overwhelmingly reported that they had no plans to implement pasteurization (Daly, 1997).

Fresh juice producers are currently searching for alternative processes that allow production of a safe product and compliance with FDA regulations while maintaining characteristics of fresh, unpasteurized juices. The use of ozone and antimicrobial agents offer hope of meeting these demands.

The objectives of this study were (1) to determine the efficacy of ozone treatment (0.9 g/h) at 4, ~20, and 50°C for inactivating *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without preservatives, (2) to determine the survival of *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without preservatives treated with ozone (4°C, 0.9 g/h) in combination with dimethyl dicarbonate (250 ppm) and hydrogen peroxide (300 ppm), and (3) to determine the survival of *E. coli* O157:H7 (mixed strains) and *Salmonella* spp. (mixed strains) in unpasteurized apple cider and pasteurized orange juice without preservatives treated with ozone (4°C, 0.9 g/h) in combination with dimethyl dicarbonate (250 ppm, 500 ppm) and hydrogen peroxide (300 ppm, 600 ppm) and stored at 4°C.

The results of these studies show that direct application of ozone was effective for reducing *E. coli* O157:H7 and *Salmonella* populations in apple cider and orange juice. However, the magnitude of reductions was influenced by the temperature and duration of ozone treatment. During ozone treatment at 4°C, populations of *E. coli* were reduced by 4.8 (apple cider) and 5.4 log cfu/ml (orange juice) in 180 and 240 min, respectively, as determined by recovery on tryptic soy agar (TSA). Therefore, under the protocols used in this study, processing times of 3 to 4 hours at 4°C were necessary to approach the 5-log reduction standard required by FDA regulations. Mild heating (50°C) during

treatment with ozone reduced treatment time to achieve greater than 5-log reductions of *E. coli* O157:H7 to 45 and 75 min in apple cider and orange juice, respectively. However, reductions of *E. coli* O157:H7 and *Salmonella* spp. in apple cider and orange juice were less than 5 log cfu/ml when juices were treated at 20˚C.

Differences in recovery of *E. coli* O157:H7 and *Salmonella* on non-selective (i.e., TSA) versus selective (i.e., hemmorhagic coli, modified eosin methylene blue, sorbitol MacConkey [*E. coli*] and bismuth sulfite, XLT4 [*Salmonella*]) media indicate that both of these pathogens were sublethally injured during ozone application, regardless of treatment temperature. Bacterial injury caused by ozone treatment of foods has not been widely reported and further investigation of ozone induced injury of microorganisms in foods is needed.

Survival of *E. coli* O157:H7 and *Salmonella* spp. during ozone treatment is affected by temperature and duration of treatment. Additionally, recovery of pathogenic bacteria via direct plating varied depending on recovery media used. Results indicate that a combined environment of acidic conditions (i.e., juice) and ozone treatment induce sublethal injury. This fact illustrates the importance of media selection when determining effectiveness of ozone treatment in juices as part of HACCP validation.

No combination of treatments in Objective 2 studies resulted in a 5-log cfu/ml reduction of either *E. coli* O157:H or *Salmonella* in either apple cider or orange juice during 90 min of treatment. For Objective 3, all treatment combinations (O_3/DMDC 500 ppm, O_3/DMDC 250 ppm, O_3/HP 600 ppm, or O_3/HP 300 ppm) caused greater than 5-log reductions for *E. coli* O157:H7 and *Salmonella* in apple cider and orange juice after 24 h storage at 4˚C, with the exception that O_3/DMDC (250 ppm) in orange juice reduced

populations of *E. coli* O157:H7 less than 5-log cfu/ml during ozone treatment and subsequent storage. These results indicate that the antimicrobial activity of DMDC and HP continues to cause reductions in pathogen populations during storage for 24 h at 4°C.

Greater reductions in populations of *E. coli* O157:H7 and *Salmonella* were observed in ozone and antimicrobial treated apple cider than orange juice for both studies, with the exception that in Objective 2 studies, there was no difference between juices for the HP (300 ppm) plus ozone treatment. The orange juice used in this study was a "homestyle" type that contained added pulp. This orange juice contains more ascorbic acid and presumably other organic matter than apple cider. Organic matter is known to have a quenching effect on ozone (Restaino et al., 1995) and antimicrobials, resulting in reduced availability for interaction with microorganisms. For example, the antimicrobial activity of HP is primarily oxidative and may be diminished by ascorbic acid (a reducing agent), resulting in reduced effectiveness.

In Objective 2 studies, O_3/DMDC (250 ppm) was more effective after 90 min treatment than HP (300 ppm), while in Objective 3 studies, O_3/HP (300 ppm) was more effective than O_3/DMDC (250 ppm) for reducing *E. coli* O157:H7 and *Salmonella*. Objective 2 samples were taken during 90 min treatment only, whereas Objective 3 samples were taken during treatment and after 24 h of refrigerated storage. Therefore, O_3/DMDC (250 ppm) appears to act rapidly to inactivate microorganisms, while HP requires longer contact time to inactivate microoganisms.

These studies demonstrate that ozone is a potential alternative to traditional thermal pasteurization to control *E. coli* O157:H7 and *Salmonella* in fresh juices. Combining ozone and antimicrobial treatment followed by refrigerated storage may potentially be

used to achieve a 5-log reduction of *E. coli* O157:H7 and *Salmonella* populations in apple cider and orange juice. Survival of both pathogens is influenced by juice and antimicrobial characteristics. However, ozone treatment-related processing problems such as juice foaming, off-gasing of ozone, and difficulties determining ozone concentration in juice must be solved before practical application of ozone for the treatment of fruit juice can be utilized.

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Singh, B.R, Kulshreshtha, S.B., and Kapoor, K.N. 1995. An orange juice-borne diarrhoeal outbreak due to enterotoxigenic *Escherichia coli*. J. Food Sci. Technol. 32:504-506.

APPENDIX

EXPERIMENTAL DESIGN AND STATISTICAL ANALYSIS

Experimental parameters for each study described in this dissertation are represented in table form. Statistical analysis programs that were used follow. All statistical analyses were performed using Statistical Analysis Software (SAS; Cary, NC) version 8.1.

I. Effect of Ozone and Treatment Temperature on Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in apple cider and orange juice.

micro	*Escherichia coli* O157:H7						*Salmonella* spp.					
juice	Apple Cider			Orange Juice			Apple Cider			Orange Juice		
temp	4	20	50	4	20	50	4	20	50	4	20	50
time	0	0	0	0	0	0	0	0	0	0	0	0
	30	30	15	30	30	15	30	30	5	30	30	5
	60	60	30	60	60	30	60	60	10	60	60	10
	90	90	45	90	90	45	90	90	15	90	90	15
	120	120	60	120	120	60	120	120	20	120	120	20
	150	150	75	150	150	75	150	150	25	150	150	25
	180	180		180	180		180	180	30	180	180	30
		210		210	210			210		210	210	
		240		240	240			240		240	240	
media	tsa hc memb smac	tsa hc memb smac	tsa hc memb smac	tsa hc memb smac	tsa hc memb smac	tsa hc memb smac	tsa bsa xlt-4	tsa bsa xlt-4	tsa bsa xlt-4	tsa bsa xlt-4	tsa bsa xlt-4	tsa bsa xlt-4
count	x 2	x 2	x 2	x 2	x 2	x 2	x 2	x 2	x 2	x 2	x 2	x 2
rep	3	3	3	3	3	3	3	3	3	3	3	3

Statistical design: Randomized bock design, nested treatment arrangement, repeated measures with sampling, blocked on rep.

Statistical program:

```
/*
proc access dbms=xls ;
  create work.beef.access;    ** must be first- creates descriptor;
    path='e:\temp\apojsas fix.xls';
    worksheet="Sheet1";
    skiprows=1;              ** skip 1 row (which has variable names) ;
    getnames=yes;            ** read variable names from row 1;
    scantype=no;             ** scan columns to decide if numbers or
        text?;
                             ** unfortunately, blanks are treated as char;
                             ** if no, use formats in first row of data;
    mixed=yes;               ** convert numbers to char in char fields;
    type time=n temp=n; *var1=c var2=n;
    unique=yes;              ** create unique SAS variables names??;
    list all;                ** display column info in log;

  create work.beef.view;     ** start view creation;
    select all;              ** use all observations;
run;

proc access dbms=xls viewdesc=work.beef out=raw;
run;
*/
data raw; infile 'e:\temp\apojsas txt.txt' expandtabs firstobs=2;
 input micro$     temp  juice$      rep   time  media$      count;
run;

proc sort data=raw; by micro media temp juice rep time;
proc means noprint; by micro media temp juice rep time;
 var count;
 output out=two mean=count;
 run;

data zero; set two; if time=0;
 rename  count =ctrl;
run;
proc sort; by micro temp juice rep ; run;
proc means noprint; by micro temp juice rep;
 var ctrl;
 output out=xxx mean=ctrl;
 run;
proc sort data=two; by micro temp juice rep;
data one; merge two xxx; by micro temp juice rep;
  cntreduc=100*(count - ctrl)/ctrl;
run;

proc print; run;

%include 'pdmix800.sas';

proc sort data=one out=temp; by micro juice media time temp;
proc means noprint; by micro juice media time temp;
 var cntreduc;
 output out=mmm mean=rawmean std=stddev;
```

```
run;
proc print;
 title2 "Check equality of stddev for cntreduc";
run;

 proc sort data=one; by juice micro temp;
 proc mixed data=one; by juice micro temp;
  title2 "Mixed ANOVA for cntreduc- compare media within
        juice/micro/temp";
    class rep  time media;
    model cntreduc =  media| time / outp=rrr;
    random rep rep*media rep*time*media;
    lsmeans media| time / pdiff;
    ods output diffs=ppp;
    ods output lsmeans=mmm;
    ods listing exclude diffs;
    ods listing exclude lsmeans;
run;
  title2 "Mean separation for cntreduc";
%pdmix800(ppp,mmm);
proc univariate plot normal data=rrr;
  title2 "Check on Normality for cntreduc";
 var resid;
run;

data tsa; set one; if media='tsa';

 proc sort data=tsa; by juice micro;
 proc mixed data=tsa; by juice micro;
  title2 "Mixed ANOVA for cntreduc- compare temp within juice/micro";
    class rep  time temp;
    model cntreduc =  temp time(temp) / outp=rrr;
    random rep rep*temp rep*time(temp);
    lsmeans  temp time(temp) / pdiff;
    ods output diffs=ppp;
    ods output lsmeans=mmm;
    ods listing exclude diffs;
    ods listing exclude lsmeans;
run;
  title2 "Mean separation for cntreduc";
%pdmix800(ppp,mmm);
proc univariate plot normal data=rrr;
  title2 "Check on Normality for cntreduc";
 var resid;
run;

 proc sort data=tsa; by micro temp;
 proc mixed data=tsa; by micro temp;
  title2 "Mixed ANOVA for cntreduc - compare juice within micro/temp";
    class rep  juice  time temp;
    model cntreduc =  juice time(juice) / outp=rrr;
    random rep rep*juice  rep*time(juice);
    lsmeans  juice  time(juice ) / pdiff;
    ods output diffs=ppp;
    ods output lsmeans=mmm;
    ods listing exclude diffs;
```

```
    ods listing exclude lsmeans;
run;
  title2 "Mean separation for cntreduc";
%pdmix800(ppp,mmm);
```

II. Effect of Ozone in Combination with Dimethyl Dicarbonate or Hydrogen Peroxide on Survival of *Escherichia coli* O157:H7 and *Salmonella* spp. in Apple Cider and Orange Juice

Study 1

micro	*Escherichia coli* O157:H7		*Salmonella* spp.	
juice	Apple Cider	Orange Juice	Apple Cider	Orange Juice
rep	3	3	3	3
time	0 15 30 45 60 75 90	0 15 30 45 60 75 90	0 15 30 45 60 75 90	0 15 30 45 60 75 90
anti	DMDC H_2O_2	DMDC H_2O_2	DMDC H_2O_2	DMDC H_2O_2
count (x 2)	2 plates	2 plates	2 plates	2 plates

Antimicrobials: DMDC 250 ppm
H_2O_2 300 ppm

Statistical design: Randomized block design, factorial treatment arrangement, repeated measures with sampling, blocked on rep.

Statistical program:

```
proc access dbms=xls ;
  create work.beef.access;    ** must be first- creates descriptor;
    path='c:/stats2/dmdc h2o2.xls';
    worksheet="Sheet1";
    skiprows=1;              ** skip 1 row (which has variable names) ;
    getnames=yes;            ** read variable names from row 1;
    scantype=no;             ** scan columns to decide if numbers or
        text?;
```

```
                          ** unfortunately, blanks are treated as char;
                          ** if no, use formats in first row of data;
    mixed=yes;            ** convert numbers to char in char fields;
**  type var1=c var2=n;
    unique=yes;           ** create unique SAS variables names??;
    list all;             ** display column info in log;

  create work.beef.view;  ** start view creation;
    select all;           ** use all observations;
run;

proc access dbms=xls viewdesc=work.beef out=one;
run;
data one; set one; if time ne .;
drop var7;
run;

proc sort data=one; by anti temp micro juice rep time;
proc means noprint; by anti temp micro juice rep time;
 var count;
 output out=two mean=count;
 run;

data zero; set two; if time=0;
run;
proc sort; by anti temp micro juice rep ; run;
proc means noprint; by anti temp micro juice rep;
 var count;
 output out=xxx mean=ctrl;
 run;
proc sort data=two; by anti temp micro juice rep;
data one; merge two xxx; by anti temp micro juice rep;
  cntreduc=100*(count - ctrl)/ctrl;
run;

proc print; run;



%macro rbdfact(dset,yvar);
proc sort data=&dset out=temp; by anti juice micro time temp;
proc means noprint; by anti juice micro time temp;
 var &yvar;
 output out=mmm mean=rawmean std=stddev;
run;
proc print;
 title2 "Check equality of stddev for &yvar";
run;

 proc mixed data=&dset;
  title2 "Mixed ANOVA for &yvar";
    class rep anti juice micro time temp;
    model &yvar =  anti| juice| micro| time  / outp=rrr;
    random rep rep*anti*juice*micro ;
    lsmeans  anti| juice| micro| time / pdiff;
```

```
    ods output diffs=ppp;
    ods output lsmeans=mmm;
    ods listing exclude diffs;
    ods listing exclude lsmeans;
run;
%include 'c:/stats/pdmix800.sas';
  title2 "Mean separation for &yvar";
%pdmix800(ppp,mmm);
proc univariate plot normal data=rrr;
  title2 "Check on Normality for &yvar";
 var resid;
run;
%mend;

%rbdfact(one, cntreduc);


**************************by anti ********************************;
%macro byfact(dset,yvar);
 proc mixed data=&dset noprofile; by anti;
  title2 "Mixed ANOVA BY MICRO for &yvar";
    class rep anti juice micro time temp;
    model &yvar =  juice| micro| time / outp=rrr;
    random rep rep*juice*micro;
    lsmeans  juice| time|micro  / pdiff;
 ods output diffs=ppp;
 ods output lsmeans=mmm;
 ods listing exclude diffs;
 ods listing exclude lsmeans;
run;
%include 'c:/stats/pdmix800.sas';
  title2 "Mean separation for &yvar";
%pdmix800(ppp,mmm);
%mend;

proc sort data=one; by anti;
%byfact(one,cntreduc);


/*  */
```

Study 2

micro	*Escherichia coli* O157:H7		*Salmonella* spp.	
juice	Apple Cider	Orange Juice	Apple Cider	Orange Juice
anti **level** = (x)	Air Ozone only Oz + DMDC (250) Oz + DMDC (500) Oz + H_2O_2 (300) Oz + H_2O_2 (600)	Air Ozone only Oz + DMDC (250) Oz + DMDC (500) Oz + H_2O_2 (300) Oz + H_2O_2 (600)	Air Ozone only Oz + DMDC (250) Oz + DMDC (500) Oz + H_2O_2 (300) Oz + H_2O_2 (600)	Air Ozone only Oz + DMDC (250) Oz + DMDC (500) Oz + H_2O_2 (300) Oz + H_2O_2 (600)
rep	3	3	3	3
time	0 min 30 min 60 min 24 h	0 min 30 min 60 min 24 h	0 min 30 min 60 min 24 h	0 min 30 min 60 min 24 h
count (x 2)	2 plates	2 plates	2 plates	2 plates

Statistical design: Randomized block design, factorial treatment arrangement, repeated measures with sampling, blocked on rep.

Statistical program:

```
proc access dbms=xls ;
  create work.beef.access;    ** must be first- creates descriptor;
    path='c:/stats2/dmdc h2o2.xls';
    worksheet="Sheet1";
    skiprows=1;             ** skip 1 row (which has variable names) ;
    getnames=yes;           ** read variable names from row 1;
    scantype=no;            ** scan columns to decide if numbers or
        text?;
                            ** unfortunately, blanks are treated as char;
                            ** if no, use formats in first row of data;
    mixed=yes;              ** convert numbers to char in char fields;
**  type var1=c var2=n;
    unique=yes;             ** create unique SAS variables names??;
    list all;               ** display column info in log;

  create work.beef.view;    ** start view creation;
    select all;             ** use all observations;
run;

proc access dbms=xls viewdesc=work.beef out=one;
run;
data one; set one; if time ne .;
```

```
drop var7;
antilevel=anti||level;
run;

proc sort data=one; by antilevel temp micro juice rep time;
proc means noprint; by antilevel temp micro juice rep time;
 var count;
 output out=two mean=count;
 run;

data zero; set two; if time=0;
run;
proc sort; by antilevel temp micro juice rep ; run;
proc means noprint; by antilevel temp micro juice rep;
 var count;
 output out=xxx mean=ctrl;
 run;
proc sort data=two; by antilevel temp micro juice rep;
data one; merge two xxx; by antilevel temp micro juice rep;
  cntreduc=100*(count - ctrl)/ctrl;
run;

proc print; run;



%macro rbdfact(dset,yvar);
proc sort data=&dset out=temp; by antilevel juice micro time temp;
proc means noprint; by antilevel juice micro time temp;
 var &yvar;
 output out=mmm mean=rawmean std=stddev;
run;
proc print;
 title2 "Check equality of stddev for &yvar";
run;

 proc mixed data=&dset;
  title2 "Mixed ANOVA for &yvar";
    class rep antilevel juice micro time temp;
    model &yvar =  antilevel| juice| micro| time  / outp=rrr;
    random rep rep*antilevel*juice*micro ;
    lsmeans  antilevel| juice| micro| time / pdiff;
    ods output diffs=ppp;
    ods output lsmeans=mmm;
    ods listing exclude diffs;
    ods listing exclude lsmeans;
run;
%include 'c:/stats/pdmix800.sas';
  title2 "Mean separation for &yvar";
%pdmix800(ppp,mmm);
proc univariate plot normal data=rrr;
  title2 "Check on Normality for &yvar";
 var resid;
run;
%mend;
```

```
%rbdfact(one, cntreduc);


*************************by anti ******************************;
%macro byfact(dset,yvar);
 proc mixed data=&dset noprofile; by antilevel;
  title2 "Mixed ANOVA BY MICRO for &yvar";
    class rep antilevel juice micro time temp;
    model &yvar =  juice| micro| time / outp=rrr;
    random rep rep*juice*micro;
    lsmeans  juice| time|micro  / pdiff;
 ods output diffs=ppp;
 ods output lsmeans=mmm;
 ods listing exclude diffs;
 ods listing exclude lsmeans;
run;
%include 'c:/stats/pdmix800.sas';
  title2 "Mean separation for &yvar";
%pdmix800(ppp,mmm);
%mend;

proc sort data=one; by antilevel;
%byfact(one,cntreduc);


/*  */
```

The macro "pdmix800.sas"* was provided courtesy of Dr. Arnold Saxton.

*Saxton, A.M. 1998. A macro for converting mean separation output to letter groupings in Proc Mixed. In Proc. 23rd SAS Users Group Intl., SAS Institute, Cary, NC, pp1243-1246.

VITA

Robert Charles Williams was born on December 22, 1970 in Morristown, TN. He graduated from Morristown Hamblen High School West in May 1989. He attended Carson-Newman College (Jefferson City, TN) and received the B.S. degree in Biology, with a concentration in Microbiology, from Tennessee Technological University (Cookeville, TN) in June 1994. In August 1994, he entered the Department of Food Science and Technology at the University of Tennessee. He received the M.S. degree in Food Science and Technology, with a concentration in Food Microbiology, in August 1998. The Ph.D. degree was awarded in August 2001.