The Efficacy of Antimicrobials for the Control of *Alicyclobacillus acidoterrestris* in Fruit and Vegetable Juices

By

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(ABSTRACT)

The efficacy of antimicrobials for control of *A. acidoterrestris* spoilage in juices was analyzed. Apple and tomato juices were inoculated with 4 log spores/ml. Antimicrobials were added at: 1000, 500 and 250 ppm (sodium benzoate, potassium sorbate, and sodium metabisulfite); 500, 250, and 125 ppm (cinnamic acid, dimethyl dicarbonate, and ascorbic acid); 125, 75 and 25 ppm (lysozyme); and 5, 3, and 1 IU/ml (nisin).

In apple juice, *A. acidoterrestris* population reductions were caused by the following antimicrobials (reduction in log CFU/ml): lysozyme - all levels and nisin - 5 IU/ml (5.1), nisin - 3 IU/ml (4.2), cinnamic acid - 125 ppm (3.1), cinnamic acid - 250 ppm (2.6), potassium sorbate - 250ppm (2.5), nisin - 1 IU/ml (2.4), potassium sorbate - 500 and 1,000 ppm (2.3), dimethyl dicarbonate - 500 ppm (1.9), cinnamic acid - 500 ppm (1.4). In tomato juice, *A. acidoterrestris* population reductions were caused by the following antimicrobials (reduction log CFU/ml): nisin - all levels and lysozyme - 125 ppm and 75 ppm (4.4), lysozyme - 25 ppm (3.8), potassium sorbate - 500 ppm (2.6), cinnamic acid - 500 ppm (2.5), cinnamic acid - 250 ppm (2.4), cinnamic acid - 125 ppm (2.1), potassium sorbate - 1,000 ppm (1.9), and potassium sorbate - 250 ppm (1.6).

Antimicrobial treatments: nisin - ≥ 1 IU/ml, lysozyme - ≥ 25 ppm, cinnamic acid - ≥ 125 ppm, and potassium sorbate - ≥ 250 ppm may be appropriate controls to prevent *A. acidoterrestris* spoilage in juices or juice containing beverages.

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INTRODUCTION/JUSTIFICATION

Pasteurized shelf-stable juices make up a large part of the juice market, therefore spoilage of these types of products may translate to large costs for producers. According to Rathwell (1997a; 1997b), 42% of fruit consumption is in the form of fruit juices, with consumption increasing about 2.5% annually during the late 1990's. Consumption of juices has grown due to increased awareness of health and nutrition among consumers. In response, many companies have started producing new juice products and beverages containing fruit juice.

Recently, fresh juices, pasteurized shelf stable juices, and juice blends have been implicated in several spoilage incidences. It was concluded that the microorganism responsible, *Alicyclobacillus acidoterrestris*, is able to withstand the pasteurization and hot-fill processes as well as the acidic nature of juices that prevents other microorganisms from growing (Eguchi, 1999). *A. acidoterrestris* causes off-odors and off-flavors in the juices that are often undetected by producers until consumer complaints are received (Walls and Chuyate, 2000).

A. acidoterrestris has been found throughout many stages of processing, from the incoming fruit to water used to finish the beverages. Therefore, one ingredient or stage in processing is not the only factor associated with spoilage, making removal more difficult (Eguchi, 1999). Although it has been shown that producers recognize this organism as a problem, the amount of impact it has had on the juice industry is unknown due to unreported spoilage and difficulty in detection (Walls and Chuyate, 2000). The reduction of *A. acidoterrestris* has been a demanding challenge because the organism is ubiquitous, has the ability to survive high temperatures for a period of time, and can survive thermal concentration of juices. In addition, *A. acidoterrestris* is highly resistant to many cleansers, sanitizers, and other physical treatments (Parrish, 2000). Therefore, changes in processing alone may not inhibit this microbe in all products without causing excessive energy costs or decreased organoleptic qualities of the juices.

A survey of juice producers showed that 35% of respondents reported at least one spoilage incident that is characteristic of *A. acidoterrestris* spoilage over a five-year span. Reported spoilage caused loss of more than half of their product, on average (Walls and Chuyate, 2000). In addition, *A. acidoterrestris* spoilage is not recognized under normal quality control visible inspection procedures and producers are unaware of the spoilage until the product reaches

the consumer. However, since consumer complaints do not occur with every spoiled juice, the problem may be larger than recognized and consumers may stop buying brands that have detectable *A. acidoterrestris-associated* off-odors and off-flavors. Spoilage by *A. acidoterrestris* may translate to scrapping costs, customer dissatisfaction, loss of brand quality image, and increased processing costs to remove the microorganism from production environments (Borlinghaus, 1997; Eguchi, 1999; Jensen, 1999b; Walls and Chuyate, 2000). Therefore, a successful intervention is needed to prevent *A. acidoterrestris* spoilage in juices, juice blends, and beverages containing juices.

One way that certain juice and beverage producers prevent spoilage is through the addition of either natural or chemical antimicrobial agents. Antimicrobials have been used effectively for decades to prevent bacteria, yeasts, and molds from spoiling food products or causing human illness, without resulting in considerable quality defects (Jay, 2000). Therefore, the use of certain antimicrobials may be an effective control method for *A. acidoterrestris* spoilage in fruit juices. Determining the efficacy of antimicrobials on *A. acidoterrestris* and defining the effective level needed to inhibit both its vegetative cells and spores may allow prevention of A. *acidoterrestris* spoilage in juices. Inhibition of this organism through the use of antimicrobials would benefit the beverage industry and could be used as a control method in fruit and vegetable juices, teas, or canned tomatoes that are susceptible to *A. acidoterrestris spoilage*. Studies are needed to determine if antimicrobials may be an effective control for *A. acidoterrestris* and which antimicrobials are most effective while having the least negative effect on juices.

Four juices were examined in this study because they have been commonly associated with spoilage caused by *A. acidoterrestris*. Apple, orange, white grape, and tomato juices were investigated to determine growth patterns of *A. acidoterrestris* under optimum growth conditions and to determine the length of time until the organism reaches a concentration that is associated with taint. Differences between spores combined with vegetative cells and spore only inoculant were also tested to determine if growth patterns differed based upon initial cell state. Although growth studies have been performed, little information exists on the growth patterns in these juices at optimum conditions.

Antimicrobials were chosen as a control measure because of the ineffectiveness of increased heat, fruit washing procedures, and sanitation methods to prevent *A. acidoterrestris*

survival. In addition, preservatives have the ability to prevent growth when used at levels that do not negatively impact juice quality. In this study, eight antimicrobials were tested due to their history of use in beverages or their effectiveness for inhibiting or inactivating Gram-positive spoilage organisms. Consequently, nisin, lysozyme, cinnamic acid, ascorbic acid, sodium benzoate, potassium sorbate, sodium metabisulfite, and dimethyl dicarbonate were tested to determine if the antimicrobials could decrease the amount of viable spores and vegetative cells and prevent outgrowth of *A. acidoterrestris* up to 29 days.

Antimicrobials may degrade naturally, in the presence of high temperatures, or in the presence of juice components. Therefore, growth curves are necessary to determine if the antimicrobial would be effective in preventing the germination and growth during the storage of the juice. Determining appropriate levels of antimicrobials is important so that the lowest effective amount of antimicrobial may be used to inhibit or inactivate *A. acidoterrestris.*

CHAPTER I
LITERATURE REVIEW

Alicyclobacillus spp.

In 1967, Uchino and Doi isolated an aerobic, acidophilic, spore-forming bacterium from hot water springs in Japan. The bacterium was first classified as *Bacillus coagulans* due to observed tolerance to low pH and heat. However, DNA-based identification tests by Darland and Brock in 1971 suggested that the organism was a new species which they named, *Bacillus acidocaldarius*. In 1981, Hippchen described an acid and heat tolerant, spore-forming thermophilic isolate from garden soil that differed from *B. acidocaldarius* based upon a lower growth temperature, biochemical characterization, and DNA based composition. This study illustrated that this type of organism could be found in non-thermal, neutral environments, and was more widespread than originally thought (Jensen, 1999b; Jensen, 2001; Walls and Chuyate, 2000). Another strain of a thermotolerant spore-former was isolated from garden soil, but its omega fatty acid profile was different than previously isolated strains. In 1987, Deinhard and others used taxonomic studies on strains isolated by Poralla and Cerny and recognized two new species: *Bacillus cycloheptenicus* and *Bacillus acidoterrestris,* respectively. The genus *Alicyclobacillus* was created in 1992 and is based upon the presence of ω-alicyclic acids in the bacterial cellular membrane and the DNA sequence of the organism, which was found to be unique in that it differs from other *Bacillus* spp. (Brown, 1995; Eguchi, 1999; Jensen, 1999a). The first case of juice spoilage by an *Alicyclobacillus* species was confirmed in 1982 when Cerny and others isolated an acidophilic bacillus from spoiled, pasteurized apple juice. Further inquiry revealed that this strain was similar to the strain found by Cerny et al. (1984) in garden soil in 1981.

There are currently seven species in the *Alicyclobacillus* genus: *A. acidiphilus*, *A. acidocaldarius*, *A. acidoterrestris, A. cycloheptenicus*, *A. herbarius*, *A. hesperidium*, and *A. sendaiensis*. *A. acidocaldarius* is acidophilic and thermophilic with an optimum growth pH of 2 to 6 and an optimum temperature growing range of 45 to 70˚C. In 1998, a new subspecies of *A. acidocaldarius* was isolated from an Antarctic volcano. This new *A. acidocaldarius* subspecies *rittmannii* was isolated from the crater of Mt. Rittmann, at over 2000 m above sea level, near steaming vents with ambient temperatures of -20˚C and ground temperatures of 60˚C (Nicolaus, 1998).

A. cycloheptenicus is generally found in soil and has an optimum temperature and pH for growth of 48°C and pH 3.0 to 5.5, respectively (Nicolaus, 1998). The cytoplasmic membrane of *A. cycloheptenicus* contains ω-cycloheptane as opposed to ω-cyclohexane that are found in the cytoplasmic membranes of *A. acidoterrestris* and *A. acidocaldarius.* The hydrocarbon ω-cyclohexane may increase the stability of the cell membrane, which reduces its permeability in thermal environments, therefore, promoting survival under such conditions (Jensen, 1999a).

A. hesperidium is a thermophilic spore-former that was recovered from solataric soil at Furnas on the Island of Säo Miguel in the Azores (Albuquerque et al., 2000). The *A. sendaiensis* species was isolated from the soil of Aoba-Yama Park in Sendai Japan. Its optimum growth temperature is 55°C at a pH of 5.5 and it is most closely related to A. *acidocaldarius*, phylogenetically based on 16S rDNA (Tsuruoka, 2002.). *A. herbarius* was isolated from an herbal tea made from the dried flowers of the hibiscus plant; however it has not been associated with spoilage of beverages. *A. herbarius* requires an aerobic atmosphere and its optimum pH for growth is 4.5-6.0 at 55-60°C. It contains ω-cycloheptane fatty acids similar to those found in *A. cycloheptenicus* (Goto, 2002).

Of the sevens species in the *Alicyclobacillus* genus, only two have been associated with spoilage of juices, juice-containing beverages, and canned tomatoes: *A. acidiphilus* and *A. acidoterrestris*. *A. acidiphilus*, a strain that is phylogenetically similar to *A. acidoterrestris*, was recently isolated from shelf-stable iced tea containing berry juice that had been pasteurized at 95°C for 30 seconds and hot-filled into cartons. A contributing factor to spoilage by this microorganism was a slow rate of product cooling. *A. acidiphilus* was isolated from spoiled tea, as well as rose hips and hibiscus tea ingredients, but it was not found in the black or green tea, or in the berry concentrate (Duong and Jensen, 2000). When orange juice was inoculated with this isolate, a small amount of the taint compound, guaiacol, was produced (Matsubara, 2002).

A. acidoterrestris has been associated with spoilage of a wide variety of beverages throughout the world. Currently, *A. acidoterrestris* has been recognized as the cause of most cases of *Alicyclobacillus*-related spoilage in beverages and has become a problem for beverage producers.

A. acidoterrestris

Alicyclobacillus acidoterrestris is a Gram-positive, rod-shaped, aerobe that is an acidothermophilic, spore-forming spoilage microorganism. This organism is 2.9 to 4.3 μm long and 0.6 to 0.8 μm wide. It produces spores that are oval, subterminal or terminal with a sporangium that may be swollen (Jensen, 1999a). *A. acidoterrestris* colonies on laboratory media are circular, white/yellow colonies that may appear opaque or translucent. The fatty acids that are found in its cellular membrane are predominately made up of ω-cyclohexane, however hopanoids and sulpholipds are also present (Eguchi, 1999).

Ecology

A. acidoterrestris are widespread in nature, often survive typical pasteurization and thermal concentration methods, are strictly aerobic, and are thermo-acidophilic (Parrish, 2000). This microorganism can grow at 26 to 55°C (optimum: 42 to 53°C) and from pH 2.0 to 6.0 with an optimum pH of 3.5 to 5.0 (Eguchi, 1999; Jensen, 1999a).

Heat and Acid Tolerance

Spores and vegetative cells of most molds are inactivated within 5 minutes at 60°C and the typical microflora of fruit juices is not heat resistant (Silva, 2000). Pasteurization inactivates most non-spore-forming microorganisms in juices. Generally, the acidic nature of juice prevents germination of surviving spores. Therefore, for control of most spoilage bacteria in juices, pasteurization is an acceptable means to result in shelf-stable juices, however *A. acidoterrestris* may survive pasteurization and grow during storage (Pontius, 1998; Silva, 2000). Generally, juice processors use the hot-fill and hold process that holds the product at 88 to 96°C for two minutes. This process is very effective for inactivating most bacteria but is not sufficient to destroy *Alicyclobacillus* (Pontius, 1998). *Alicyclobacillus* spores may survive the pasteurization treatment given to most shelf-stable juices and pasteurization may actually activate *A. acidoterrestris* spores to begin germination (Brown, 1995; Krueger Labs, 2001). Since *A. acidoterrestris* survives pasteurization, it was important to determine the heat resistance of this organism to determine if additional heating time could be used as a control method.

D values for *A. acidoterrestris* in juices are shown in the following table:

Juice	ºBrix	pH	Temperature (ºC)	D value (minutes)	Z value (ºC)	Source
Apple	11.4	3.5	85	56.0	7.7	Splittstoesser et al., 1994
			90	23.0		
			95	2.8		
Apple	11	3.55	88	22.7-35.8	6.4 - 7.5	Previdi et al., 1997
			90	13.9-18.8		
			92	7.6-11.3		
			95	2.2		
Concord Grape	15.8	3.3	85	573.3	7.2	Splittstoesser et al., 1994
			90	16.0		
			95	2.4		
Concord Grape	30	3.3	85	76.0	6.6	Splittstoesser et al., 1994
			90	18.0		
			95	2.3		
Concord Grape	65	3.3	95	276.0	7.4	Splittstoesser et al., 1994
			90	127.0		
			95	12.0		
Apple-grape-raspberry	4.8	3.5	87.8	11.0	7.2	McIntyre et al., 1995
			91.1	3.8		
			95	1.0		
Orange	11.2	3.82	88	30.2-44.2	6.5-7.4	Previdi et al., 1997
			90	16.2-21.9		
			92	9.7-12.6		
			95	3.4-3.6		
Orange juice drink	4.1	5.3	95	5.3	9.5	Baumgart et al., 1997

These values indicate that the D values for *A. acidoterrestris* are much higher than the hold time for traditional heat and fill process and increasing soluble solid concentration (°Brix) increases the microbe's heat resistance (Splittstoesser, 1998).

When *Alicyclobacillus* cells were subjected to 90°C, there was only 1 log CFU/ml reduction of *A. acidoterrestris* after 15 minutes of treatment (Previdi, 1997). Using a model fruit-juice system (12% glucose; pH 2.8 to 4.0; adjusted with malic, citric, or tartaric acid), researchers found that the type of acid did not significantly affect heat resistance (Pontius, 1998). At 97°C, a D value of 8-9 minutes was observed. This is far more than two minute hold time used by juice processors (Pontius, 1998). By altering soluble solids and temperature, it was shown that a small temperature increase caused a considerable D value decrease and the D value increased slightly with increasing soluble solids and pH, especially at a lower pH of 2.5. However, at temperatures near 97°C, an effect associated with soluble solids and pH was not observed (Silva, 1999).

Biochemistry of Heat and Acid Tolerance

A. acidoterrestris contain ω-cyclohexane fatty acids, rarely present in other microorganisms, in their cellular membrane (Eguchi, 1999). At least 90% of the *A. acidoterrestris* membrane is comprised of 11-cyclohexylundecanoic acid and 13-cyclohexyltridecanoic acid. The unique composition of the *Alicyclobacillus* membrane, i.e., it contains ω-alicyclic fatty acids and squalene hopene cyclase, contributes to its survival and growth at acidic pH and high temperatures (Palop, 2000). It has been shown that as growth temperature increases, content of the ω-alicyclic compounds increase. It is believed that these lipids contribute to the heat tolerance of *Alicyclobacillus* because they stabilize the cell membranes, and reduce membrane permeability that leads to cell leakage and death (Jensen, 1999a).

One species of *A. acidocaldarius*, produces thermo-acidophilic 55-70kDa α-amylases. Compared to relative species of bacteria, *A. acidocaldarius* has 30% fewer charged amino acid residues, which are replaced by neutral, polar residues that are believed to be located at the surface of the protein, α-amylase. It has been proposed that these proteins are more tolerant to the acidic environment due to reduced density of both the positive and negative charges at their

surface. This prevents electrostatic repulsion of charged groups at low pH and thereby contributes to the acidostability of these proteins (Schwermann et al., 1994).

Acidophiles, like *A. acidoterrestris*, maintain their cytoplasmic pH at a value close to neutrality and thereby tolerate pH changes of up to five units in their environment. Acidophilic properties are only necessary for proteins that are in contact with an acidic environment. Typically, under acidic conditions, carboxylic groups at the water/protein interface become protonated. This has negative effects on the conformation and stability of the protein (Schwermann et al., 1994). However, few of the surface proteins of acidophiles have positive or negative charged amino acids, which ensure that the stability and structure of the protein is maintained (Jensen, 1999a).

Maintenance of cytoplasmic pH is an active process driven by outward pumping of protons across the cell membrane, and the subsequent exchange of inwardly moving K^+ ions. Low numbers of acidic residues and basic residues result because protonation of acidic groups increases if pH decreases below the iso-electric point of proteins. If proteins possess a large content of positively charged residues, the positive charges at the surface would repel each other leading to the unfolding of the protein. Consequently, this group of proteins contains a reduced number of positively charged residues. However, if such a protein possessed a large number of negative groups, unfolding may also occur above the iso-electric point of the protein. In order to be stable and active in a broad pH range, these groups of proteins also possess a reduced number of acidic residues (Matzke, 1997; Schwermann et al., 1994).

Additionally, these microorganisms retain divalent cations, such as calcium, more strongly than other bacterial spores. This could explain their high resistance to demineralization and the minimal effect of pH change and alteration of other characteristics of the heating medium on their heat resistance (Palop, 2000).

Enumeration and Identification Techniques

Since *A. acidoterrestris* are strictly aerobic, and require high temperature and low pH to grow, they are not able to grow on neutral culture media commonly used for thermophiles and mesophiles, such as Tryptone Glucose Yeast Extract Agar (TGYE) or Dextrose Tryptone Agar (DTA). However, acidified Potato Dextrose Agar (PDA), Orange Serum Agar (OSA), *Bacillus*

acidocaldarius Medium (BAM), Thermoacidurans Agar (TA), Malt Extract Agar (MEA), and WORT Agar may be used (Eguchi, 1999; Jensen, 1999a).

According to Eguchi (1999), K medium and OSA are both suitable growth media for *A. acidoterrestris*. Five strains of *A. acidoterrestris* isolated from various juices and canned tomatoes were streaked onto OSA, Tomato Juice Agar Special (TJAS), PDA (each adjusted to pH 3.5, 4.0, 4.5,and 5.0), DTA (pH 7.4), and K medium (pH 3.7). All five isolates grew on OSA (pH 5.0), and K medium (pH 3.7), at 35ºC (Eguchi, 1999). According to Pettipher (2000), *Alicyclobacillus* failed to grow on nutrient agar and tryptone soy agar at pH 7.3, but grew well on BAM medium, PDA, and OSA. Of these media, it was determined that OSA allowed the highest recovery. Previdi (1995) also stated that all *A. acidoterrestris* strains tested were able to grow on TA (pH 4.91), OSA (pH 5.09), and MEA (pH 4.0).

On OSA, A. *acidoterrestris* has a distinctive appearance. Colonies have a very flat interior with raised annulus and button center. Colonies are translucent, and initially cream colored, followed by darkening. The colonies then become umbonate and increasingly opaque with age. Colonies take approximately two days to develop above 40˚C, but generally take longer to form if heat treatments have been used. Jensen (1999) showed that OSA with 0.5% added sucrose performed better as an enumeration medium than OSA alone. However, according to Walls and Chuyate (2000), when *A. acidoterrestris* spores were inoculated onto K Agar (pH 3.7), semi-synthetic medium (pH 4.0), OSA (pH 3.5) and minimal salts medium (pH 4.0) and incubated at 24, 35, 43, and 55°C, the highest recovery of spores were obtained on K agar or semi-synthetic medium incubated at 43°C. Additionally, peptone has been shown previously to inhibit *A. acidoterrestris* (Splittstoesser et al., 1998); however, it was not inhibitory when present in K agar (Walls and Chuyate, 2000).

Walls and Chuyate (1998) reported *A. acidoterrestris* growth in Orange Serum Broth at pH 2.5 to 5.0 over a temperature range of 20 to 55ºC. Deinhard et al. (1987), reported the optimum pH range for *A. acidoterrestris* growth of the organism in BAM was 2.5 to 5.8 over a temperature range of 35 to 55ºC with an optimum at 42 to 53ºC, and McIntyre et al., (1995) reported growth of *A. acidoterrestris* on PDA over a pH range of 3.0 to 5.3 at 30 to 55ºC.

According to Splittstoesser (1998), peptone may inhibit *Alicyclobacillus*. However, Jensen (1999) stated that a suitable diluent is 0.1% peptone at pH 4 with 0.5% glucose, glycerol,

or sucrose, with sucrose giving the highest recovery. Typically, an acidified diluent is required containing a protectant to reduce the osmotic shock on *A. acidoterrestris* cells (Jensen, 1999a).

In addition to testing for *A. acidoterrestris* cells, taint compounds formed by *A. acidoterrestris* may also be detected. Using solid phase micro extraction (SPME), as little as two ppb of 2-methoxyphenol (taint compound) can be detected in 3 ml of sample. Generally, this concentration of taint compound correlates to 50-100 natively growing *A. acidoterrestris* cells/ml. Using previous methods, 500 ml of sample were needed in order to detect the spoilage compounds. Additionally, SPME reduces detection time from up to seven days to one hour and is much less expensive than previous methods (Gannon, 1998).

Sources

The primary reservoir for *A. acidoterrestris* is soil, however, it has been isolated from washed and unwashed fruit, fruit juices (apple, apple-cranberry, mixed berry, pear, orange, grape fruit, and pineapple juices), nectars, juice-containing beverages, canned diced tomatoes, liquid sugar used in beverages, "diet" fruit juices and drinks, condensate water used in finished juices, processing plants or equipment, and in a condensate recovery system that is used to make concentrates (Eguchi, 1999; Parrish, 2000; Splittstoesser, 1998; Vieria et al., 2000; Walls and Chuyate, 2000; Worobo and Churey, 1999). Various strains of these thermo-acidophiles have been isolated from spoiled acidic juices, isotonic water, lemonade, fruit juice blend, and fruit-carrot blend (Yamakazi, 1996).

The most likely source of *A. acidoterrestris* contamination is soil that contaminates fruit during harvest or through windfall (Brown, 1995). It seems reasonable that fruit in contact with the soil is more susceptible to contamination (Splittstoesser, 1998). Since *Alicyclobacillus* has been isolated from soil and sound and decaying fruits, spores in the processing plant environments may be introduced into the process by unwashed or poorly washed fruit (Borlinghaus, 1997; Brown, 1995). However, contaminated fruit may not be the only source of *A. acidoterrestris* in the plant. The organism may be present in raw and processing materials in extremely low numbers. In addition to being found on incoming fruit and in processing plants, these bacteria have been found in the condensate water recovery system of thermal evaporators used to produce concentrated juices (Parrish, 2000).

Another possible source of contamination is the water used to dilute concentrates to full-strength juice (Palop, 2000). McIntyre (1995) recently isolated A. *acidoterrestris* from water that had been used as an ingredient in a finished beverage that had later spoiled. This illustrates that the source of *A. acidoterrestris* in spoiled juices may not always be the fruit; water or other sources may be implicated as well. Certain other beverage ingredients are also potential sources of contamination. Recent unpublished studies revealed the presence of *A. acidoterrestris* spores in liquid sugar (Worobo and Churey, 1998). Therefore, control of this microorganism in the processing environment may be quite problematic for processors because it is widespread in fruit and vegetable growing and processing environments and it has proven difficult to eliminate from the processing plant (Eguchi, 1999).

In processing plants, *A. acidoterrestris* spores may be transferred to juice products from a variety of sources and may be widely distributed in the processing plant environment. Wisse and Parrish (1998) studied the occurrence of spore-forming thermo-acidophilic bacteria, including *Alicyclobacillus* and *Sulfobacillus*, in citrus fruit growing and processing environments. Isolates were obtained from seven of eighteen soil samples taken from citrus orchards, surfaces of unwashed fruit at eight of ten processing plants, on surfaces of six of nine washed fruits, and in condensate water used to wash fruits in six of seven facilities examined. Finding these bacteria in condensate water generated in the processing of citrus juice concentrates is significant because it indicates that the microorganisms are likely to be present in the water used to wash fruits (Wisse, 1998). MPN - based population estimates of washed and unwashed fruits showed that approximately 46 spores/fruit of spore-forming, thermo-acidophilic rods (STAR) were detected. Isolation of STAR from fruit surfaces was expected due to cross contamination with soil or other contaminated fruits during fruit growing and fruit harvesting/handling procedures. However, researchers were surprised that STAR were found on washed fruit from six plants; although this may be due to the fact that there were substantial numbers of STAR spores in condensate water used for fruit washing.

Condensate water is collected during evaporation of the water from juice during thermal processing used to produce juice concentrates. It is condensed and used for a variety of purposes, such as fruit washing. At two plants, condensate water taken directly from an evaporator contained STAR.

STAR were not isolated from single-strength juice deposited into the evaporator of the ten test plants but were found in concentrate samples in the evaporator of one facility. This implies that in single-strength juice, STAR bacteria were below present detectable level concentrations until the juice was concentrated. STAR were also found in four of eleven samples of frozen orange juice concentrate contained in tanker trucks when tested by the MPN method. In this study, STAR were present at <30, 150, 230, and 430 spores/g in the juice concentrate (65°degrees Brix). Additionally, one of 12 frozen orange juice samples in 210-liter drums used for bulk commerce was positive (40 spores/g). Two pear concentrate samples taken from 210-liter drums were positive (<30 spores/g), one of one retail package of pear juice (<3 spores/ml), and one of one retail packages of orange juice nectar (1.1×10^2 spores [MPN]; 1.7×10^4 CFU/ml [ALI agar]) was positive for STAR. An increase in total microflora and STAR spore counts of condensate water between the evaporator and storage tanks or spray nozzles indicate that heavy contamination and/or growth occurs in the condensate water system. Additionally, testing washed and unwashed fruits showed that washing reduced counts of aciduric microflora by only 0.2 to 1.5 log CFU/ml. However, in five of the processing plants tested, populations were greater (0.2 to 1.2 log CFU/ml) in washed fruit as compared to unwashed fruit.

Research has shown that at maximum cleaning efficiency of most fruit wash systems, a 90 to 95% reduction in populations of STAR may be achieved. However, less-than optimum cleaning may result in only a 60% reduction of fruit surface microflora (Wisse and Parrish, 1998). The rinsing of clean equipment (extractors, pipelines, evaporators, and blending tanks) with condensate water containing STAR spores may contaminate juice during transport to the evaporator. Furthermore, heat treatment in the evaporator is not sufficient to kill STAR spores. Temperatures reached in later stages of the evaporator process may actually support germination and outgrowth of *A. acidoterrestris*.

Since bacterial attachment can occur on surfaces such as pipelines and equipment, the widespread presence of STAR spores in soil, on fruit surfaces in the processing environment, and in juice products suggests that the complete elimination of *A. acidoterrestris* in final product may be difficult and/or impractical. However, improvements in cleaning protocols and condensate water systems may reduce STAR contamination (Wisse and Parrish, 1998).

A. acidoterrestris has been found in many different parts of the world and over a wide range of temperature zones (Palop, 2000; Pinhatti, 1997). It has been found in Europe, Asia, South America, and the United States, in the domestic supply of juices and concentrates and in imported concentrates (Gannon, 1998). When Pinhatti analyzed juices and concentrates from around the world, she showed that 23 of 34 contained *A. acidoterrestris* (Orr et al., 2000a). *A. acidoterrestris* has also been isolated from commercial juice products present in the retail environment (Palop, 2000; Pinhatti, 1997). Kang (2001) found more than 20% of apple juice products or concentrates obtained from the Pullman, Washington area contained *A. acidoterrestris*. This finding alone suggests that the widespread distribution of *A. acidoterrestris* in juices puts these products at a high risk of spoilage if held under conditions that would allow growth of this bacterium.

A. acidoterrestris was detected in a majority of juices from various countries that were analyzed; 11 of 11 concentrated orange juice samples that originated from various locations around the world were positive for *A. acidoterrestris* (Pinhatti, 1997). Four of four concentrated commercial orange juices, lemonades, and limeades were positive, and eight of 19 commercial ready-to-drink juices were positive (Pinhatti, 1997). These results indicate that *A. acidoterrestris* is present in juices on a wide scale, since they are present in a variety of juices, from different regions globally (Pinhatti, 1997).

Even in light of the worldwide occurrence of *A. acidoterrestris* in juices and the rapid growth and sporulation of the microorganism under appropriate conditions, spoilage of juices by *A. acidoterrestris* appears to be incidental and requires a combination of conditions for growth of the organisms in the juice, such as low pH (3.5-5.0) and high temperatures (42-53ºC) for a sufficient period of time (Pinhatti, 1997). This hypothesis may help to explain the episodic nature of *A. acidoterrestris* spoilage of juice products in Europe, which is observed most often in juices stored under ambient temperatures in years with very warm summers (Pinhatti, 1997).

Juice components or characteristics may have a positive or negative effect on *A. acidoterrestris* growth. When *A. acidoterrestris* was inoculated at high (4 log CFU/ml) and low (3 log CFU/ml) concentrations into apple juice, orange juice, white grape juice (no metabisulfites), salsa, grapefruit juice, tomato juice, and pear juice at 35°C, both the low and high inoculum germinated and grew within 1-2 weeks in white gape (with no metabisulfites), orange, apple, pear, and tomato juices. Growth of the microorganism was not detected in

grapefruit juice until the eighth week of incubation and did not grow in other products tested such as white grape juice (with sulfites) and salsa. Spoilage occurred at 20°C in orange juice only at the high inoculum level, at 23°C in pear juice at high inoculum level, and in white grape and tomato juices at both the high and low inoculum. Both orange and pineapple juices had similar pH values (3.6 and 3.1, respectively), but the microbe grew in orange juice, not pineapple. In addition, the pH of salsa (3.92) was closer to the microorganisms optimum pH compared to orange juice, but *A. acidoterrestris* did not grow in salsa. Walls and Chuyate (2000) theorized that ingredients in salsa or pineapple juice may have inhibited spore germination or the solids level in those products may have negatively influenced spore germination and vegetative cell growth.

Apple juice and tomato juice consistently supported growth of many *A. acidoterrestris* strains while other juices, such as apple-orange-pineapple, grapefruit, orange, and pineapple allowed growth of only one strain tested. No *A. acidoterrestris* growth was observed in apple-grape, apple-grape-cherry, two brands of Concord grape juice, cranberry juice, or prune juice (Splittstoesser, 1998). When heat-activated spores were inoculated into commercial juice beverages, the highest amount of growth was observed in tomato juice, with apple being second. The higher pH and lower soluble-solids content of tomato juice may explain why it is a more favorable growth medium for *A. acidoterrestri*s. This is explained by the fact that the optimum pH for A. *acidoterrestris* is 3.5-5.0 and a high concentration of soluble solids (16.1°Brix) has been shown to inhibit the microorganism (Splittstoesser, 1994).

The potential for *A. acidoterrestris* to cause extensive spoilage of acidic shelf-stable products should not be underestimated (Komitopoulou, 1999). Eiora (1999) tested 65 concentrated orange juice samples, and found that 14.7% of the samples contained *A. acidoterrestris*. In addition, concentrates used to make a carbonated fruit drink were found to contain A. *acidoterrestris* at low levels (i.e., approx 14-21 CFU/20 mls). Traditional plating methods revealed that full-strength juice made from the concentrate contained 1-500 viable *A. acidoterrestris* organisms/20 ml., but this level was lower than 5 log CFU/ml, the level of *A. acidoterrestris* that typically leads to taint compound formation. However, using DEFT, a membrane filtration-epiflourescence microscopy method, very high numbers of rod-shaped bacteria, >5.7 log CFU/ml, were present in the product. The most likely explanation of these results is that after dilution of the concentrates, spores germinated and the number of *A.*

acidoterrestris increased to levels that led to taint. However, the addition of carbon dioxide inactivated vegetative cells while taint compounds were still present (Pettipher, 2000). Furthermore, it is known that when concentrates are diluted to make juice, *A. acidoterrestris* will quickly begin to multiply (Pettipher, 2000).

Spoilage

Although it has never been associated with illness and there has been no poisoning by its metabolic by-products formed in juices, *A. acidoterrestris* is considered to be a global problem in juice products (Cerny et al., 1984). This microorganism is problematic due to its ability to cause spoilage of traditionally shelf-stable juices.

Spoilage of fruit juices and drinks by *A. acidoterrestris* is now recognized as endemic internationally (Borlinghaus, 1997). Spoilage by *A. acidoterrestris* is limited to traditionally shelf-stable juices or juice-containing beverages and is characterized by a disagreeable off-flavor with a lack of carbon dioxide production (Parrish, 2000). *A. acidoterrestris* spoils juice by creating an off-odor and off-flavor with occasional turbidity or sedimentation, although no gas is produced and no pH change occurs (Eguchi, 1999; McIntyre, 1995). Generally, no obvious visual signs of spoilage result. However, in rare cases, *Alicyclobacillus* spoilage may cause darkening of apple juice and separation of tomato juice that is covered with a white film (Eguchi, 1999). Therefore, spoilage caused by *A. acidoterrestris* is often first recognized by consumers due to off-flavors and off-odors present in the product. Generally, companies do not recognize that a spoilage incident exists until consumer complaints have been received (Walls and Chuyate, 2000).

A. acidoterrestris presence in the finished product is clearly undesirable due to the potential for spoilage, associated costs, and loss of a brand's quality image with consumers (Borlinghaus, 1997). According to Jensen (1999), spoilage in Europe and USA has proven expensive to producers. Therefore, *A. acidoterrestris* has been recognized as a spoilage agent in a variety of ambient-stored fruit juices, shortening shelf-life (Komitopoulou, 1999).

The majority of cases of *A. acidoterrestris* spoilage have occurred during the spring or summer, especially when the temperatures were unusually high and prolonged. Numerous studies have shown that when warehouse and product temperature reaches approximately 32°C, contaminated product has the potential to spoil within two days to one week (Eguchi, 1999;

Krueger Labs, 2001; Walls and Chuyate, 2000). A small number of viable organisms have the potential to contaminate large volumes of juice resulting in numerous spoilage incidents and financial losses for the producers affected (Pinhatti, 1997). In the US, spoiled juice was found to contain 76 spores/236 ml and in Australia 240 and 790 CFU/ml vegetative cells were found (Jensen et al., 2001; Lusardi, 2000). In the USA, spoilage has been observed primarily in apple juice, but has also been observed in pear juice, orange juice, fruit juice blends, and canned diced tomatoes. Under ideal conditions, low levels of spores (approximately 1 spore per 10 ml of juice) were sufficient to cause spoilage in apple and white grape juices (Walls and Chuyate, 1999).

In Australia, there have also been several incidences of *A. acidoterrestris* contamination and/or spoilage. In 1990, a 40% apple drink packaged in a Tetra-Pak was identified as having a halogen type off-flavor after 10 weeks of storage. In 1995, hot-filled orange juice in 2-liter cartons was shown to contain *A. acidoterrestris*, although no taint was observed even after nine months of storage. The source of contamination was believed to be orange juice concentrate. In 1995, an apple drink was spoiled by *A. acidoterrestris* after eight weeks. In 1996, a 35% tropical fruit drink containing orange, passion fruit, and three other juices developed halophenolic off-odors after seven weeks. This spoilage incident was traced to the imported orange juice used in the product. In 1997, a 25% tropical fruit drink was found to contain *A. acidoterrestris* and the taint compound, guaiacol. Contamination of the tropical fruit drink occurred due to contaminated apple juice used in the formulation. In 1999, 50% apple drink and 25% tropical Tetra-packed drinks were spoiled, and the source of contamination was apple juice added during production of the beverages. The use of passion fruit or passion fruit flavor may have contributed to contamination with *A. acidoterrestris* cells in tropical fruit drinks. In 2000, hot filled apple-lime juice in PET bottles developed taint (guaiacol) after eight weeks at room temperature and the *A. acidoterrestris* contamination was traced to the apple concentrate. In 2001, guaiacol formation was observed in hot-filled apple juice after 6 weeks of storage. Again the source of contamination was traced to apple concentrate used in the product (Goldberg, 2001). This data suggests that producers have had accounts of *A. acidoterrestris* spoilage in a variety of products with different sources of concentrate and juice contamination.

Today, *A. acidoterrestris* spoilage is a growing problem because *A. acidoterrestris*-associated spoilage in juices is now being reported more frequently than in previous years

(Duong and Jensen, 2000; Gannon, 1998). Fruit juices that have most commonly been spoiled by *A. acidoterrestris* include fresh juices, aseptically packaged juice, and pasteurized juices that are stored without preservatives at room temperature. However, ultra high temperature (UHT) juices have not been recognized as likely candidates for spoilage by *Alicyclobacillus* (Komitopoulou, 1999; Splittstoesser, 1994).

Slow cooling of heat-treated juice products may contribute to the development of spoilage by allowing *A. acidoterrestris* spores sufficient time to germinate and initiate growth. Pinhatti and others (1997) cite similar examples in Europe where hot-filled products in large volumes were allowed to cool "naturally" under high ambient (i.e., summer) temperatures. Presumably, the storage temperature must have been sufficiently high to allow growth and taint formation to occur.

A. acidoterrestris spoilage compound formation generally occurs within 7-10 days at 25°C, however if the temperature is higher (i.e., 42ºC), spoilage rates may be increased dramatically (Brown, 1995). Growth of *A. acidoterrestris* was observed at 37 and 45°C in 7 to 14 days in juices. Incubating fruit juices at temperatures above 37°C may allow significant increase in *A. acidoterrestris* within 14 days (Cerny et al., 2000). Unpreserved apple and orange juice spiked with *A. acidoterrestris* vegetative cells contained very high levels (6 – 7 log CFU/ml) of *A. acidoterrestris* after six to ten days at 44ºC (Pettipher, 2000).

A. acidoterrestris has been found in juice concentrates in low numbers (<50 per ml). However, spoilage has not been a concern in concentrates above 30° Brix because *A. acidoterrestris* vegetative cells are not able to grow at these levels. Unfortunately, spores of this microorganism have the ability to survive in the concentrates for over three months. When concentrates containing spores are diluted to make full-strength juice and pasteurized, *A. acidoterrestris* spores may germinate and spoilage may occur if optimum conditions, such as elevated storage temperatures are present. Spoilage of juice may occur when concentrates or juices are contaminated with less than 10 spores per ml and the juice is stored at an elevated temperature for a period of time (35˚C for 1 week) (Evancho and Walls, 2001; Pinhatti, 1997).

In a survey completed by the National Food Processors Association (NFPA), approximately 35% of 34 juice processors surveyed reported spoilage that is consistent with A. *acidoterrestris*. The majority of these companies had only a few cases (< 3) of *A. acidoterrestris* spoilage in the past five years, although the spoilage incidents resulted in 55% loss of their

product for the affected lots. However, one respondent had anywhere from 7-98% loss of product from affected lots due to spoilage. It was determined that the observed spoilage generally occurred in early spring or summer and was not a result of processing changes (Walls and Chuyate, 2000).

Since widespread *A. acidoterrestris* contamination of juices exists, it is important to determine the cell concentration that is associated with development of taint in juices. Sensory tests have determined that an off-flavor or off-odor may be detected in juices when *A. acidoterrestris* spores were present at a concentration of 1×10^3 CFU/ml (Eguchi, 1999) while Pettipher and Osmundson (2000) stated that a level of 1×10^5CFU/ml was required for taint to be detected. However, populations of less than 2 log CFU/ml also may cause taint (Gannon, 1998).

Spoilage Compounds

In early to mid 1990's, Australian chemists were consulted about "sanitizer taints" in fruit juices and canned vegetables. Ultimately, bromophenols and chlorophenols were found but were caused by microbial growth, not sanitizers. The cause of observed off-flavor and aroma was attributed to formation of the chemical compounds, guaiacol (2-methoxyphenol) and 2,6 dibromophenol by *A. acidoterrestris* in the juices (Eguchi, 1999). Two hypotheses exist on how these taint products are formed in juices. Using GC-MS, Krueger Laboratories (2001) determined that *A. acidoterrestris* acts upon vanillin naturally found in juices to form guaiacol and 2,6 dibromophenol, while Lusardi et al. (2000) stated that guaiacol may arise from the decomposition of lignin, phenolic acids, and ferulic acid found in fruits (Pettipher, 2000).

Since guaiacol and 2,6 dibromophenol have been established as off-odor and off-flavor compounds in *A. acidoterrestris*-associated spoilage incidents, many studies have been performed to confirm human threshold levels for these compounds. The compound, 2,6-dibromophenol, can be detected at 0.5 ppt in water and 1 ppt in fruit juices (Borlinghaus and Engel, 1997; Lusardi et al., 2000). However in apple juice, researcher stated that 2,6-dibromophenol has a taste threshold of 0.004 ppb (Leatherhead Food, 2000). Inoculation studies with *A. acidoterrestris* have demonstrated production of approximately 20 ppt of 2,6-dibromophenol in apple juices, a level that is nearly 40 times the threshold for human detection (Lusardi, 2000; Pettipher, 2000).

A. acidoterrestris spoilage has been shown to produce up to 100 ppb of guaiacol in juice, but the taste threshold is generally 2 ppb in orange and apple juice (Kang, 2001). The threshold of detection for guaiacol in juices varies with the matrix. In pear nectar, the threshold is 0.1µg/kg (parts per billion), and in orange juice it is 10µg/kg (ppb). The difference may be due to the interference by the limonene present at high levels among the volatile components of citrus juices (Lusardi et al., 2000). Generally guaiacol (1-100 ppb) and taint were detected at levels of 5 log CFU/ml and higher. Pettipher et al., (2000a) added different amounts of guaiacol to juices to determine threshold levels. This study showed that when juice contained at least 5 ppb of guaiacol, 80% of panelists correctly identified the odd sample in a Triangle Test (Orr et al., 2000a).

Although there have been several studies on human thresholds for guaiacol and 2,6-dibromophenol and the concentration of cells needed to cause taint, there is still debate on what descriptors should be used for off-flavor and off-odors in the spoiled juices. An Eguchi study (1999) described the aroma and flavor as "antiseptic" or "disinfectant-like", while in another study; panelists described the off-flavor and odor as "nasty", "acidic" and/or "putrid" (Eguchi, 1999). Orr et al. (2000a) stated that guaiacol spoiled the juice with a "sweetish" and "fermented" note, while inoculating with 2,6-dibromophenol resulted in a pungent " medicine-like" off-odor (Orr et al., 2000a). *A. acidoterrestris* also produces a "hammy" off-odor much like smoky bacon crisps (Pettipher, 2000).

Control Measures

Due to the impact that *A. acidoterrestris*-associated spoilage has caused producers, control measures need to be explored. Certain components of juices or extrinsic factors applied to juices may be used as a control method for *Alicyclobacillus*. Since *A. acidoterrestris* is strictly aerobic, oxygen may be a limiting factor for growth. Approximately 1% residual oxygen in a juice drink allowed the *A. acidoterrestris* to grow for 6 days but eventually caused death of the cells, due to decreased oxygen available for *A. acidoterrestris* to grow. At an oxygen level of 0.1%, no outgrowth of spores was detected, although decreased oxygen levels may be difficult to achieve in juices (Cerny, 1984).

In a carbonated beverage, the carbonation process inactivates vegetative cells but is not effective for elimination of spores (Pettipher, 2000). In a study performed by Pettipher (2000),

carbonation of juices decreased vegetative cells of *A. acidoterrestris* in orange juice and apple juice from an initial concentration of 580 CFU/ml to less than 100 CFU/ml in 10 days and decreased 680 spores/ml to 90 spores/ml in 3 months, respectively.

It has also been determined that *A. acidoterrestris* has the capability of growing in white grape juice but not red grape juice. Furthermore, removal of neutral phenolics eliminated from red grape juice eliminated the antimicrobial effect in red grape juice. Among several neutral phenolic compounds added to apple juice, catechin gallate was determined to be inhibitory to *Alicyclobacillus* at levels above 1,000 ppm (Splittstoesser et al., 1994). However, since red grape juice contains a variety of neutral phenolics, it is likely that other phenolics may also have an effect on growth. When *A. acidoterrestris* spores were incubated at 43°C for one hour and then heated at 70°C for 15 minutes, vegetative cells and remaining spores in white and red grape juices exhibited similar destruction rates. This finding suggests that red grape phenolics have some effect other than inhibiting spore germination (Splittstoesser et al., 1994).

Other control measures for *A. acidoterrestris* include storage of product below 20°C and filtration (Jensen, 2001). Sugar concentrations above 18° Brix, such grape juice and apple juices with sugar added (high Brix), and ethanol levels above 6% are also inhibitory to *A. acidoterrestris* (Splittstoesser, 1998).

Certain sanitation practices to prevent *A. acidoterrestris* in fruits and processing equipment have also been explored. Approximately 1% hydrogen peroxide, 200 ppm chlorine, and 500 ppm acidified sodium chloride applied to a pure suspension of *Alicyclobacillus* for 10 minutes at 23°C resulted in a 0.4 to 2.4 log CFU/ml reduction in viability. Treatments with 8% trisodium phosphate or 80 ppm Tsunami™ (hydrogen peroxide and peroxyacetic acid) were not very effective in reducing *A. acidoterrestris* in pure suspension. Treatments with 500 ppm chlorine or 1200 ppm acidified sodium chloride for one minute reduced the amount of viable dried spores on the surface of unwaxed Roma Beauty apples; however, reductions were less than 1 log CFU/ml and the spores were not killed. Hydrogen peroxide (2%) was ineffective for killing spores remaining on the apple skins after treatment. The observation that chemical sanitizers are less effective for killing *A. acidoterrestris* on the surface of the apples as compared to pure suspensions is likely due to inaccessibility of cells in cracks and crevices on the skin. In addition, the diminished level of the active form of treatment chemicals may occur due to interference with components on the skin. Because the surface of the apples is hydrophobic and

contains a cutin, it protects attached spores from contact with aqueous sanitizers (Orr and Beuchat, 2000b).

Approximately 40 ppm free chlorine dioxide reduces *A. acidoterrestris* populations in lab media by 0.1, 0.7, and 4.4 log CFU/ml, when treated for 0.5, 1, and 5 minutes, respectively. Approximately 80 ppm chlorine dioxide reduced populations of *A. acidoterrestris* by 1.0, 0.8, and >5.4 log CFU/ml when samples were treated for 0.5, 1, and 5 minutes, respectively. A concentration of 40 ppm free chlorine dioxide reduced *A. acidoterrestris* spores on four apple cultivars by 1.5, 3.2, 4.5, and >4.8 log after 1, 2, 3, and 4 minutes, respectively. Approximately 120 ppm chlorine dioxide reduced spore populations by >4.8 log CFU/ml after only 1 minute treatment (Gray, 2002). When using Oxonia Active ®, a hydrogen peroxide and peroxyacetic acid sanitizer, at 20°C, the D value ranged from 7.59 to 31.54 minutes with 0.05% Oxonia Active® and 3.9 to 14.28 minutes with 0.1% Oxonia Active®. Therefore, to achieve a 5 to 6 log CFU/ml reduction, would require more than 60 minutes contact time at 20°C. However, at 40°C, a D value of 1.18 to 3.76 minutes with 0.1% Oxonia Active® was needed to reduce *A. acidoterrestris* spores in water. Allowing spores to dry on steel plates before Oxonia Active® treatment did not significantly increase D values. Therefore, frequent sanitation of processing plants may reduce the risk of contamination, and ultimately, deterioration of the finished product (Previdi, 1999).

Physical and chemical reduction methods have been explored for prevention of *A. acidoterrestris* growth in juices. The MIC for inhibition of *A. acidoterrestris* was >0.5% for sucrose stearate, ≥0.5% for sucrose palmitate, 0.05% for sucrose laurate L1695, 0.1% for sucrose laurate LWA1570, and 0.01% for Lauricidin. In tomato juice, a 3 log CFU/ml reduction of *A. acidoterrestris* was observed when 0.005% sucrose laurate L1695 was combined with a 10-minute treatment of 392 MPa and a 45˚C heat treatment. When sucrose laurate was increased to 0.01%, spore outgrowth occurred with or without the combination of mild heat and pressure. Treatments of 392 MPa for 10 minutes at 45°C resulted in a 2 log CFU/ml reduction with no additional reduction associated with sucrose laurate. Sucrose laurate at 0.045% plus pressure (392 MPa) and mild heat treatment (45°C) resulted in a 5.5 log CFU/ml reduction. The addition of sucrose laurate at <1.0% had no obvious effect on the appearance or odor of the food products but caused foaming during mixing (Shearer et al., 2000). At 25°C, high pressure (90 Kpsi) did not inhibit spoilage bacteria/spores. However, mild temp (45°C) and high pressure (60 Kpsi)

reduced numbers from 6.5 log CFU/ml to 2 log CFU/ml in 1 minute, to 1.5 log CFU/ml in 5 minutes, and to 1 log CFU/ml in 10 minutes (Lee, 2001).

Commercial, pasteurized apple juice (pH 3.7) was inoculated with a two strain cocktail of *Alicyclobacillus* (6 log CFU/ml), and treated at 0, 207, 414, and 621 MPa at room temperature, as well as 45, 71, and 90°C. Spore viability was not appreciably reduced by high pressure alone at room temperature. However, the viability of spores treated with high pressure and heat (45, 71, and 95°C) was significantly reduced. Treatment with 207 MPa at 45°C for 10 minutes or at 71°C for 1 minute resulted in more than a 3.5 log CFU/ml reduction in viable spores. Treatment of 414 or 621 MPa at 71°C for 1 minutes resulted in a 4 log CFU/ml reduction, and a treatment of 414 or 621 MPa at 71°C for 10 minutes reduced spores to undetectable levels (<1 CFU/ml), a > 5.5 log CFU/ml reduction. Greater reductions in viability occurred as treatment time increased at 45 or 71˚C; however, there was not a significant difference among 207, 414, and 621 MPa treatments on spore viability. There was no significant reduction in spore viability when spores were heated at 90°C without high pressure for 1 minute. However, treatments of 414 or 621 MPa for one minute, or 207 MPa for five minutes at 90°C reduced spores to undetectable levels, more than a 5.5 log CFU/ml reduction. Therefore, high pressure alone did not reduce levels of spores in apple juice, but high pressure was more effective as temperature increased. The authors concluded that spores of *A. acidoterrestris* are relatively resistant to pressure (Lee et al., 2002).

In addition, certain antimicrobials have been investigated to prevent *A. acidoterrestris* growth in juices. The MIC needed for *A. acidoterrestris* is as follows: 458 ppm malic acid, 707 ppm tartaric acid, 539 ppm lactic acid, 458 ppm citric acid, and 61 ppm benzoic acid (Hsiao, 1999). Sorbic (300 ppm), benzoic (150 ppm), and sorbic and benzoic acid plus carbonation reduced numbers of *A. acidoterrestris* from 580 CFU/ml to less than 100 CFU/ml in 10 days (Pettipher, 2000). Also, a crude preparation of a bacteriocin of *Lactococcus* CU216 reduced levels of *Alicyclobacillus* growth (Oh and Churey, 2000).

Although some control measures have been established for *A. acidoterrestris*, many of the methods available are excessively costly to the producer or result in decreased quality of juice products. However, additions of some antimicrobials have shown potential in preventing growth and spoilage of *Alicyclobacillus*. Therefore using antimicrobials for the control of *A. acidoterrestris* in juices need to be further studied.

Antimicrobials

Antimicrobials have been used for centuries to help prevent spoilage and pathogenic bacteria in foods. If applied in accordance with industry and government levels, flavor and quality of the juice may be maintained.

Nisin

Chemical Description and Approval

Nisin, a polypeptide produced by *Lactococcus lactis*, was first characterized in 1928 by Rogers and Whittier (Delves-Broughton, 1990; Hoover, 1993). The name nisin was derived from "N inhibitory substance" given by Matak and Hirsch (Hoover, 1993). This antimicrobial is a pentacyclic peptide that consists of three unusual amino acids including: dehydroalanine, lanthionine, and β-methyl lanthionine and exists as a dimer with a molecular weight of 7,000 (Davidson and Juneja, 1990; Hoover, 1993).

Bacteriocins, such as nisin, may be perceived by consumers to be more natural than chemical preservatives making it a more attractive choice for preservation (Harlander, 1993). Since nisin is produced by *Lactococci* that reside in raw milk, and have likely been consumed for centuries, it is considered to be safe for consumption. The median lethal dose is 7g/kg, similar to the maximum allowed dose of NaCl (Davidson and Juneja, 1990; Hoover and Steenson, 1993). In addition, it is odorless, tasteless, can be broken down in the human gastrointestinal tract, and cannot be detected in the saliva beyond 10 minutes after consumption (Hoover, 1993; Komitopoulou, 1999; Task Force, 1998). Nisin received "Generally Regarded as Safe" (GRAS) status from the Food and Drug Administration in 1988, to be used in pasteurized cheese spreads to prevent *C. botulinum* spore outgrowth and toxin formation. Nisin is generally used at a maximum concentration of 250 ppm in cheese spread in accordance with Good Manufacturing Practices (Food and Drug Administration, 2001a).

Nisin has been legally used in 47 countries including the UK and the US for over 30 years. Although in the United States, nisin is only allowed in cheese spreads; worldwide it has been used in a variety of food products including: pasteurized, flavored, and long life milks, aged and processed cheeses, frozen desserts, liquid egg products, dressing, canned vegetables, alcohol, meats, fish, bakery products, soups, and more recently, to inhibit lactic acid bacteria in wine

(Davidson and Juneja, 1990; Delves-Broughton, 1990; Task Force, 1998; Whole Foods Market, 2000). The Nisaplin-brand of nisin is also approved for liquid egg products, dressings, and sauces (Bayer News, 2001). Since nisin is effective against many Gram-positive microorganisms, it has been used in teat dips to treat mastitis, in toothpaste, mouthwash, soap, skin care products, and acne treatment cosmetics (Hoover and Steenson, 1993).

To determine the activity of nisin in a product, Tamer and Fowler suggested the use of 1 Reading Unit (RU) as the amount of activity present in 1μg of pure nisin. This was later changed to International Units (IU) with the conversion of 40 IU=1RU (Hoover, 1993). Up to 400 IU/g food, about 10 ppm nisin, is typically recommended for food preservation (Hoover and Steenson, 1993; Hurst, 1983). Normally, levels of 2.5 to 12.5 ppm of nisin are used to preserve food. This range of concentrations is equivalent to 100 to 500 ppm Nisaplin, a product made up of nisin, hydrolyzed milk solids, and salt (Weeks Publishing Company, 1999).

Mechanism of Action

Nisin has shown bactericidal activity against a wide range of Gram-positive microorganisms (especially spore-formers) but has shown little to no effect on some Gram-negative microorganisms, yeasts, and molds. However, with the addition of a chelating agent, some Gram-negative bacterial inhibition may occur (Delves-Broughton, 1990; Hoover and Steenson, 1993). Nisin is effective against *Staphylococcus, Streptococcus, Micrococcus, Lactobacillus, Listeria monocytogenes*, and the majority of spore forming species of *Bacillus* and *Clostridium* (Hoover and Steenson, 1993).

Nisin causes disruption of the cytoplasmic membrane of vegetative cells (Hoover and Steenson, 1993). In vegetative bacterial cell walls, it acts as a surfactant absorbed strongly on the plasma membrane of sensitive cells, where it destabilizes the cellular membrane, binds vital sulfhydryl groups, and inactivates enzymes such as Coenzyme A (Daeschel, 1999; Ray, 1992; Task Force, 1998). Cell death following adsorption of nisin is associated with the release of cytoplasmic materials, leakage of ATP, and in some strains, cell lysis (Delves-Broughton, 1990; Hoover and Steenson, 1993; Ray, 1992). Nisin at concentrations of 5 μg/ml or greater resulted in the loss of total proton-motive force in cells at external pH values of 5.5 and 7.0 (Hurst, 1993). Nisin may also produce poration complexes in the cell membrane resulting in leakage of cellular components and losses of membrane electrical potential (Task Force, 1998). The loss of

membrane potential and gradient destroys the ability of the cell to generate energy through electron transport (Hansen, 1993). At a higher concentration (e.g. 1500 IU/ml) nisin can inhibit peptidoglycan synthesis and cell wall synthesis (Ray, 1992; Task Force, 1998). A concentration of 1500 IU/ml caused 50% inhibition of enzymes related to peptidoglycan synthesis of *B. stearothermophilus* and *E. coli*.

The physical presence of nisin in the membrane, as well as covalent modification of membrane proteins, may disrupt the structural integrity of the membrane and its ability to participate in energy transductions. Membrane leakage and collapse of gradients would then lead to the death of the cell (Hoover, 1993). Nisin's bactericidal effect is likely produced by its interference with membrane functions. A recent study confirmed that nisin treatment causes a rapid efflux of amino acids from the cytoplasm of sensitive Gram-positive bacteria. It also decreases the membrane potential of cells and thereby deprives them of the components required for proton motive force and biosynthesis of cellular materials (Ray, 1992). Gram-negative bacteria remain unaffected by nisin because their extra outer membrane is impermeable to nisin. Therefore, nisin does not inhibit *E. coli*, *Salmonella*, *Campylobacter*, and *Yersinia*, but does inactivate *Bacillus*, *Clostridium*, and *Listeria* (Landy, 2001).

Nisin causes the loss of cellular material followed by lysis of *L. monocytogenes* (Daeschel, 1999). The bacteriostatic or bactericidal action of nisin against vegetative cells of bacteria is applied on the phospholipid component of the cytoplasmic membrane, where it acts as a membrane-depolarizing agent, causing cell membrane leakage. Nisin is believed to break the membrane permeability barrier of sensitive bacteria through pore formation rather than a non-specific detergent-like membrane destabilizing effect (Task Force, 1998).

Among spores of *Bacillus* species, M Spores (*B. subtilis*), i.e. the spores that rupture their envelope by mechanical pressure, were more sensitive to nisin than L spores, such as those of *B. cereus,* which grow by lytic mechanisms (Ray, 1992). The cytoplasmic membrane is apparently destroyed immediately after spore germination (Davidson and Juneja, 1990). While low heat treatment will not destroy endospores of spoilage bacteria, nisin prevents germination by acting early in the endospores germination cycle (Jay, 2000). With strains of *Bacillus* spp., Hitchens (1963) and Gould (1964) observed that germination was unaffected by nisin, but post-generation swelling and subsequent stages of spore development were inhibited (Meghrous et al., 1999).

In spore formers, nisin affects the development of spores by inhibiting pre-emergent swelling and preventing vegetative cell formation (Delves-Broughton, 1990; Komitopoulou, 1999; Prittjarvi, 2001). The action of nisin against spores is considered to be sporostatic instead of sporicidal, and therefore a residual amount of nisin is needed in the product to inhibit germination (Delves-Broughton, 1990).

Scientists suggested that nisin was sporostatic and that it prevented the outgrowth of spores of *B. coagulans*. However, studies by other researchers have shown that spores of sensitive *Bacillus* strains were killed by nisin (Ray, 1992). It had been shown that germination swelling was not inhibited by nisin, whereas the pre-emergence swelling was oxygen dependent and was inhibited by nisin (Hurst, 1993). The cytoplasmic membrane is apparently destroyed by nisin immediately after spore germination (A and Z Food Additives). Other researchers have strongly argued that nisin does not inhibit germination swelling, but inhibits pre-emergence swelling prior to emergence of cells from spore envelopes, and that this was followed by the commencement of growth (Ray, 1992).

An alternative mechanism that has been suggested is that dehydro residues act as an acceptor to react with nucleophilic groups such as sulfhydryl groups in the target spore, similar to vegetative cells. It has been shown that nisin inactivates sulfhydryl groups in the membrane of germinated bacterial spores so that they no longer react with iodoacetate. It has also been reported that unmodified membrane sulfhydryl groups are necessary to permit spore outgrowth (Hoover, 1993).

Interaction with Juice Components

The antimicrobial action of nisin is influenced by factors such as pH, sodium chloride concentration, and storage temp (Task Force, 1998). Nisin is relatively heat stable at acidic pH, and the beneficial effects of its inclusion prior to pasteurization enhance the effect of the heat process. Since nisin is not degraded by heat, there would also be a nisin residual; which, even at low levels, prevents the outgrowth of surviving spores (Komitopoulou, 1999). Nisin in small amounts increases sensitivity of spores to heat, reducing the temperature needed to inactive spores. Nisin effectiveness against bacterial spores is enhanced after injury due to heating (Task Force, 1998).

Nisin is an acidic molecule and exhibits the greatest stability under acidic conditions and is considered to be more soluble at lower pH (such as pH 3.0) (Delves-Broughton, 1990). The solubility of nisin is 12% and 4% at pH 2.5 and 5.0, respectively, but the molecule is insoluble at neural and alkaline pH (Daeschel, 1993; Davidson and Juneja, 1990). It has recently been shown that nisin is not completely stable at pH 2, but is most stable at pH 3.0 and maintains more than 70% antibacterial activity at pH 4 even when autoclaved at 115ºC (Yamakazi, 2000). Tramer (1964) reported that nisin was stable when autoclaved at 121ºC at pH 2 but 40% of its activity was lost at pH 5 and more that 90% was lost at pH 6.8 (Daeschel, 1993; Delves-Broughton, 1990). The successful application of nisin requires that the food be acid to ensure stability during both processing and storage (Hurst, 1993).

Inactivation due to nisin is less dramatic in foods than in buffer. Loss of nisin occurs during food storage, however losses are increased when the food has high a pH and is stored at high temperatures (Daeschel, 1993; Delves-Broughton, 1990). The efficacy of nisin on outgrowth of spores of *C. botulinum* in bacterial medium was increased by lowering the pH and increasing the length and temperature of the heat shock process (Meghrous et al., 1999). Nisin is proportionately more inhibitory to sensitive bacteria in liquid than in solid or semisolid foods (Ray, 1992). The main reason for this observation is likely due to barrier functions of non-liquid systems (Ray, 1992). Henning et al. (1989) indicated that emulsifiers have an antagonistic effect on the antimicrobial activity of nisin in a tomato juice system (Daeschel, 1993). Therefore the food environment such as higher pH, high lipid content, larger particle size, extended storage time, and non-uniform distribution of nisin in food may reduce the antibacterial effectiveness of nisin (Ray, 1992).

Certain food components may either act synergistically or antagonistically with nisin, so it is important to account for these factors when adding nisin to certain foods. Gross and Morrell (1970) reported that certain amino acids in the nisin molecule compete with sulfhydryl-containing enzymes. It is hypothesized that the decrease in nisin activity seen in red wines may be the result of nisin interactions with phenolic compounds that are absent in white wines. Daeschel et al. (1993) observed that nisin retained activity in white wines but decreased to less than 90% of its original activity in red wines within 4 months. In addition, polymeric phenols (e.g., tannins) may have a higher affinity for binding or inactivating nisin (Daeschel et al., 1993). However, nisin may bind with polyphenols in apple juice and may increase bacterial inhibition

due to the synergy of the polyphenols and nisin together on the microorganism (Yamakazi, 2000).

Sodium chloride at 3 and 4% either enhanced bacteriocin activity or resulted in greater spore sensitivity. However, an opposite effect was observed by Bell and DeLacy (1985) during studies with heat activated *Bacillus lichenformis* spores. The inclusion of various combinations of salt (1-5% w/v) and nisin (1-100 IU/ml) in spore suspensions ($1x10^6$ CFU/ml) consistently showed a protective effect of salt on inhibition of spore outgrowth by nisin. These authors concluded that salt prevents nisin from absorbing to the spores (Ray, 1992).

Nisin may also react with certain food additives in the product. Synergistic effects were reported between nisin and acetic acid, lactic acid, citric acid, or glucono-delta lactone on *Bacillus* spores (Ray, 1992). However, nisin does not appear to be compatible with sorbic acid, since this acid inhibits the antibacterial activity of nisin (Hoover and Steenson, 1993). Nisin is a hydrophobic compound, and can be degraded by metabisulfite, titanium oxide, and certain proteolytic enzymes (Jay, 2000).

Previous Work

The Minimum Inhibitory Concentration (MIC) of nisin for *Bacillus* vegetative cells is 2 to 4 IU/ml and 0.25 to 80 IU/ml for *Clostridia* spores (Hoover and Steenson, 1993). Small spore *Bacillus* species were inhibited by 5 IU/ml nisin, while large spore species were inhibited by 100 IU/ml or more nisin (Hoover and Steenson, 1993). Nisin A had an MIC of 0.01 to 10.5 mg/ml (4.2 mg/ml average) MIC for *Bacillus* spp. and 0.1 to 4.2 mg/ml (average of 1.1 mg/ml) MIC on *Clostridium* species (Mota-Meira et al., 2000). Nisin completely inhibited the outgrowth of *C. botulinum* spores at a concentration of 500 IU/ml or higher, but at least 75-100 µg/ml was needed for inhibition of *C. sporogenese* spores (Meghrous et al., 1999). Beuchat et al. (1997) observed that the inhibitory effects of nisin were greater on vegetative cells of *B. cereus* than on its spores in beef gravy; this effect increased considerably as temperature decreased. Incorporation of 100 to 200 IU/g nisin in high acid food, such as canned tomato products, greatly controlled growth and spoilage by acid tolerant *C. pasteurianum* and *B. coagulans* (Ray, 1992). The inhibitory range for *Bacillus* spp. in microbiological medium ranged from 1.6 to 80 IU/ml (Davidson and Juneja, 1990; Whole Foods Market, 1990). Although generally ineffective on Gram-negative organisms, nisin decreased *S. Typhimurium* by 1.3 to 2.4 log CFU/ml in

pasteurized orange juice, 0.1 to 0.19 log CFU/ml in fresh-squeezed juice with pulp, and 0.1 to 0.2 log CFU/ml in fresh squeezed juice without pulp (Liang, 2002).

Several studies have suggested that nisin may be used as a highly effective control measure for *A. acidoterrestris* in juices. According to a study by Komitopoulou (1999), *A. acidoterrestris* vegetative cell growth was completely inhibited using 100 IU/ml of nisin in grapefruit juice, apple juice, and orange juice when juice was incubated at 43˚C. However, using a process that more realistically compares to the normal commercial practice, amounts as low as 5 IU/ml were effective for inhibition. According to Yamakazi (2000), vegetative cells were inhibited by 1 to 100 IU/ml nisin, and <0.78 to 100 IU/ml was needed to inhibit spore outgrowth. *A. acidoterrestris* was sensitive to 1.25×10^{-4} µg nisin when applied directly to an inoculated agar plate (Prittjarvi, 2001). The MIC of nisin on mYPGA plates was 0.78 to 12.55 IU/ml and from 25 to 100 IU/ml at pH 3.4 and 4.2, respectively. The levels of nisin inhibition against vegetative cells were higher than those of spores. When *A. acidoterrestris* spores were heated and added to acidic clear apple drink and orange drink, the spore's heat resistance declined gradually as the nisin concentration in both drinks was increased. The $D_{90°C}$ values in clear apple juice were 20.8, 19.3, 15.5, and 14.8 minutes at 0, 50, 100, and 200 IU/ml nisin, and in orange drink, the $D_{90°C}$ values were 23.1, 20.4, 20.6, and 17.6 minutes at 0, 50, 100, and 200 IU/ml, respectively (Yamakazi, 2000). When 200 IU/ml Nisaplin was present (or 5ppm as active nisin) the spores were reduced by 71 and 76% in a clear apple and orange drink compared to the control (Yamakazi, 2000). The outgrowth of spores was inhibited by the addition of 25 to 50 IU/ml nisin in both the orange juice and fruit-mixed drinks but was not inhibited by the higher-level addition in clear apple drink (Yamakazi, 2000).

Sodium Benzoate

Chemical Description and Approval

Benzoic acid is a phenylformic acid that is naturally found in cranberries, plums, prunes, cinnamon, cloves, and other berries. It is considered to be one of the most widely used preservatives in the cosmetic, food, and drug industries (Chipley, 1993; Davidson and Juneja, 1990).

In 1986, sodium benzoate was the first chemical preservative that was approved for use in foods by the FDA. It was approved for use up to a maximum concentration of 0.1% and is

now commonly used in foods because it is inexpensive, easy to apply food products, a lack of attributed color, and low toxicity (Chipley, 1993; Davidson and Juneja, 1990; Food and Drug Administration, 2001c; Task Force, 1998). Foods in which benzoate is used as a preservative include: fruit products, beverages, bakery products, fruit juices and drinks, fruit salads and cocktails, salads and salad dressings, pickles, relishes, olives, sauerkraut, dried fruits and preserves, jams and jellies, and margarine (Task Force, 1998). Sodium benzoate is used as an antimicrobial in carbonated and still beverages (0.02 to 0.05%), syrups (0.1%), cider (0.05 to 0.1%), fruit juices and concentrates (0.05%)(Chipley, 1993). At concentrations above 0.1%, benzoic acid may have a negative impact on flavor, causing a "peppery" or burning taste (Jay, 2000). However, benzoic acid is generally used in combination with sorbic acid or another preservative to synergistically increase bacteriostatic effect while avoiding levels that result in a flavor impact (Chipley, 1993).

Mechanism of Action

Benzoic acid concentrations of 1,000 to 3,000 ppm have a bacteriostatic effect, but are generally not bactericidal. While pathogenic and spore-forming bacteria may be inhibited by 0.01% to 0.02% undissociated acid, a larger amount of undissociated acid is needed to inhibit spoilage bacteria (Chipley, 1993). The antimicrobial activity of benzoate is greater against yeasts than against bacteria and molds but depends on food pH, water activity, and microorganisms present (Task Force, 1998). Pathogenic bacteria that have reported susceptibility to benzoate include *V. parahemolyticus*, *S. aureus*, *B. cereus*, and *L. monocytogenes* (Task Force, 1998). However, benzoate can be metabolized by bacteria such as *Enterobacteriaceae*, *Psuedomonas*, *Corynebacterium glutamicum*, and by certain thermophilic *Bacillus* species (Task Force, 1998).

Benzoic acid prevents bacterial growth by decreasing the permeability of the cell membrane and reducing the internal pH (Chipley, 1993). The potency of weak acids, such as benzoic acid, as food preservatives is related to their capacity to reduce the intracellular pH. Although both the undissociated and dissociated forms of these acids cause the intracellular pH to drop, growth inhibition is predominately due to the undissociated acid portion of the antimicrobial. However, the dissociated compound has also shown some antimicrobial activity (Brannen, 1993; Task Force, 1998).

Benzoates act against microbes by inhibiting cellular uptake of substrate molecules. In the undissociated form, they facilitate proton leakage into cells and increased energy directed out of cells in an effort for the cells to maintain an optimum internal pH. With this disruption of membrane activity, amino acid transport is adversely affected (Jay, 2000). In *B. subtilis*, inhibition of amino acids moving across the membrane results in starvation of the cell (Chipley, 1993). Inhibition of transport in turn results from the destruction of the proton-motive force caused by the continuous shuttle of protons into cells by benzoic acid. Benzoic acid removes the negative charge of ions within the interior of cells and thus increases membrane mobility. Benzoate has also been shown to inhibit amino acid uptake in *P. chrysogenum*, *B. subtilis*, *E. coli*, and *P. aeroginosa* (Chipley, 1993).

Benzoic acid may also inhibit specific enzyme systems within cells. In many bacteria and yeasts, enzymes involved in acetic acid metabolism and oxidative phosphorylation can be inhibited. Among enzymes in the citric acid cycle, α-ketoglutarate and succinate dehydrogenases appear to be quite sensitive to benzoic acid, which may cause an effect on the citric acid cycle of the sensitive bacteria (Chipley, 1993). Benzoates may also serve as scavengers for free radicals by inhibiting D-amino acid oxidases, poly (ADP ribose) polymerase, passive anion transport, and nitrosamine formation (Chipley, 1993; Davidson and Juneja, 1990). In spores, it negatively affects the shedding of the spore wall (Jay, 2000).

Interaction with Juice Components

It is the undissociated molecule of benzoic acid that is believed to be responsible for its antimicrobial activity (Chipley, 1993). Therefore, antimicrobial activity increases as the pH value of the food decreases near the pKa of 4.19 and maximal antimicrobial activity occurs at pH values of 2.5 to 4.0. At pH 6.0, antimicrobial activity is only 1% of that at pH 4.0 (Brannen, 1993; Task Force, 1998). Although the antimicrobial activity is increased at a lower pH, as an acid, benzoic acid is soluble to a limited extent in water (Brannen, 1993). However, sodium benzoate has solubility of 50 grams/100 mls of water at 25°C (Brannen, 1993).

Certain food components and additives may impart an increased antimicrobial effect when combined with benzoic acid. Sodium chloride has a considerable synergistic effect on sodium benzoate activity (Furia, 1968). Combinations of sorbic acid and benzoic acid behave synergistically to inhibit several bacterial strains better than either sorbic or benzoic acid alone.

Synergistic effects have also been reported for benzoic combined with carbon dioxide and boric acid (Chipley, 1993).

Previous Work

Food poisoning and spore-forming bacteria are generally inhibited by 0.01% - 0.02% undissociated benzoic acid, but many spoilage bacteria are much more resistant. The MIC for benzoates on *B. cereus* (pH 6.3) and *Lactobacillus* spp. (pH 4.3-6.0) is 500 ppm and 300 - 1800 ppm, respectively. It was found that benzoic acid at concentrations of approximately 1000 - 3000 ppm had a strong bacteriostatic effect, but relatively modest bactericidal activity against cells in liquid minimal medium (Chipley, 1993).

There has been little work with benzoic acid for the prevention of *A. acidoterrestris*, but two studies have been performed with the use of benzoic acid in media and juice. Research by Pettipher (2000) revealed that benzoic acid at 150 ppm was shown to decrease vegetative cells at 5.8×10^2 CFU/ml to <100 CFU/ml in ten days and reduced $6.8 * 10^2$ spores/ml to 1.4×10^2 CFU/ml in three months in apple juice and orange juice, in combination, 300mg/L sorbic acid + 150mg/L benzoic acid reduced 580 vegetative cells/ml to <100 cells/ml in ten days and 680 spores/ml to 130 cells/ml in 3months (Pettipher, 2000). However, in bacteriological media, Hsiao (1999) stated that the MIC for benzoic acid was 61 ppm for *A. acidoterrestris* vegetative cells.

Potassium Sorbate

Chemical Description and Approval

Sorbic acid is a straight chain unsaturated monocarboxylic fatty acid that, in 1859, was isolated from the rowanberry (Davidson and Juneja, 1990). Sorbic acid has a molecular weight of 112.13 (Sofos, 1989).

Sorbic acid has been commonly used in food such as dairy products, bakery products, fruit and vegetable products, fat emulsion products, certain meat and fish products, and confectionary items, with levels present at approximately 0.02 to 0.3% sorbic acid (Davidson, 1993b; Sofos and Busta, 1993). In beverages, wines, carbonated and non-carbonated beverages, and fruit drinks, sorbate is generally used at levels of 0.02% to 0.1%, which imparts some antimicrobial inhibition while not imparting strong off-flavors in juices (Sofos, 1989; Sofos and

Busta, 1993; Task Force, 1998). However, sorbates above 0.10% may result in undesirable off-flavors in fruit products (Sofos, 1989).

Sorbic acid and its salts have been considered to be GRAS since 1978 by USDA, and this status has been reaffirmed by the FDA (Food and Drug Administration, 2001d; Task Force, 1998). The Environmental Health Organization (EHO) has set the acceptable daily intake of sorbate to 25 mg/kg per body weight (Sofos and Busta, 1993). Sorbates may be used for prevention of spoilage during storage of raw materials to be used in further processing for manufacture of beverages, soft drinks, and essences (Sofos, 1989). Direct addition and mixing of sorbic acid or its salts into a food product assures control of the correct concentration used and uniform distribution throughout the product (Davidson and Juneja, 1990). Sorbates are applied to food and other products as direct additives in the formulation, through spraying or immersing the material in sorbate solution, by dusting the product with powder through an organic carrier applied to the coating or packaging material, or as a component of a multi-ingredient composition applied in a single action and as a solution (generally 2 to 40%) that is directly applied to beverage and pickled products (Sofos, 1989).

Mechanism of Action

Sorbates have been show to be effective against Gram-positive and Gram-negative, catalase positive and negative, aerobic and anaerobic spoilage and pathogenic bacteria. Some species that are inhibited by sorbate include: *Acetobacter, Campylobacter, Enterobacter, Klebsiella, Lactobacillus, Listeria, Micrococcus, Mycobacterium,* and *Psuedomonas* (Davidson, 1993b; Task Force, 1998). Important pathogenic bacteria inhibited by sorbates include *Salmonella*, *E. coli*, *Staphylococcus*, *V. parahemolyticus*, *Bacillus* spp, and *C. botulinum*. Concentrations of sorbate that is inhibitory to bacteria range from 0.001% to 1.0% with certain species such as *Lactobacillus* spp. and *Clostridium* spp., being more resistant than others (Sofos, 1989).

Sorbate concentrations used in foods (i.e., < 0.3%) typically inhibit microorganisms, while higher amounts may result in cell death. Since sorbic acid is bacteriostatic at levels less than 0.3%, residual sorbic acid must be maintained in treated foods to prevent outgrowth (Sofos, 1989; Sofos and Busta, 1993). Consequently, when the sorbate hurdle is removed or reduced, the microorganisms that have remained viable may resume growth and spoil the food (Sofos and Busta, 1993). Levels above 0.3% sorbic acid is bactericidal, however, this level is impractical in

food preservation because of adverse effects on sensory characteristics (Davidson and Juneja, 1990). Traditionally, sorbate has been considered to be more effective as an antimicrobial agent than other common food preservatives (e.g., benzoate and propionate) (Davidson and Juneja, 1990).

The mechanism of inhibition by sorbate is based on the theory that lipophilic, weak acid preservatives starve microbial cells of compounds that are transported actively by the proton-motive force across the cell membranes (Sofos and Busta, 1993). Sorbate can also have a direct inhibitory effect on division of vegetative cells of bacteria. Inhibition of microbial cell metabolism by sorbate may be attributed to several mechanisms including negative alterations of morphology, integrity, and function of cell membranes, inhibition of cell transport and nutrient uptake, and inhibition of enzymatic activity (Task Force, 1998). Inhibition of nutrient uptake may be due to neutralization of the proton motive force, inhibition of electron transport system, inhibition of synthesis, depletion of ATP, inhibition of transport enzymes, and inhibition of metabolic energy utilization by the amino acid transport systems (Sofos, 1989).

Melnick postulated that sorbic acid inhibits dehydrogenase involved in fatty-acid oxidation. Sorbic acid has also been shown to be a sulfhydryl enzyme inhibitor. York and Vaughn (1964) suggested that sorbate reacted with sulfhydryl enzymes through the addition of thiol groups of cysteine. Other suggested mechanisms for sorbate have included interference with enolase, proteinase, and catalase, or inhibition of respiration by competitive action with acetate during the formation of acetyl CoA (Davidson and Juneja, 1990). Sorbates inhibit the activity of several enzyme systems, which may lead to disruption of vital processes involved in transport functions, cell metabolism, growth, and replication (Sofos, 1989). Sorbate has been shown to inhibit the uptake of glucose and amino acids into the cell, and interfere with its electron transport system. Several studies have reported extension of lag phase, and to a lesser degree, reduced growth populations and growth rate (Davidson, 1993b; Sofos, 1989). However, death of microorganisms exposed to high concentrations of preservatives such as sorbate, has been attributed to the generation of holes in cell membranes.

The effects of sorbate on spore-forming bacteria may be targeted toward spore germination, outgrowth, and/or vegetative cell division (Davidson, 1993b; Sofos and Busta, 1993). In spore-formers such as *Bacillus* spp. and *Clostridium* spp., the molecular mechanisms of microbial inhibition are not well defined. It has been shown that *s*orbate prevents the emergence

of vegetative cells (commitment to germinate) from the spores during outgrowth, inhibits sporelytic enzymes, or interacts with the spore membrane. However, larger concentrations of sorbic acid are needed inactivate spores as compared to vegetative cells (Sofos, 1989; Task Force, 1998).

Published data have suggested that sorbate acts as a competitive and reversible inhibitor of amino acid-induced germination. At least one study has suggested that sorbate inhibits spores that have been triggered to germinate or after germinant binding (Sofos and Busta, 1993). Another study concluded that sorbate inhibits spore commitment to germination, not triggering. Inhibition of spore germination may be taking place through inhibition of sporeolytic enzymes involved in germination or through the interaction of sorbate with spore membranes that leads to membrane increased fluidity (Sofos and Busta, 1993).

It is generally believed that the first event in germination process, after spore activation, is the trigger or initiation reaction through which spores become committed to germinate. Triggering or commitment to germinate is followed by a poorly defined lag period, where "connecting reactions" take place. It is postulated that sorbates acts as a competitive and reversible inhibitor of amino acid induced germination (Sofos, 1989). Specifically, it is postulated that sorbate competes directly with the germinant (L-amino acid) for a binding site in the germination-trigger-receptor site or for an active site on an enzyme involved in the germination process (Sofos, 1989). The addition of sorbate (50 to 1000 μg/ml, depending on pH) to the germination medium resulted in reduction of germination rates at low amino acid concentrations (Sofos, 1989). As the concentration of amino acid increased, the positive effect of sorbate on germination decreased. However, sorbates may act at a site or stage different from the D- and L-alanine binding sites (Sofos, 1989). Researchers have stated that there may be a mechanism of inhibition of reactions involved in early stages of germination that may be metabolic in nature, i.e., reactions involving electron and cation transport, or lytic reactions. In addition, changes in membrane permeability that may occur during triggering of germination could be sites that are susceptible to sorbate interference (Sofos, 1989). According to other scientists, sorbate binds with L-alanine for a receptor site on the spore, then combines with L-alanine, making it unavailable for the spore, or inhibits an enzyme on the spore that initiates L-alanine metabolism (Sofos, 1989).

The involvement of cation transport is also unclear, however it has been suggested that sorbate may alter spore membrane permeability, and thus, inhibit essential cation transport functions. Since triggering of spore germination may alter the permeability of inner spore membranes, sorbate may be acting on monounsaturated fatty acids, gases, alcohols and carbon dioxide, and inhibiting germination by interacting with spore membranes in a manner that increases membrane fluidity (Davidson and Juneja, 1990; Sofos, 1989).

Sorbate inhibits emergence of vegetative cells from spores during outgrowth. Additionally, a few studies have demonstrated inhibition of cell division in spore-forming bacteria. Against germinating endospores, sorbate prevents outgrowth of vegetative cells (Jay, 2000). Studies using agar microslides indicate that 0.015 to 0.05% sodium sorbate at pH 6.0 allowed emergence and elongation of vegetative cells from *Bacillus* spp. spores, but prevented multiplication of these cells (Davidson and Juneja, 1990).

Sorbate may also inhibit lytic enzymes such as hexoseaminidase, which are involved in initial stage of germination by causing cortex hydrolysis in vitro. Sorbic acid has also been reported to be weakly genotoxic. Although sorbate displays no mutagenic activity, it may form mutagenic reaction products with sodium nitrite or sulfur dioxide (Davidson and Juneja, 1990; Sofos, 1989).

Interaction with Juice Components

Sorbic acid has been shown to be more effective in lower pH foods, because the majority of its antimicrobial activity is present when the acid is in the undissociated form (Task Force, 1998). Antimicrobial activity of sorbate increases as the pH value of the food decreases toward the pKa of 4.76, thus, sorbate is more effective as a preservative in food products with lower pH (< 6.5) values (Sofos, 1989; Sofos and Busta, 1993; Task Force, 1998). Increasing inhibition by sorbate with reducing pH has been reported for several species of microorganisms, including *B. subtilis*, *C. botulinum,* and *S. aureus* (Sofos, 1989). Smoot and Pierson reported that potassium sorbate was a strong inhibitor of bacterial spore germination at pH 5.7 but it was much less inhibitory at pH 6.7 (Davidson and Juneja, 1990). Although the undissociated form of the acid is known as the highly active species, microbial inhibition also has been attributed to the dissociated molecule; but its activity is 10 to 600 times less than that of undissociated acid (Davidson, 1993b; Task Force, 1998).

Interactions of sorbate with heat may also affect the rate and extent of microbial destruction during heating as well as dormancy and recovery of heated microbes (Sofos and Busta, 1993). Sorbates become volatile above 60°C, so in pasteurized juice, sorbates should be added after pasteurization to avoid loss of the antimicrobial (Davidson and Juneja, 1990; Sofos, 1989).

The solubility of sorbic acid in water also increases with temperature, however, potassium sorbate may be preferred due to its high solubility in water, (58.2% at 20°C) (Davidson and Juneja, 1990). Although the potassium salt is used to increase solubility, the activity of the salt is 74% compared to the acid (Sofos, 1989). Sorbic acid is more soluble in lipid materials than in water and its characteristic of increased solubility with increasing pH is the result of partial conversion of sorbic acid to its more soluble salts (Davidson and Juneja, 1990).

In general, losses of sorbate are slower in solid food than in aqueous solutions. In pasteurized fruit juices (e.g. apple, black currant, and grape) decomposition of sorbic acid was <3% after 320 days at 20°C, while initial decomposition in aqueous solutions under the same conditions was 25% and 80% after 5 months storage, respectively (Davidson and Juneja, 1990; Sofos, 1989; Task Force, 1998).

Salt, sugars, and other soluble food components will also reduce the concentration of sorbate in the aqueous phase. The synergistic activity of sorbate with sugar and salt, however, may offset the negative effects of these solutes on the solubility of sorbate (Davidson and Juneja, 1990). In high sugar products, such as jams and jellies, a smaller quantity of sorbates are needed due to the synergistic effect of sorbate with sugar (Chipley, 1983).

Presence of acids may reduce the water solubility of sorbate, but they enhance the microbial inhibition by increasing the amount of undissociated sorbic acid, as a result of increasing the hydrogen ion concentration, and by their own direct antimicrobial effects. Citric and lactic acids enhance the antimicrobial action of sorbate to a greater extent than inorganic acids. Sorbic, boric acid, and sodium formate tended to be antagonistic when used in combination with other preservatives, while sodium sorbate tended to be synergistic (Davidson and Juneja, 1990).

Sorbic acid is more suitable for fruit products because of the organoleptic properties of sorbic acid (neutral taste)(Davidson, 1993b). However, combinations of sorbic acid and sulfur dioxide are commonly used in the preservation of high pulp fruit juices. In these products, sorbic

acid acts as the microbial inhibitor, and sulfur dioxide prevents oxidation and enzymatic spoilage. Sorbates may be used in combination with benzoates or propionates in foods of reduced pH. This is done to minimize off-flavors by using lower concentrations and to broaden the scope of microorganisms inhibited (Davidson and Juneja, 1990).

Previous Work

Sorbic acid was found to be superior to sodium benzoate in the preservation of apple juice. Ascorbic acid, however, was more inhibitory than potassium sorbate to microbial growth in fruit juices because it had a greater effect in reducing product pH (Sofos, 1989). Fruit products may be preserved more effectively with combinations of sorbate and ascorbate (Davidson and Juneja, 1990).

Studies have shown that 0.1% - 0. 2 % of sorbic acid could inhibit *B. subtilis* germination in media, but more than 1.0% was needed for inhibition in food (Sofos, 1989). In media, pH 6.0, a concentration > 0.04% caused inhibition but ≤ 0.05% did not inhibit *B. subtilis*. Potassium sorbate at 0.05, 0.1 and 0.15% delayed initiation of growth, prevented spore germination, and decrease the rate of growth of *P. patulum* in potato dextrose broth at 12°C (Sofos, 1989).

Little research has been performed with sorbic acid on *A. acidoterrestris*. While Splittstoesser et al. (1998) stated that levels as high as 100 ppm of sorbic acid did not cause heat sensitivity to *A. acidoterrestris* spores. According to Pettipher (2000), sorbic acid (300 mg/ml) reduced *A. acidoterrestris* vegetative cells from 580 CFU/ml to <100 cells/ml in 10 days and reduced 680 spores/ml to 145 spores/ml in 3 months in juice.

Trans-Cinnamic Acid

Chemical Description and Approval

Trans-cinnamic acid (3-phenylpropenoic acid) is a phenolic compound that is present in cranberries, prunes, cloves, cinnamon, and other plant parts that are used as spices (Anslow, 2000; Dorantes et al., 2000; Task Force, 1998). Cinnamic acid is a weak acid with a pKa of 4.37 to 4.44 (Anslow, 2000). Cinnamic acid and its derivatives are found in fruits, vegetables, tea, molasses, and other plant sources. All of the cinnamic acid-related compounds show antibacterial and antifungal activity (Davidson, 1993a; Jay, 2000). It has been hypothesized that cinnamic acid

is present in plants and fruit to provide protection against infections by pathogenic microbes (Chambel et al., 1999).

Cinnamic acid has been commonly used as a flavoring agent at concentrations of 25 to 600 ppm in cakes, drinks, ice cream, dressings, marinades, margarines, condiments, meat and dairy based foods, and chewing gum. However, it is also an effective antimicrobial agent (Anslow, 2000; Cirigliano, 2000a; Cirigliano, 2000b; Cirigliano, 2000c). It has been reported that 50 to 500 ppm of cinnamic acid and from 50 to 5,000 ppm of organic acids are used to preserve daikon (Japanese pickles), Japanese fish products, Vienna sausage, mixed bean paste and flour paste. Cinnamic acid contributes to the quality of beverages and may be used alone or in combination with mild heat treatments or reduced levels of traditional chemical preservatives such as sorbic and/or benzoic acid and their salts (Cirigliano, 2000b). Since cinnamic acid is derived from cinnamon, it gives foods a mild resinous odor similar to honey or flowers, and has a sweet, slightly spicy taste, although no flavor effect is observed when used at 10 ppm or below (Anslow, 2000). Although there is no established legal limit for cinnamic acid, in tea, the reported flavor usage within the industry is 31 ppm because at this level the flavor impact becomes undesirable (Anslow, 2000).

Cinnamic acid was given a GRAS status by FEMA (Flavoring Extract Manufacturers Association) in 1965, and as a flavoring agent in food by the Food and Drug Administration, however it has not been approved by the Food and Drug Administration as an antimicrobial in foods (Anslow, 2000; Food and Drug Administration, 2002a).

Mechanism of Action

Cinnamic acid and related compounds are effective against yeasts, molds, and spoilage bacteria in ready-to-drink tea beverages, tea beverages containing juice, fruit or vegetable extracts and/or other flavors (Cirigliano, 2000b). Cinnamic acid derivatives, such as caffeic, ferulic, and p-coumaric acids, have been shown to inhibit *E. coli*, *S. aureus*, and *B. cereus* (Task Force, 1998). They also may inhibit wine and other fruit fermentations caused by *Saccharomyces cerevisiae* (Chambel et al., 1999).

Like other lipophilic weak acids, its mechanism of action may result from the stimulation of passive influx of protons across the plasma membrane by causing an increase in membrane permeability. Antimicrobial activity of these compounds may also rely on the dissociation of the liposoluble acidic form in the cytoplasm, leading to additional acidification of the cell interior

(Chambel et al., 1999). Trans-cinnamic acid inactivates bacteria by transporting across the cytoplasmic membrane in the undissociated form and dissociating inside the cytoplasm to cause a decrease in the internal pH of the cell leading to the inhibition of cellular enzymes (Anslow, 2000; Cirigliano, 2000c). Unlike benzoic and sorbic acid, cinnamic acid has an unsaturated side chain that prevents cinnamic acid from being pumped out of the cell. The efficacy of this side chain increases with the length of the side chain and the number of reactive double bonds (Cirigliano, 2000a).

Another suggested mechanism is the inhibition of phenylalanine and tyrosine utilization (Anslow, 2000). This can significantly affect the activity of many crucial enzymes, leading to cell death. With regard to *Listeria*, cinnamic acid may inhibit enzymes involved in glucose uptake and ATP production (Dorantes et al., 2000).

Interaction with Juice Components

Trans-cinnamic acid is effective at both ambient and refrigerated temperatures, however its efficacy is unknown at higher temperatures (Cirigliano, 2000b). Cinnamic acid is best applied to food prior to the addition of an acidulant. If cinnamic acid is added after an acidulant, it tends to precipitate out and there is consequential loss of activity (Anslow, 2000).

The combination of cinnamic acid and benzoic acid is favored when the pH of the tea is less than pH 3.0. Whereas, the combination of cinnamic acid with sorbic acid is recommended when the pH is less than pH 3.4 (Anslow, 2000). It has been reported that the combination of cinnamic acid with another organic acids (e.g., citric, acetic, malic, fumaric, sorbic, tartaric, or lactic) results in a synergistic antibacterial action (Anslow, 2000).

Since cinnamic acid is soluble in essential oils but not in tea, a solubility-enhancing step may be needed before adding this compound to tea. This may be performed by spray drying the cinnamic acid onto a carrier powder which may be sugar based, for example, and adding the powder to tea. Other methods to increase solubility include converting the acid to its salt, or dissolving the cinnamic acid in a small quantity of organic solvents such as ethanol, or propylene glycol (Anslow, 2000).

Previous Work

According to Lipton, trans-cinnamic acid has been shown to inhibit yeasts, molds, acid tolerant bacteria, thermophilic spore formers (i.e., *Bacillus coagulans* and *Alicyclobacillus*) and

non-spore forming bacteria at 25 to 600 ppm alone or in combination with sorbic and benzoic acid (Cirigliano, 2000c). It has been shown that cinnamic acid and m-coumaric acid found in peppers inhibit *Listeria monocytogenes*, *Staphylococcus aureus*, *Salmonella* Typhimurium and *Bacillus cereus* (Dorantes et al., 2000).

When a tea beverage at pH 3.0 and pH 2.8 was preserved with cinnamic acid (125 ppm) and EDTA (30 ppm), there was no spoilage detected 14 weeks. No tea spoilage was observed within 14 weeks when 200 ppm of benzoic acid (or 100 ppm benzoic and sorbic acid combined) was combined with 100 ppm cinnamic acid and 30 ppm of EDTA at pH 2.8 (Anslow, 2000; Cirigliano, 2000b). Nagel and Baranowsli et al. (1980) suggested that allyl hydroxycinnamates, compounds that are quite similar to the phenolic derivatives, found in allspice (eugenol), clove (eugenol), cinnamon (cinnamic aldehydes and eugenol), oregano (carvacol), thyme (thymol), and savory (thymol) spices inhibited the growth of S. *cerevisiae* and *Psuedomonas florescens* (Davidson, 1993a).

Sodium Metabisulfite

Chemical Description and Approval

Sodium metabisulfite has a chemical formula of $Na_2S_2O_5$, and is commonly used in the salt form due to its high solubility in water. Sulfur dioxide, the active antimicrobial form, is a colorless gas that was first used to treat wines in Rome and was used to treat cider in the 17th century (Davidson and Juneja, 1990).

Sulfites are commonly used as antimicrobials in dried fruits, fruit juices, wines, sausage, fresh shrimp, and acid pickles, but also protect against browning reactions and chemically induced color changes (Davidson and Juneja, 1990). Generally, sulfur dioxide is used at 0.01% to 0.2 % to temporarily preserve fruit products and at 50 to 100 ppm in grape juices used for wine. Sulfites are allowed in vegetable juices at a level of 100 ppm and are allowed at 1,000 ppm in fruit juices. Suggested levels of sulfur dioxide in juice concentrates are from 350 to 600 ppm sulfur dioxide (Furia, 1986; Zoecklein et al., 1995). In 1986, the FDA required labeling on any product containing more than 10 ppm sulfites (Davidson and Juneja, 1990; Ough, 1993b; Warner et al., 2000). In addition, sulfites may not be legally used in meats or raw fruits or vegetables (Davidson and Juneja, 1990; Food and Drug Administration, 2001e). The maximum level of sulfur dioxide allowed in wine was set at 350 mg/L by the regulating body for the US alcoholic

beverage industry and Bureau of Alcohol, Tobacco, and Firearms. The oral lethal dose (LD_{50}) is 1000 to 2000 mg sulfur dioxide per kilogram body weight (Davidson and Juneja, 1990; Zoecklein et al., 1995).

Mechanism of Action

Potassium metabisulfite is most effective at a pH below 4.0, because of the more active, undissociated form (Ough, 1993b). Although the undissociated acid contributes the majority of the antimicrobial activity, the bound portion of sulfur dioxide at levels greater than 50 ppm may be inhibitory to certain bacteria including lactic acid bacteria (Zoecklein et al., 1995). The sulfites are used primarily in fruit and vegetable products to control three groups of microorganisms: acetic acid producing and malolactic bacteria, fermentation and spoilage yeasts, and molds on fruits (Davidson and Juneja, 1990; Wedzicha, 1984). It has been shown to inhibit juice and wine bacteria, yeasts, and molds, however yeasts and molds are more resistant to this antimicrobial (Ough, 1993b; Zoecklein et al., 1995). Sulfur dioxide is reported to be more inhibitory to Gram-negative rods such as *E. coli* and *Psuedomonas* as compared to Gram-positive rods (Davidson and Juneja, 1990).

Sulfur dioxide is not only used as an antimicrobial but also has other functions such as protection against oxidative, enzymatic, and non-enzymatic browning reactions and inhibition of chemically induced color loss. Sulfur dioxide is also used to sanitize equipment. In wine, the concentration of sulfur dioxide used depends on the cleanliness, maturity, and general condition of grapes, but 50-100 ppm is generally employed (Davidson and Juneja, 1990).

Growth inhibition and lethal effects of sulfur dioxide are most intense when the acid is in the molecular free (un-ionized) form. Bound forms of sulfur generally have lowered antimicrobial activity. In grape juice, bound forms of sulfurous acid have about $1/30^{th}$ the antimicrobial effectiveness of the free form of sulfur dioxide (Ough, 1993b; Zoecklein et al., 1995). Against some bacteria, concentrations of 1-2 ppm are bacteriostatic, but higher concentrations are needed to be bactericidal. Levels of 1-10 μg/ml sulfite prevent lactic acid bacterial spoilage in fruit products at pH 3.5 or less (Davidson and Juneja, 1990; Ough, 1993b).

The antimicrobial action of sulfur dioxide is based on its interference with various cell components (Davidson and Juneja, 1990). Cell damage may occur from the interaction with sulfhydryl groups and linkages in the structural proteins and interactions with enzymes,

cofactors, vitamins, nucleic acids, and lipids (Furia, 1986). Sulfur dioxide cleaves disulfide linkages in proteins and changes the molecular confirmation of enzymes, which modifies the active site or destroys its coenzymes. Sulfites may also damage cell metabolism and membrane function by peroxidizing lipids. One or more of these factors may result in inhibition or death of the microbial cell (Ough, 1993b; Davidson and Juneja, 1990).

Sulfur dioxide may also react with end products or intermediate products and inhibits enzyme chain reactions. It destroys the activity of thiamine and thiamine-dependent enzymes by cleavage and produces cytotoxic effects by cross-linking individual nucleic acid residues or nucleic acid residues and proteins. Sulfur dioxide may react with RNA leading to interference in protein synthesis resulting in inhibition of growth, and in *B. subtilis*, its mechanism of action may result from DNA breakdown (Wedzicha, 1984; Zoecklein et al., 1995). It has also been suggested that the antimicrobial action of this compound is due to its strong reducing power that results in a reduction in oxygen tension to a point below that of which aerobic microorganisms can grow.

Interaction with Juice Components

In juices, the total amount of bound sulfur dioxide depends on pH, temperature, and the amount of sulfite added (Wedzicha, 1984). Binding of sulfites can also be dependent on pH and temperature. A gradual increase in binding capacity of citrus juices can be prevented by avoiding temperatures above 30°C during processing or storage (Wedzicha, 1984). However, the addition of heat caused the D-value to be divided by half for *Bssoychlamys* with sulfur dioxide (Wedzicha, 1984). Equilibrium of sulfur dioxide of binding is generally dependant on pH (Wedzicha, 1984). Potassium metabisulfite is most effective at pH below 4.0, because it is most able to transfer across cell membranes when it is undissociated (Ough, 1993b). Researchers found that at pH 3.5, two to four times as much sulfur dioxide was needed to inhibit growth as compared to pH 2.5. Enhanced effectiveness at low pH may result from more extensive penetration of the cell wall (Furia, 1986).

Sulfites react with various food constituents including nucleotides, sugars, disulfide bonds, aldehydes, proteins, and aldo-sugars (Jay, 2000; Zoecklein et al., 1995). Binding of sulfite by glucose affects its bacterial activity since “bound” sulfite is not effective as a preservative. Approximately 50-70% of total sulfur dioxide may be bound by sugars present in

fruit juices (Zoecklein et al., 1995). Sulfur dioxide is bound by sugars in decreasing order of: arabinose > glucose > maltose > lactose > fructose > and saccharose. However, the equilibrium constant for reactions between sulfur dioxide and glucose is lowest between pH 3.0-5.5 (Wedzicha, 1984). Glucose is by far the most abundant source of reactive aldehydes and ketones in most fruit juices with which sulfur dioxide reacts. Compared to model solutions, natural fruit juices always bind more sulfur dioxide than would be calculated from the glucose present in the juice (Ough, 1983b). Higher levels of sulfur dioxide may be needed if numerous amounts of sugars or other sulfur dioxide binding materials are present (Furia, 1986).

Sulfur dioxide may also react with other food constituents such as the oxygen and phenol content in juice. Sulfur dioxide may also bind with aldehydes and ketones at a much faster rate compared to sugars (Zoecklein et al., 1995). Additionally, loss of free sulfur dioxide is affected by the amount of dissolved oxygen present, with the loss of free sulfur dioxide being proportional to the amount of dissolved oxygen (Zoecklein et al., 1995). Furthermore, sulfur dioxide can loosely bind to anthocyanins found in the juices (Ough, 1983b; Zoecklein et al., 1995).

Sulfur dioxide may also interact with other preservatives added to juice products. Antimicrobial efficacy of sulfur dioxide may be reduced when sorbic acid and sulfur dioxide are added to juices simultaneously, however this effect may be synergistic in grape juice (Jay, 2000; Wedzicha, 1984). It has also been suggested that sulfites may inhibit the antimicrobial activity of cinnamic aldehyde (Wedzicha, 1984).

Previous Work

The addition of sodium metabisulfite (a source of sulfur dioxide) delayed *C. botulinum* outgrowth in perishable canned, commuted pork that was temperature abused at 27ºC (Wedzicha, 1984). Levels above 120 mg/L of total sulfur dioxide decreased the incidence of malolactic fermentation, with lower pH values increasing the effectiveness of the sulfur dioxide. Orange juice treated with 84 ppm sulfur dioxide combined with 360 ppm benzoic acid or 632 ppm sorbic acid had better protection against microorganisms and color darkening than juice treated only with 300 ppm sulfur dioxide (Schroeter, 1966). In wine, a 10,000-fold reduction of lactic acid bacteria and other spoilage microbes was achieved when 0.8 ppm molecular free sulfur dioxide was present (Zoecklein et al., 1995). Approximately 10 ppm sulfur dioxide was

lethal to *Leuconostoc*, however *Lactobacillus* and *Pediococcus* were shown to be more tolerant to sulfur dioxide (Zoecklein et al., 1995).

There currently is little research relating to the antimicrobial ability of sodium metabisulfite on *A. acidoterrestris*. Splittstoesser et al (1998) stated that sulfur dioxide did not sensitize *A. acidoterrestris* spores to heat at 100 ppm and below. However, Walls and Chuyate (2000) stated that *A. acidoterrestris* spores at 4 log spore/ml did not grow in white grape juice that contained metabisulfites, but in grape juice without metabisulfite, spores germinated within 1-2 weeks when inoculated at levels 2 and 4 log spores/ml into juices.

Lysozyme

Chemical Description and Approval

Lysozyme is an enzyme that is believed to function as an endogenous antimicrobial substance in bacteria, fungi, plants and almost all animal tissues. The highest levels were found in secretions, including milk, mucus, saliva, and tears, and eggs (Food Task Force, 1998). Lysozyme has also been found in ficus and papaya latex, in hen egg whites (lysozyme c), and domestic goose hen eggs (lysozyme g) (Worthington Biochemical Corporation). Lysozyme accounts for approximately 3.5 percent of the total protein of the domestic hen egg whites and exists at a level of 2,250 - 3,270 ppm (Davidson et al., 1993b; Food and Drug Administration, 2001c). Lysozyme from a hen egg is a single polypeptide of 129 amino acids, with a molecular weight of approximately 14,700 Da, and is linked in four places with disulfide bridges (Davidson et al., 1993b).

Lysozyme has many characteristics that make it ideal for use as an antimicrobial in foods. It has little or no toxicity in humans, doesn't cause color, flavor, or aroma changes, low concentrations are effective, is economical to produce, remains stable under acidic and high temperatures, and has a very specific target (i.e., peptidoglycan) in bacteria (Task Force, 1998). Lysozyme has probably been the most widely applied to foods, specifically for its preservative properties, although its use in the food industry is still limited (Davidson et al., 1993b). Potential applications include its use in heat-sterilized products to reduce thermal requirements, and its use in foods such as poultry, shrimp, sausage, and sake as a preservative (Hughey, 1987). Lysozyme is also used to extend shelf life of a variety of processed foods including pickles, dairy products, meats, fresh vegetables, tofu, fresh fruit, fish surfaces, wine and sake, sausage, and seafood

(Daeschel, 1999; Task Force, 1998). According to Inovatech (2002), lysozyme is effective against spoilage and pathogenic bacteria in beer, wine, bread, canned foods, cheeses, meat, and rice (Davidson et al., 2002).

Lysozyme received GRAS approval from the FDA in March 1998 for use in cheese production to prevent gas formation and cracking caused by butyric forming *Clostridia*, however due to allergen issues the declaration of egg whites must be put on the label when lysozyme is added as an ingredient (Food and Drug Administration, 2001b). Cheese manufactured with egg white lysozyme contains a maximum of 400 ppm of lysozyme, at least 8 times less than eggs on a weight/weight basis. However, proteins derived from egg whites do raise allergenicity concerns, especially in children. This does not however establish that ingestion of egg white lysozyme in cheese will actually cause a clinically significant allergic reaction (Food and Drug Administration, 2001b). The International Office of Vine and Wine, has approved lysozyme in wine up to a level of 500 ppm.

Mechanism of Action

Lysozyme is effective against *Micrococcus luteus*, as well as nonpathogenic strains of *Clostridium, Lactobacillus, Bacillus, Bifidobacterium, Corynebacterium,* and *Streptococcus*. Of the Gram-positive bacteria, *Micrococcus* and *Sarcinia* are more susceptible to lysozyme than *Lactobacillus* and *Bacillus* (Conner, 1993; Davidson, 1993b; Task Force, 1998). Thermophilic, flat sour bacteria, such as *B. stearothermophilus*, are perhaps sensitive because the structure of the cell walls contributes to their high sensitivity to lysozyme (Hughey, 1987). Gram-positive bacteria are more susceptible to the action of lysozyme than Gram-negative bacteria, which contain a lipoprotein-lipopolysaccharide layer that limits access of lysozyme to polysaccharide layers of the cell wall (Conner, 1993; Davidson et al., 1993b).

Lysozyme is attractive as a preservative because of its specificity of action toward bacterial cell wall components (Davidson et al., 1993b). Lysozyme hydrolyzes preferentially the β-1, 4 glucosidic linkages between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG), which exists as 90% of the peptidoglycan in the cell wall structure of Gram-positive microbes (Task Force, 1998; Worthington Biochemical Inc.). This hydrolysis can damage the cell wall and result in lysis of the cell (Liang, 2002). Lysozyme leaves a punctured cell wall, which may lead to cell lysis in hypotonic media (Task Force, 1998).

Lysis of several Gram-negative pathogenic and spoilage bacteria was enhanced and consistently obtained when lysozyme was used in combination with EDTA. EDTA may allow partial removal of the lipopolysaccharide layers and promote penetration of lysozyme into peptidoglycan (Hughey, 1987). Compounds acting in synergy and enabling lysozyme to penetrate the peptidoglycan substrate enhance the activity of lysozyme against bacteria (Task Force, 1998)

Interaction with Juice Components

Lysozyme can withstand boiling for 1 to 2 minutes at acidic pH and no loss at pH 5.0 when heated at 65°C for 1 hour (Task Force, 1998). Lysozyme also resists high temperatures (up to 80°C) at acidic pH, although its optimum activity occurs at pH 6.0 and at 55-60°C (Lagarde, 1997). Egg white lysozyme remains stable despite exposure to a number of food processing activities. Ibrahim et al., (1996) found that lysozyme subjected to reduced activity through heating at 80°C at pH 6.0 still exhibited strong bactericidal activity against Gram-negative and Gram-positive bacteria suggesting action is dependent on catalytic functions (Davidson et al., 2002). Lysozyme may also bind or be sequestered by food components, which can decrease its activity; therefore it may be most useful in rather homogenous food systems such as juices and alcoholic beverages (Task Force, 1998).

Previous Work

Approximately 20 ppm of lysozyme inhibited *B. stearothermophilus* growth for 7 days and was completely inactivated by lysozyme at 70°C (Hughey, 1987). Since lysozyme has excellent heat resistance at low pH, it may be possible to reduce the thermal energy requirements during canning by including lysozyme in the process.

Although, lysozyme is typically ineffective on Gram-negative bacteria, in pasteurized orange juice, lysozyme decreased *S.* Typhimurium by 1.4 to 3 log CFU/ml. In pasteurized orange juice, lysozyme and nisin combination decreased *S.* Typhimurium by 1.8 to 3.2 log CFU/ml. In unpasteurized juice with pulp, lysozyme decreased the microorganism by 0.08 to 0.18 log CFU/ml, while the combination of nisin and lysozyme decreased it by 0.12 to 0.18 log CFU/ml. In unpasteurized juice without pulp, lysozyme decreased growth by 0.5 to 0.20 log

CFU/ml and in combination with nisin by 0.1 to 0.2 log CFU/ml. The effect of pasteurization tended to increase the log decrease (Liang, 2002).

The addition of lysozyme to media containing *A. acidoterrestris* contributed to the reduction of thermal resistance in citrate buffer at pH 4.0. *A. acidoterrestris* spores in the presence of 10 ppm lysozyme were decreased to about 70% as compared to that of the control when heated to 89ºC (Yamakazi, 1997)

Dimethyl Dicarbonate

Chemical Description and Approval

Dimethyl dicarbonate was first synthesized in 1952 by Kovalenko (Ough 1983). Dimethyl dicarbonate is a dicarbonic acid ester that is a colorless, fruity smelling liquid. It has a melting point of 15.2˚C, and a boiling point of 123-149˚C (Fisher and Golden, 1998; Ough, 1993a).

The acute toxicity of dimethyl dicarbonate is 330-900 mg/kg body weight. However, Ames mutagenicity tests have been negative and the compound has been shown to be safe using short-term toxicological studies (Ough, 1993a). Furthermore, no adverse effects were observed after feeding juice and alcoholic beverages to mice with 400 ppm of dimethyl dicarbonate for 3 months (Ough, 1993a). However, in alcohol, the ethanol reaction product, ethyl methyl carbonate, must not exceed 2 mg/L in treated wine (Ough, 1993a).

Dimethyl dicarbonate is approved (Food and Drug Administration, 2002b) as an inhibitor of yeast in wine, in ready-to-drink teas at ≤ 250 ppm, in carbonated or non-carbonated, non-juice containing, flavored or unflavored beverages with electrolytes at ≤ 250 ppm, and in carbonated, dilute beverages containing juice, fruit flavor, or both with less than 50% juice at ≤ 250 ppm and more recently in single-strength 100% juice. On June 20, 2001, Bayer showed that Velcorin meets U.S. FDA specifications in non-carbonated juice beverages containing up to and including 100% juice (Bayer News, 2001). However, the label must indicate that “dimethyl dicarbonate” is added (Davidson et al., 2002; Food and Drug Administration, 2002b).

For more than 10 years, Velcorin® has been used to protect alcoholic and nonalcoholic wines against lactic acid bacteria, and is currently used to protect juice sparklers, sports drinks, and ready-to-drink teas. Velcorin provides the option to reduce, or in some cases eliminate the need for other chemical preservatives in non-carbonated juice beverages as well (Bayer

Corporation, 2001). Velcorin not only helps to eliminate microbes coming from various ingredients of juices, but also helps to eliminate contamination from other sources once it contacts bottles, cans, closures, and filling equipment (Bayer News, 2001).

Mechanism of Action

Velcorin® is a microbial control agent that controls a wide range of microbes, including a variety of yeasts, molds, and bacteria. The antimicrobial activity produced by dimethyl dicarbonate is believed to be related to the inactivation of enzymes (Ough, 1978; Ough, 1983a). Protein modification, through reaction of nucleophilic groups such as imidazole, amines or thiols, can readily occur with the dicarbonate. The point of attack is one of the central carbon atoms. The residual portion of the dicarbonate is unstable and decomposes rapidly (Ough, 1993a). Upon hydrolysis, the compound yields methanol and carbon dioxide (Jay, 2000). Enzyme inhibition results from active site blocking and conformational changes. Diethyl dicarbonate (DEDC), a compound that is similar to dimethyl dicarbonate, has been hypothesized to inhibit nuclease, alcohol dehydrogenase, and the enzyme lactase dehydrogenase (Ough, 1983a). Dimethyl dicarbonate is an effective inactivator of several glycolytic enzymes (Duadt and Ough, 1980). In yeast, the reaction with a histidine molecule is extremely fast. If this reaction occurs at an enzyme activity site, it can inactivate the enzyme and may also cause conformational changes of the enzyme. If any of the enzymes that the yeast needs to survive are inactivated, then the yeast will die (Duadt and Ough, 1980).

Interaction with Juice Components

Dimethyl dicarbonate is reported to react with several food components including amino acids, ammonia, primary amines, secondary amines, polyphenols, and ascorbic acid (Bizri and Wahem, 1994; Ough, 1993a). Reactions of dimethyl dicarbonate with phenols, phenol glucosides, organic acids, and with vitamins may also be significant (Ough, 1975; Ough, 1983a). Dimethyl dicarbonate hydrolyzes rapidly upon reaction with a number of substances including, water, alcohols, phenols, thiol groups, carboxylic acids alkyl and aromatic amines, and sulfhydryl groups (Davidson et al., 2002; Ough et al., 1978). However, it was reported that dimethyl dicarbonate did not become hydrolyzed in the presence of sugars or artificial sweeteners (Fisher and Golden, 1998).

The hydrolysis rate of dimethyl dicarbonate is dependent on temperature, the higher the temperature, the more rapid the rate of hydrolysis. In contrast, Turtura (1966) found that increased temperature increased the effectiveness of diethyl dicarbonate as a sterilant, it was twice as effective at 27°C as at 0°C (Ough, 1983a). Dimethyl dicarbonate is only slightly soluble in water but has greater solubility in organic solvents (Ough, 1993a). DEDC is only slightly soluble in water, with a solubility of about 0.6g/100 g at 18°C. The compound also hydrolyzes rapidly in water (Ough, 1983a).

Van Zyl (1962) noted the synergistic effect of sulfur dioxide with DEDC (Ough, 1983a). However, Fisher and Golden (1998), reported that an increased amount of dimethyl dicarbonate was needed to inactivate *E. coli* O157:H7 in apple cider when combined with sodium bisulfite. Ough (1983) reported that diethyl dicarbonate reacts with malic acid.

The addition of dimethyl dicarbonate resulted in decreasing availability of beta carotene, which may have diminished its protection against oxidation of ascorbic acid (Bizri and Wahem, 1994). When 250 ppm dimethyl dicarbonate was inoculated into tomato juice the juice became darker (smaller L), more yellow (larger hue angle), and less vivid color (smaller chroma) than juice treated with sorbate or benzoate (Bizri and Wahem, 1994).

Previous Work

While dimethyl dicarbonate is most commonly used as a fungicide, dimethyl dicarbonate has been shown to be bactericidal to *Lactobacillus buchneri* at 30 ppm, 20 ppm for *Lactobacillus brevis*, and 30 ppm for *Lactobacillus* and *Pediococcus cerevisiae*. Diethyl dicarbonate, which has a similar bactericidal activity to dimethyl dicarbonate, is considered to be bactericidal for *B. megatherium* and *B. mediosporum* and at 3,000 ppm is bactericidal for *B. subtilis* (Ough, 1993a). Dimethyl dicarbonate is bactericidal at 30-400µg/ml to a number of species including *Acetobacter pasteurianus*, *E. coli*, *Psuedomonas aeroginosa*, *Staphylococcus aureus*, several *Lactobacillus* species, and *Pediococcus cerevisiae* (Davidson et al., 2002).

Approximately 30 ppm of dimethyl dicarbonate prevents yeast growth in wine (Ough et al., 1978). *Saccharomyces* are inhibited at 25 to 50 ppm of dimethyl dicarbonate in wine, however at high pH values (3.8 or above) dimethyl dicarbonate is not very effective without the addition of other antimicrobials (Ough et al., 1977). With the addition of 25 ppm free sulfur dioxide and 50 ppm dimethyl dicarbonate, yeast and bacteria in wine (pH of 3.6) may be properly controlled (Ough et al., 1978).

Dimethyl dicarbonate (250 ppm) was applied to tomato juice at pH 3.7 and stored at 5°C was more effective in decreasing aerobic plate count, mold and yeast counts, compared to addition of sorbate or benzoate in juices acidified to pH 3.7 and stored at 5°C and 20°C. However tomato juice that was treated with dimethyl dicarbonate had lower amounts of ascorbic acid, total amino acids, fructose, glucose, lycopene, and beta-carotene (Bizri and Wahem, 1994). In apple cider at 4°C and 10°C, dimethyl dicarbonate was found to be more effective than sodium bisulfite and sodium benzoate as an inhibitor of *E. coli* O157:H7 (Fisher, 1998).

Ascorbic Acid

Chemical Description and Approval

Ascorbic acid is a six carbon, monobasic acid also known as Vitamin C. Ascorbic acid was first isolated in the early 1920-30's in adrenal glands, cabbage, oranges, paprika, and lemons and was later found in other fruits and vegetables (Bauernfeind, 1982; Zoecklein et al., 1995). Ascorbic acid is water soluble, and found in the aqueous portion of the fruit, while apples contain more ascorbic acid in the skin compared to the pulp (Nobile, 1981)

Ascorbic acid is generally added to foods as a vitamin, but can also be added as a preservative, oxygen acceptor, an acidulant, to prevent non-enzymatic browning, or as an antioxidant (Bauernfeind, 1982). Vitamin C is commonly used in meat for curing and pickling, in beer as a stabilizer, in flour to improve baking quality, and in soft drinks and wine at 150-600 mg/L as a stabilizer and antioxidant (Bauernfeind, 1982). Toxicological studies indicate that 4 g/day can be safely consumed by humans. In the US, ascorbic acid is GRAS using GMP's when used as a nutrient or food processing aid (Bauernfeind, 1982; Kläui, 1974).

Mechanism of Action

Although ascorbic acid is not normally considered to be bacteriostatic, in aqueous solutions at higher concentrations, it confers to limited antimicrobial activity (Bauernfeind, 1982). Ascorbic acid does not have the same type of antimicrobial properties that other organic acids have. It generally inhibits microbes due to its sequestering ability or the ability to decrease available oxygen (Bauernfeind, 1982). Ascorbic acid is able to scavenge oxygen out of solution at a ratio of 3.4 - 3.6 mg/cm^2 of headspace of air. Ascorbic acid functions as an antioxidant with some substrates by protecting double bonds and scavenging oxygen (Bauernfeind, 1982).

Interaction with Juice Components

In absence of oxygen and other oxidizing agents, ascorbic acid is heat stable, especially at low pH. However, pasteurization reduces the ascorbic acid present in juice. Therefore, chemical preservatives such as sulfites are often used to protect Vitamin C from oxidation during pasteurization of juices (Farnworth et al., 2001; Nobile, 1981). Ascorbic acid is also more stable at pH 3-4.5 compared to pH 6.0-7.0 (Furia, 1968; Nobile, 1981).

Oxygen permeable containers accelerate degradation of Vitamin C in orange drink. Orange drink concentrate packaged in polyethylene lost 100% ascorbic acid in 1 year at room temperature (Furia, 1986). Commercial apple juice fortified with 30-60 mg/100ml juice retained 73-80% after 1 year at 70°F versus 54-60% at 85°F (Furia, 1986). Storage of commercial fruit juices in closed containers at room temperature for 4 months resulted in ascorbic acid loss ranging from 29 to 41% (Kabasaklis et al., 2000). Light exposure of bottled orange juice drink caused 35% loss in 3 months at room temperature (Furia, 1986).

Previous Work

When used alone, ascorbic acid is only weakly inhibitory to many microbes with the exception of *Psuedomonas* and *Enterobacteriacae* (Giannuzzi, 1993; Kläui, 1974). It has commonly been used to inhibit *C. botulinum* growth in cured meats when combined with nitrites due to the sequestering of metal ions in the meat. When used to inhibit total viable microbes, *Enterobacteriacae, Psuedomonas, Lactobacillus*, molds, yeasts, *Clostridium* sulfite reducers, psychotrophs, aerobic and anaerobic spores, 10,000 ppm of ascorbic acid showed antimicrobial action. In addition, 2,700 ppm citric acid combined with 2,000 ppm ascorbic acid displayed antimicrobial activity. When ascorbic acid was compared to citric acid and lactic acid at refrigeration temps (4 and 10°C) in media (pH 4), ascorbic acid produced higher inhibitory action on *L. monocytogenes* compared to both citric and lactic acid due to ascorbic acid's higher pKa value (Giannuzzi, 1996).

While little work has performed to study the inhibition of *Alicyclobacillus* by ascorbic acid, at least one study has shown that addition of 150 ppm ascorbic acid inhibited growth, while 100 ppm actually increased growth. Since ascorbic acid reduces the redox potential by

absorbing oxygen, decreased redox potential and increased absorbed oxygen levels in the juice caused by ascorbic acid may help to inhibit *Alicyclobacillus* in juices (Cerny et al., 2000).

RESEARCH OBJECTIVES

The objective of this study was to determine the growth characteristics of *Alicyclobacillus acidoterrestris* in different fruit juices and to examine whether antimicrobials could be used as an effective control for *A. acidoterrestris*. Four types of juices were analyzed: apple, grape, tomato, and orange juice to see if juice components may have had an impact on spore germination and vegetative cell growth. The time it takes to reach a spoilage taint level and whether the organism can grow over a period of time to increase taint compound formation during storage were analyzed.

Nisin, cinnamic acid, potassium sorbate, dimethyl dicarbonate, sodium benzoate, ascorbic acid, lysozyme, sodium metabisulfite were used in the second portion of the study because they either have been used effectively in beverages against spoilage bacteria, yeast or molds or have been used effectively against gram positive organisms. These antimicrobials were compared in apple and tomato juice at optimal growth temperature (42°C) to determine which antimicrobial and which level of antimicrobial are most effective in reducing spores and preventing spore outgrowth over a period of 30 days. These antimicrobials could be used to prevent spoilage in juices, juice containing beverages, and teas, or possibly in concentrates or other sources of *A. acidoterrestris*, such as liquid sugar.

RESEARCH OBJECTIVES:

1. Determine the optimum medium and diluent combination for enumerating *A. acidoterrestris* spores and vegetative cells.
2. Determine the growth rate and characteristics of *A. acidoterrestris* spores and vegetative cells in apple, white grape, orange, and tomato juices.
3. Determine most effective antimicrobials and the optimum level of antimicrobial to prevent spoilage and spore outgrowth of *A. acidoterrestris* in apple and tomato juices.

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Fate of *Alicyclobacillus acidoterrestris* in Apple, Orange, Grape, and Tomato Juices at an Optimum Growth Temperature

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KEYWORDS: *Alicyclobacillus acidoterrestris*, apple juice, grape juice, tomato juice, orange juice

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ABSTRACT

The purpose of this study was to determine the optimum medium and diluent combination for recovery of *Alicyclobacillus acidoterrestris* spores and vegetative cells. Using the optimum medium/diluent combination of Orange Serum Agar and peptone (pH 4.0), the fate of *A. acidoterrestris* spores and vegetative cells in apple, grape, orange, and tomato juices was also analyzed. Vegetative cell and spore suspensions of *A. acidoterrestris* were diluted in peptone (PN), peptone at pH 4 (PN4), or Bacillus Broth (BB) and spread plated onto Orange Serum Agar (OSA), Bacillus Agar (BAM), or K Agar (KA). All plates were incubated at 42°C for four days. In study II, orange, apple, tomato, and white grape juices were inoculated with either *A. acidoterrestris* spores or vegetative cells and incubated at 42°C. Juice samples were withdrawn every other day, for 30 days, surface plated to OSA and incubated at 42°C for four days.

In study I, the highest levels of spores enumerated were 3.73 log CFU/ml and 3.74 log CFU/ml when OSA with PN4 and KA with PN were tested, respectively. Enumeration of *A. acidoterrestris* spores combined with vegetative cells showed OSA with PN4 allowed the highest recovery, 6.35 log CFU/ml.

In study II, using only spores to inoculate juices, the growth of *A. acidoterrestris* in juices was in the following order: apple = tomato > orange = grape ($p<0.05$). Spores decreased below the detection limit after two and four days in grape and orange juice, respectively. *A. acidoterrestris* populations reached 5 log CFU/ml, an upper limit associated with taint compound formation, at day 8 for the spore only inoculant in tomato and apple juices. The growth of spores and vegetative cells in juice followed the order: tomato = apple > grape = orange juice ($p<0.05$). *A. acidoterrestris* populations in orange and grape juices decreased to below the detection limit after two days. *A. acidoterrestris* populations reached 5 log CFU/ml at day four and six for tomato and apple juices, respectively. Growth of *A. acidoterrestris* inoculated as spores and inoculated as spores and vegetative cells juice was not significantly different ($p=0.68$).

Study I results showed that OSA or K Agar and PN or PN4 could be effectively used to enumerate *A. acidoterrestris*. Study II results show that shelf-stable apple and tomato juices tested were susceptible to spoilage by *A. acidoterrestris*, with levels reaching 5 log CFU/ml *A. acidoterrestris* within four to eight days. Continued survival and growth over 30 days suggests

that taint compound formation may be increasing over a period of time, potentially resulting in increased detection of spoilage in the product by consumers. However, orange juice and white grape juices tested were not susceptible to spoilage by ATCC strain 49025 under the conditions tested.

INTRODUCTION

Historically, the process of pasteurizing juices at 85 to 95ºC for two minutes followed by the hot-fill-hold process has proven adequate in preventing spoilage of juices, with the occasional exception of spoilage caused by heat resistant molds. Additionally, few juice processors experienced problems with bacterial spoilage of juices since most bacteria are unable to grow below pH 4.0. However in 1984, Cerny and others isolated an acidophilic bacillus from spoiled, pasteurized apple juice. This spoilage microorganism was identified as *Alicyclobacillus acidoterrestris*. Subsequent studies have shown that *A. acidoterrestris* is able to withstand the pasteurization and hot-fill-hold process (85 to 95ºC for two minutes) applied to juices as well as the acidic environment characteristic of most fruit juices, that prevents other spoilage microorganisms from growing (Eguchi, 1999; Eiora et al., 1999).

The primary reservoir for *A. acidoterrestris* is soil, however, it has been isolated from washed and unwashed fruit, fruit juices, nectars, juice-containing beverages, canned diced tomatoes, liquid sugar used in beverages, "diet" fruit juices and drinks, condensate water used in finished juices, processing plants and equipment, and in a condensate recovery system that is used to make juice concentrates (Eguchi, 1999; Parrish, 2000; Splittstoesser, 1998; Vieria et al., 2000; Walls and Chuyate, 2000; Worobo and Churey, 1999).

A. acidoterrestris spoils juices by creating off-odors and off-flavors with occasional turbidity or sedimentation, although no gas is produced and no pH change occurs (Eguchi, 1999; McIntyre, 1995). Therefore, *A. acidoterrestris* presence in finished product is clearly undesirable due to the potential for spoilage, associated costs, and loss of brand quality image with consumers (Borlinghaus, 1997). The aroma and flavor of the spoilage compounds dibromophenol and guaiacol have been described as "antiseptic", "disinfectant-like", "nasty", "acidic", "putrid", "sweetish", "fermented" and "hammy" (Eguchi, 1999; Orr et al., 2000a; Pettipher, 2000)

Unpreserved apple and orange juice spiked with vegetative cells of *A. acidoterrestris* contained very high levels (6.5×10^4 CFU/ml) after 10 days (Pettipher and Osmundson, 2000). *A. acidoterrestris* spoilage compound formation generally occurs within 7 to 10 days at 25°C, however if the temperature is higher, spoilage rates may be dramatically higher (Brown, 1995). It has also been reported that *A. acidoterrestris* growth was observed at 37 and 45°C in 7 to 14 days. Incubating contaminated fruit juices at a temperature above 37°C may have a significant

increase in *Alicyclobacillus* by 14 days (Cerny et al., 2000). Spoilage of juice may occur when concentrates or juices are contaminated with less than ten spores per ml and stored at an elevated temperature for a period of time (35˚C for 1 week) (Evancho and Walls, 2001; Pinhatti, 1997).

Sensory tests have determined that an off-flavor or odor may be detected when approximately 1 x 10^3 CFU/ml spores are present, (Eguchi, 1999) while Pettipher and Osmundson (2000) stated that a level of 1 x 10^5 CFU/ml was required for taint production to be detected. However, populations of less than 1 x 10^2 may also cause taint (Gannon, 1998). Taint can be defined as the detectable level of the off-flavors and off-odors (guaiacol and 2,6-dibromophenol) by consumers.

Scientists have reported that several enumerating methods can be used effectively to enumerate *A. acidoterrestris*. Jensen (1999) showed that OSA with 0.5% added sucrose also performed well for enumerating *A. acidoterrestris*. However, according to Walls and Chuyate (2000), when *A. acidoterrestris* spores were inoculated onto K Agar (pH 3.7), semi-synthetic medium (pH 4.0), Orange Serum Agar (pH 3.5), and minimal salts medium (pH 4.0) and incubated at 24, 35, 43, and 55°C, the highest recovery of spores were obtained in the K agar or semi-synthetic medium incubated at 43°C (Walls and Chuyate, 2000).

Walls and Chuyate (1998) reported A. *acidoterrestris* growth in Orange Serum Broth at pH 2.5-5.0 over a temperature range of 20-55ºC. Deinhard et al. (1987), reported the optimum pH range for growth of the organism in *Bacillus acidocaldarius* medium (BAM) was 2.5-5.8 over a temperature range of 35-55ºC with an optimum temperature of 42-53ºC. McIntyre et al. (1995) reported *A. acidoterrestris* growth on Potato Dextrose Agar over a pH range of 3-5.3 at 30-55ºC.

According to Splittstoesser (1995), peptone may inhibit *Alicyclobacillus*. However, Jensen (1999a) stated that a suitable diluent is 0.1% peptone at pH 4 with 0.5% glucose, glycerol, or sucrose, with sucrose giving the highest recovery. In addition, Walls and Chuyate (2000) reported that peptone in K medium did not inhibit *A. acidoterrestris*. Jensen (1999a) reported that an acidified diluent is required containing a protectant to reduce the osmotic shock on *A. acidoterrestris* cells.

Although growth studies have been performed, little information exists on the growth patterns of *A. acidoterrestris* over time in fruit juices at optimum conditions. The objectives of this study were to determine the growth characteristics of *A. acidoterrestris* under optimum

conditions in fruit juices and determine the time required for the organism to reach concentrations where taint formation can occur. Additionally differences between spore only inoculum and inoculum that contained both spores and vegetative cells were also tested to determine if growth patterns differed. Finally, optimum media and diluent combination was determined and whether the effect of time of exposure to diluent has an effect on microorganism enumeration.

MATERIAL AND METHODS

Spore Stock Preparation

An *Alicyclobacillus acidoterrestris* (ATCC 49025) culture was streaked for isolation on Bacillus Agar (BAM; Walls and Chuyate, 2000) and grown at 42°C for 72 hours. A portion of culture was removed and examined microscopically for spores under phase-contrast. If at least 80% spores were present, the BAM plate was flooded with five mls of sterile, distilled water and the colonies were scraped off using a sterile rod to create a cell suspension. The suspension was placed into a sterile test tube and heated at 80°C for ten min to inactivate vegetative cells. Spore stock cultures were centrifuged at 11,951 x g for ten min followed by a sterile distilled water wash, followed again by centrifugation and washing three times. Finally, the spore pellet was re-suspended in sterile, distilled water and stored at 4˚C for a maximum of two months. Spore viability was assessed weekly via plating to ensure consistent inoculum levels.

Enumeration Media and Diluent Optimization

An *A. acidoterrestris* culture was grown in Bacillus Broth (BB**;** ATCC; Manassas, VA) (pH 4.0) at 42ºC for two to four days and examined microscopically for spores and vegetative cells. For the spore/vegetative (SV) enumeration, the culture was diluted in peptone (PN; Difco; Beckton-Dickson; Sparks, MD), peptone adjusted to pH 4.0 with 1N HCl (PN4), or BB and spread plated on K Agar (KA; Walls and Chuyate, 2000), Orange Serum Agar (OSA; Beckton-Dickson and Company; Sparks, MD), or BAM. To determine the optimum medium and diluent for spore only (SO) enumeration, the *A. acidoterrestris* culture in BB was heated at 80°C for ten minutes to inactivate vegetative cells. The plating method for SO was performed using the method above for the SV cell enumeration. All plates were incubated at 42°C for four days. To

determine if the culture remained viable in the diluent after 30 minutes of storage, the same procedure described above was used with the exception that only OSA was used and the diluted suspension was held in the diluents for 30 minutes before plating onto OSA.

Growth Characteristics of *A. acidoterrestris* in Juices:

Inoculum Preparation

For this experiment, commercial, shelf-stable juices were obtained from a local grocery store. All juices were preservative free with the exception of the tomato juice, which contained salt, and orange juice, which contained added ascorbic acid. For SO inoculation, the spore stock was batch inoculated into one liter of each of the four juices to achieve $1*10^2$ spores/ml of juice. Each juice (tomato, white grape, orange, and apple) was aliquoted into separate 500 ml bottles. For inoculation with the SV cells, 0.1 ml of the *A. acidoterrestris* culture was added to K medium (KM; Walls and Chuyate, 2000) and incubated for 48 hours. The SV culture grown in KM was inoculated into one liter of each juice, split into separate 500 ml glass bottles to results in 1×10^2 CFU/ml, and incubated at 42°C for two days.

Juice Sampling

A sample of juice was taken initially (day 0) and then every other day for up to 30 days. Samples were withdrawn at appropriate intervals and surface plated onto OSA and incubated aerobically at 42°C for four days. Enrichment procedures were used initially until plate counts could be achieved with enumeration and were also performed when cell numbers began to decrease or had stabilized. To enrich, approximately one ml of the juice was added to K medium and incubated at 42ºC for 24 hrs and then streaked onto OSA to detect *A. acidoterrestris* presence or absence.

Statistical Analysis

Results were statically analyzed to determine the effect of juice type and day on population (log CFU/ml). Each treatment was replicated three times. Main effects and interactions were tested using a 2-way factorial design with repeated measures. All significant (α=0.05) main effects and interactions were analyzed using Tukey's HSD to separate treatment

means. The data was analyzed using JMP statistical software (Statistical Analysis System, Cary, N.C).

RESULTS

In the enumeration of *A. acidoterrestris* spores, populations of 3.73, 3.27, and 3.26 log CFU/ml were achieved when the culture was enumerated with OSA plus PN4, PN and BB as diluents, respectively. On KA, 3.59, 3.74, and 3.59 log CFU/ml were detected using PN, PN 4.0, and BB, respectively (Figure 1). On BAM, the *A. acidoterrestris* was not recovered via direct plating. In preliminary studies, enumeration of *A. acidoterrestris* spores was the most effective when using K agar combined with PN, or OSA combined with PN4 as the diluent.

Enumeration of *A. acidoterrestris* spores/vegetative cells, showed that 6.18, 6.03, and 6.09 log CFU/ml was recovered on KA using PN4, PN, and BB, respectively (Figure 2). On OSA, PN4 had the highest detection at 6.35 log CFU/ml. However, 6.25 and 6.18 log CFU/ml was obtained using PN and BB on OSA, respectively. On BAM, no spore or vegetative cells were observed above detection limits.

Therefore, OSA combined with PN4 was the most effective at enumerating spores and vegetative cells. Although OSA and KA combined with either PN or PN4 were not significantly different, OSA and PN4 were chosen as the medium and diluent for future studies due to ease of preparation and effective enumeration of the microorganism.

In the juice growth study, spores and spores plus vegetative cells were inoculated at 2 log CFU/ml juice. The growth of spores plus vegetative cells in juice followed the order: tomato = apple > grape = orange juice ($p<0.05$) (Figure 3). *A. acidoterrestris* populations in orange and grape juices decreased to below the detection limit after two days. *A. acidoterrestris* populations in apple juice increased to approximately 5 log CFU/ml at day 6 and then stabilized at 4.5 log CFU/ml for the remainder of the 30 day sampling period. Populations in tomato juice peaked at 5.5 log CFU/ml at day 10 and remained at approximately 4.6 log CFU/ml, until an increase to 5.55 log CFU/ml was observed at day 26-29.

In the SO study, the growth rate in juices followed the order: apple = tomato > orange = grape ($p<0.05$) (Figure 4). *A. acidoterrestris* spores in grape juice decreased below the detection level after two days, and orange juice growth fell below the detection limit after four days. In both apple and tomato juice, the *A. acidoterrestris* populations increased to approximately 5.2

log CFU/ml at day 8 and then decreased to 4.1 log CFU/ml over a 8 day period. For the remaining 14 days, *A. acidoterrestris* increased to approximately 4.6 log CFU/ml.

Overall, *A. acidoterrestris* grew better in tomato and apple juices than orange and grape juices. Spore germination and cell growth in tomato juice had an increase of spore germination and vegetative cell growth, whereas apple juice increased slightly and then stabilized over a period of time. In orange and grape juices, sustained growth of *A. acidoterrestris* was not observed after 2-6 days under conditions tested.

DISCUSSION

Preliminary studies show that KA and OSA media, when used with PN and PN4 diluents are effective in enumerating *A. acidoterrestris* spores and vegetative cells. In addition, K Agar and OSA, along with all three diluents tested performed much better than BAM for enumeration. Allowing strains to stay in the diluent for 30 minutes did not significantly reduce viable populations.

Although it has previously been stated that peptone may inhibit *Alicyclobacillus* (Splittstoesser, 1998), Walls and Chuyate (2000) stated that the presence of peptone in K medium did not negatively effect *A. acidoterrestris* enumeration. In addition, Jensen (1999) stated peptone used as a diluent could be used effectively to enumerate *A. acidoterrestris*. This research showed that the use of peptone as a diluent or in KA did not negatively impact the recovery of the microorganism even when the microorganism was allowed to remain in the diluent for up to 30 minutes prior to plating. Therefore, 0.1% peptone may be effectively used as diluent or in medium to enumerate *A. acidoterrestris* without decreasing viability. Although this study showed that BAM was ineffective for enumerating both spores and vegetative cells, this media was effective for causing sporulation of *A. acidoterrestris*. Therefore, a longer incubation period may have been needed to effectively enumerate *A. acidoterrestris* spores or vegetative cells from a suspension.

Since it was determined by Splittstoesser (1998) that *A. acidoterrestris* is strictly aerobic, the ascorbic acid naturally found in the orange juice may have had a preservative effect by reducing the oxygen available to the microorganisms for growth. The Vitamin C content (380 ppm) of orange juice was slightly higher than the apple, grape, and tomato juice. According to Cerny et al. (2000), approximately 150 ppm ascorbic acid prevents growth of *A. acidoterrestris.*

Therefore, the ascorbic acid present may have reduced the environment, decreasing the oxygen available in the juice, although other factors may be present in the juice that inhibited *A. acidoterrestris*. Although this may have made a slight difference in *A. acidoterrestris* populations, there are most likely other factors or combinations of factors inhibiting growth of *A. acidoterrestris* because apple juice contained 340 ppm of Vitamin C and tomato contained 304 ppm.

Juice components and characteristics may have an effect on *A. acidoterrestris* growth and spore germination. The orange juice used in this study is a shelf-stable juice with minimal pulp and had a pH of 3.95. Since the pH of the juice was within the optimum growth conditions, it probably was not a considerable factor in preventing growth. However, in studies performed by Splittstoesser et al., (1998) only one of two strains inoculated into orange juice grew. Therefore, components such as the ascorbic acid may have had a negative effect on the strain used in this study, not allowing the strain to proliferate in the juice. Juice compounds may have prevented *A. acidoterrestris* spores from sporulating and the vegetative cells from forming spores.

According to Pettipher (2000), traditional plating methods may not be precise enough to determine the presence of *A. acidoterrestris* cells, especially if the cells are injured or are being inhibited by a constituent in the juice environment. Therefore, while spores and/or vegetative cells may have been present, plating methods used may not have been able to detect the spores.

The grape juice used in this study did not provide suitable conditions for growth of *A. acidoterrestris* spores or vegetative cells. The grape juice contained the lowest pH of all four juices at 3.07 and the highest concentration of soluble solids. According to Splittstoesser (1998), juices that contained soluble solids at a level higher than 16.1 % Brix are inhibitory to *A. acidoterrestris*. The higher level of soluble solids in the grape juice may have allowed the growth to sustain for approximately six days and then caused a decrease in populations. Since the cellular functions all occur in aqueous phase, the cellular functions may have began to decrease in the microorganism due to low Aw. In addition, the high concentration of sugar may also affect the cellular membrane since it must remain in a fluid state. Therefore, cells may be able to survive for a period of time and then convert to lag phase making them undetectable by plating (Jay, 2000). In combination with a lower pH, the soluble solids may have a synergistic inhibitory effect on *A. acidoterrestris*.

According to Splittstoesser (1998), both strains of *Alicyclobacillus* inoculated in to apple juice were able to grow. When growth was compared among juices and juice blends, including apple, tomato, orange, pineapple, concord grape, apple-grape, and apple-grape-cherry; apple juice had the second highest growth below tomato juice. This research supports Splittstoesser's findings. An optimum pH (3.5) and an acceptable Brix level (11.4° Brix) allowed spores and vegetative cells to grow by about 3.4 log CFU/ml initially and then stabilize at about 5 log CFU/ml; a level reported to be significant for spoilage taint to be detected. When SO were inoculated in the juice, a similar trend occurred as the SV cells, with growth occurring initially and then stabilizing to a level coinciding with reported spoilage taint formation. In addition, a level of 5 log CFU/ml, was achieved by day six when SV cells were present and was achieved at day eight when SO were inoculated into the juice. Therefore, under optimum conditions, apple juice containing low levels of either spores or vegetative cells has the potential to be spoiled by *A. acidoterrestris* within a week.

The tomato juice used in this study supported the highest amount of growth of the four juices tested. According to Splittstoesser (1994), the higher pH and the lower soluble solids of tomato juices may be related to better growth of *A. acidoterrestris*. This present study supports these findings. Tomato juice had the highest pH (4.21) and lowest soluble solids level (5.3° Brix) of the juices tested. Therefore, tomato juice had the most optimum growth conditions available to the organism. When SO were inoculated into tomato juice, spores were able to germinate within two to four days and the growth increased to 5.0 log CFU/ml by day four and continued to stabilize over the next 25 days. Therefore, after formation of taint may have occurred, the cell counts decreased slightly, possibly due to sporulation but then increased again, where more taint formation may have occurred. When SV were inoculated, an increase in *A. acidoterrestris* populations occurred at day two and the populations reached approximately 5.5 log CFU/ml within five days. Growth then decreased slightly but began to increase again at day 26.

This study shows that short time (two to eight days) and low level of spores or vegetative cells at an optimum temperature may provide adequate conditions for taint formation in certain juices. However certain juices, such as orange and white grape may need other factors present to sustain growth or may be able to only support growth of certain strains of the organism under optimum growth conditions. Therefore, certain juices may have a protective barrier against spoilage while other juices are highly susceptible to spoilage.

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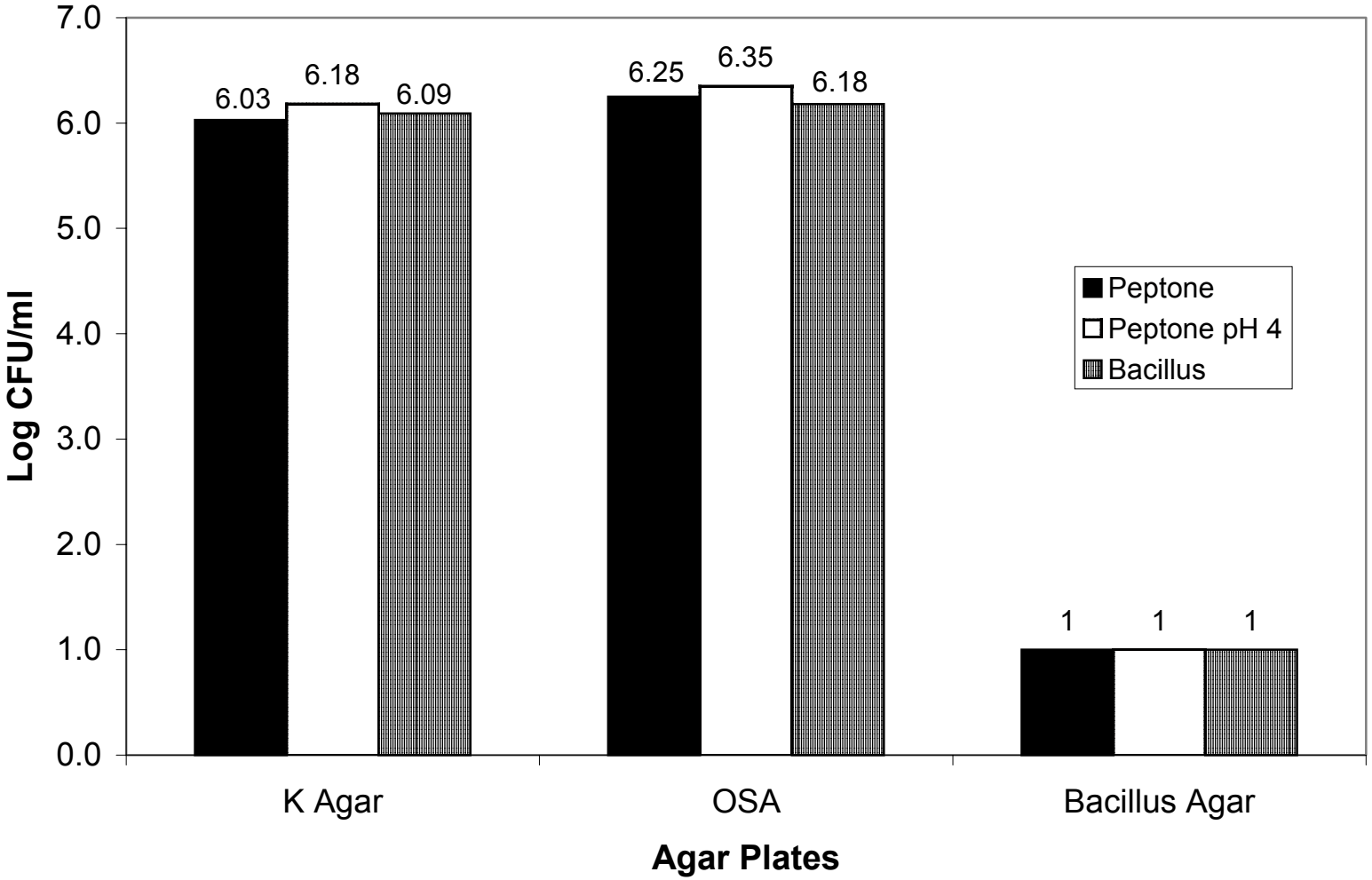

FIGURE 1: Enumeration of *Alicyclobacillus acidoterrestris* spore/vegetative cell suspensions using peptone, peptone (pH 4.0), and Bacillus Broth as diluents on Orange Serum Agar, Bacillus Agar, and K Agar (n = 108).

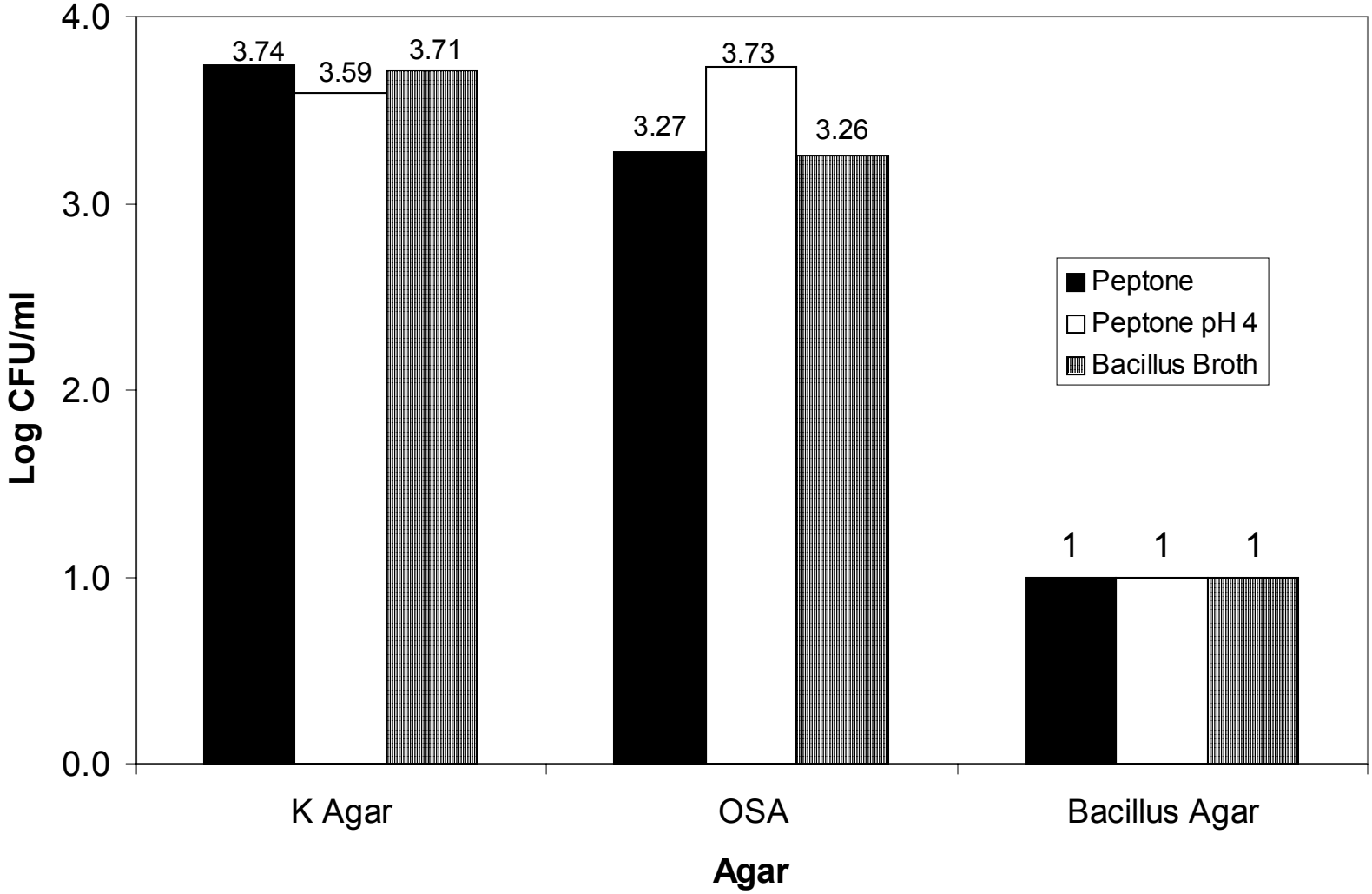

FIGURE 2: Enumeration of *Alicyclobacillus acidoterrestris* spore suspensions using peptone, peptone (pH 4.0), and Bacillus Broth as diluents on Orange Serum Agar, Bacillus Agar, and K Agar (n = 80).

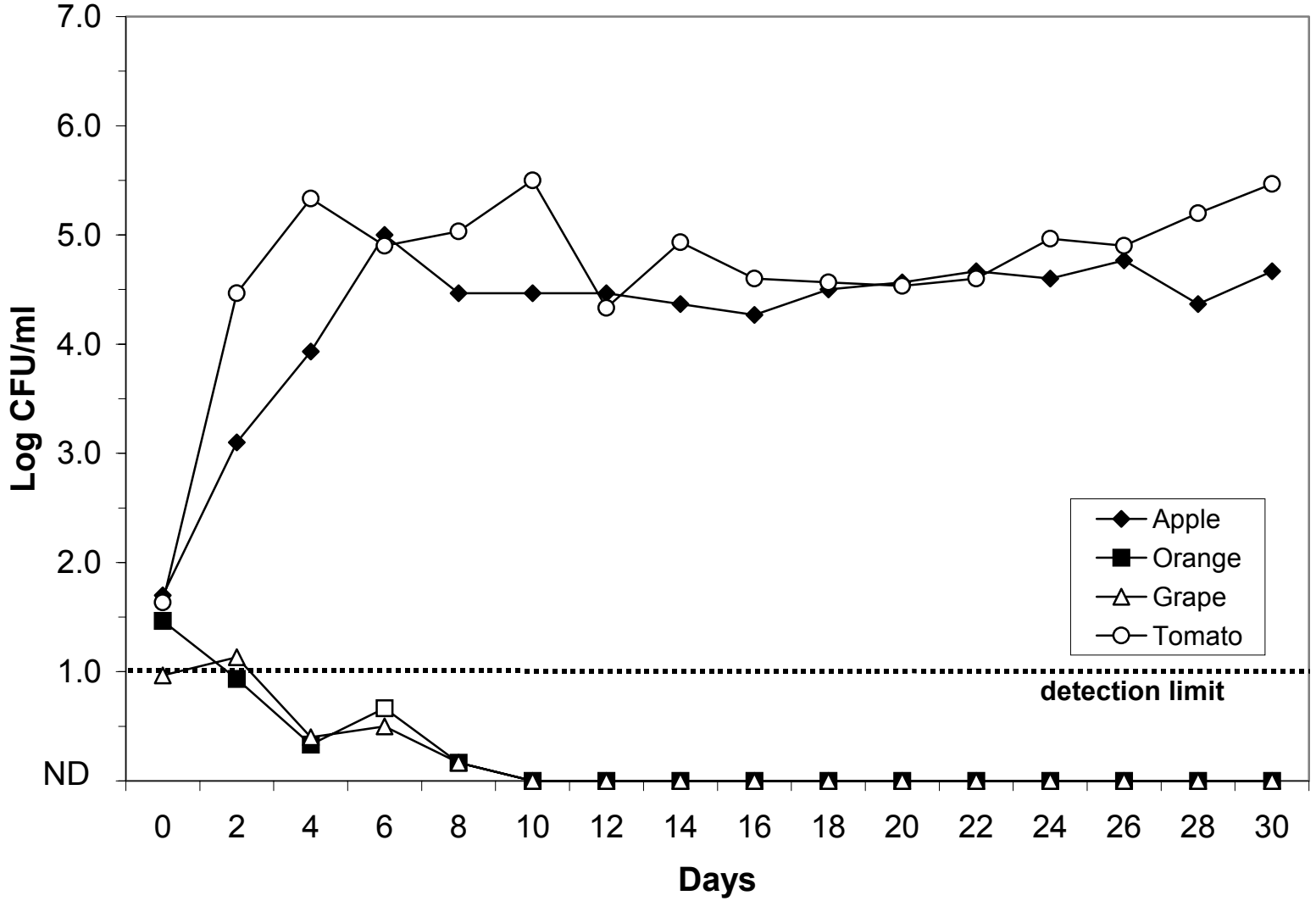

FIGURE 3: Fate of *Alicyclobacillus acidoterrestris*, inoculated as spores and vegetative cells, in apple, tomato, orange and grape juice (n = 621). ND = not detected by spiral plating or enrichment.

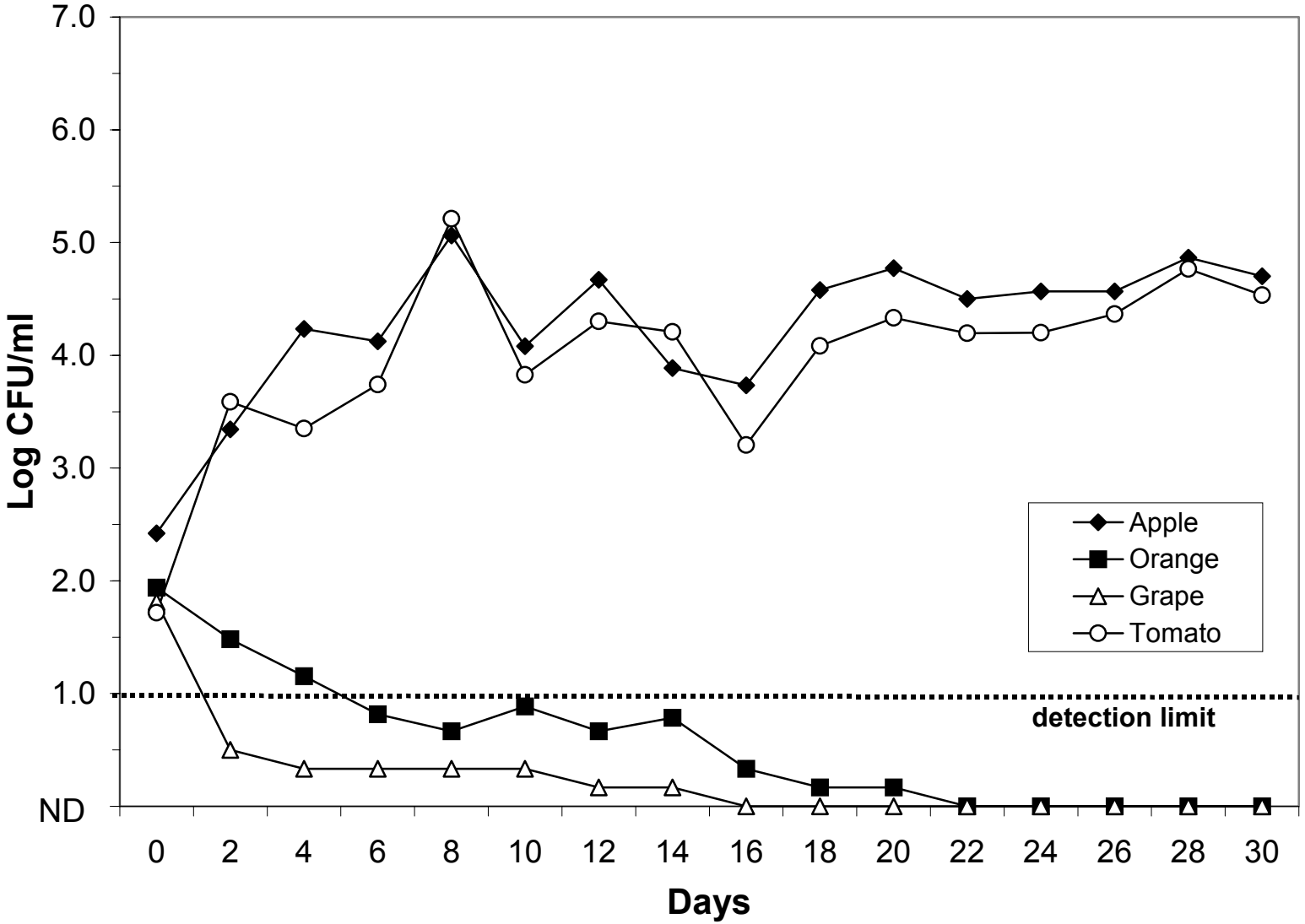

FIGURE 4: Fate of A*licyclobacillus acidoterrestris*, inoculated as spores, in apple, grape, tomato, and orange juice (n = 621). ND = not detected by spiral plating or enrichment**.**

Efficacy of Antimicrobial Treatments for the Control of *Alicyclobacillus Acidoterrestris* in Juices

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KEYWORDS: *Alicyclobacillus acidoterrestris*, juice, nisin, sodium metabisulfite, cinnamic acid, potassium sorbate, sodium benzoate, lysozyme, dimethyl dicarbonate, ascorbic acid, antimicrobials

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ABSTRACT

The acidothermophilic bacterium, *Alicyclobacillus acidoterrestris*, has the ability to spoil a variety of juice-containing beverages when optimum conditions are present. The purpose of this project was to determine the efficacy of antimicrobial agents, added directly to the juice, to inactivate or inhibit *A. acidoterrestris* in juices. Eight antimicrobials, i.e., sodium benzoate, potassium sorbate, nisin, ascorbic acid, cinnamic acid, sodium metabisulfite, lysozyme, and dimethyl dicarbonate, were evaluated for their ability to inactivate and prevent spore outgrowth of *A. acidoterrestris* in apple and tomato juices.

Shelf-stable apple and tomato juices were inoculated with *A. acidoterrestris* spores to result in a concentration of 4 log *A. acidoterrestris* spores/ml juice. Antimicrobials were added to inoculated juices to result in the following concentrations: 1000, 500 and 250 ppm (sodium benzoate, potassium sorbate, and sodium metabisulfite); 500, 250, and 125 ppm (cinnamic acid, dimethyl dicarbonate, and ascorbic acid); 125, 75 and 25 ppm (lysozyme); and 5, 3, and 1 IU/ml (nisin). Inoculated, treated juices were incubated at 42°C and sampled consecutively for 5 days, then every other day for 29 days. Juice samples were surface plated, using a spiral plater, onto Orange Serum Agar and incubated at 42°C for 48 hours.

In apple juice, *A. acidoterrestris* population reductions were caused by the following antimicrobials (reduction in log CFU/ml): lysozyme - all levels (5.1), nisin - 5 IU/ml (5.1), nisin - 3 IU/ml (4.2), cinnamic acid - 125 ppm (3.1), cinnamic acid - 250 ppm (2.6), potassium sorbate - 250ppm (2.5), nisin - 1 IU/ml (2.4), potassium sorbate - 1,000 ppm (2.3), potassium sorbate - 500 ppm (2.3), dimethyl dicarbonate - 500 ppm (1.9), cinnamic acid - 500 ppm (1.4). No reduction in *A.acidoterrestris* population was seen for sodium metabisulfite - all levels, ascorbic acid - all levels, dimethyl dicarbonate - 125 ppm and 250 ppm, and sodium benzoate - all levels. In tomato juice, *A. acidoterrestris* log reductions were caused by the following antimicrobials (reduction log CFU/ml): nisin - all levels (4.4), lysozyme - 75 ppm and 125 ppm (4.4), lysozyme - 25 ppm (3.8), potassium sorbate - 500 ppm (2.6), cinnamic acid - 500 ppm (2.5), cinnamic acid - 250 ppm (2.4), cinnamic acid - 125 ppm (2.1), potassium sorbate - 1,000 ppm (1.9), and potassium sorbate - 250 ppm (1.6). In tomato juice, *A. acidoterrestris* was not reduced when

juices were treated with dimethyl dicarbonate - all levels, sodium benzoate - all levels, sodium metabisulfite - all levels, and ascorbic acid - at all levels.

Antimicrobial treatments of nisin - ≥ 3 IU/ml and lysozyme - ≥ 25 ppm reduced *A. acidoterrestris* populations significantly and prevented spore germination throughout the duration of the study. Therefore, under the conditions of this study, these antimicrobial treatments may prevent *A. acidoterrestris* spoilage even when stored at optimum growth temperatures. Cinnamic acid - ≥ 125 ppm, nisin - 1 IU/ml, and potassium sorbate – ≥ 250 ppm may be used to prevent spoilage when ≤ 4-5 log spores/ml are present in the juice products. These antimicrobials may be used effectively under normal commercial practices; when juices are stored either below 42°C or are less contaminated (≤ 4-5 log spores/ml) than the conditions used in this study.

INTRODUCTION

Pasteurized shelf-stable juices make up a large part of the juice market in the United States. Therefore, spoilage of these types of juice products may translate to large losses for producers. In 1984, Cerny and others isolated an acidophilic bacillus from spoiled, pasteurized apple juice. It has been concluded that the microorganism responsible, *A. acidoterrestris*, is able to withstand the pasteurization and hot-fill-hold process (85 to 95ºC for two minutes) that is commonly applied to juices, as well as thrive in the acidic environment of juices that typically prevents other spoilage organisms from growing (Eguchi, 1999; Eiora et al., 1999). *A. acidoterrestris* causes off-odors and off-flavors in juices (taint), which is undesirable to consumers and may relate to problems for processors (Walls and Chuyate, 2000). Sensory tests have determined that an off-flavor or odor may be detected when approximately 1 x 10^3 CFU/ml spores are present, (Eguchi, 1999) while Pettipher and Osmundson (2000) stated that a level of 1 x 10^5 CFU/ml was required for taint production to be detected. However, populations of less than 1 x 10^2 CFU/ml may also cause taint (Gannon, 1998). Taint can be defined as the detectable level of the off-flavors and off-odors (guaiacol and 2,6- dibromophenol) by consumers.

A. acidoterrestris has been found throughout many stages of processing: from the incoming fruit to water used to finish the beverages, suggesting that one ingredient or aspect of processing is not the only route of contamination, making elimination of the microorganism more difficult (Eguchi, 1999). In addition, *A. acidoterrestris* spoilage is unable to be recognized under normal quality control visible inspection procedures, so producers are unaware that spoilage has occurred until complaints have been received by customers. However, since consumer complaints do not occur with every spoiled juice, the problem may be larger than recognized and consumers may stop buying brands that have been spoiled by this microorganism. Spoilage by *A. acidoterrestris* may increase scrapping costs, customer dissatisfaction, loss of brand quality, and cost due to removal of the organism from production environments (Borlinghaus, 1997; Abe Citrus, 1999; Jensen, 1999b; Walls and Chuyate, 2000).

The reduction of *A. acidoterrestris* contamination in juices has been a demanding challenge because the organism is ubiquitous, has the ability to survive pasteurization processes that are two to four times higher than the typical process, resists thermal condensation, and is highly resistant to many cleansers, sanitizers, and other prevention methods (Parrish, 2000). Increasing the pasteurization temperature has little effect on *A. acidoterrestris* spore destruction,

would increase energy costs, and prove detrimental to product quality (Pontius, 1998; Previdi, 1997; Splittstoesser, 1998). In addition, rinses and different washing techniques have showed some promise but do not prevent the ability of *A. acidoterrestris* to spoil juice when it is present in other sources besides unwashed fruit (Gray, 2000; Orr and Beuchat, 2000b; Previdi, 1999). Therefore, processing changes may not inhibit this microorganism in all products and instances without causing excessive energy costs or decreased organoleptic qualities of the juices.

One way that certain juice and beverage producers prevent spoilage microorganisms is through the addition of either natural or chemical antimicrobial agents to juices and beverages. Antimicrobials, when utilized at accepted levels, have been used effectively for decades to prevent bacteria from growing that may spoil food products or cause human illness, without resulting in considerable quality defects (Jay, 2000).

Therefore, the use of certain antimicrobials as a control method for *A. acidoterrestris* in fruit juices needs to be explored. Determining the efficacy of antimicrobials on *A. acidoterrestris* and defining the effective level needed to inhibit both the vegetative cells and the spores of this microorganism may provide a solution for A. *acidoterrestris* spoilage problems in juices. Inhibition of this organism through the use of antimicrobials would benefit the juice industry and could be used as a potential inhibition method in other fruit and vegetable juices, teas, or canned tomatoes that are susceptible to spoilage by *A. acidoterrestris***.**

Eight antimicrobials were chosen for testing because of either their ability to work effectively in beverage matrices to prevent spoilage bacteria, yeast, or molds or their ability to inhibit Gram-positive bacteria. Nisin, lysozyme, cinnamic acid, ascorbic acid, sodium benzoate, potassium sorbate, sodium metabisulfite, and dimethyl dicarbonate were tested to determine if the antimicrobials could decrease the amount of viable spores and vegetative cells and prevent outgrowth of the spores for at least 29 days. Antimicrobials may degrade naturally, in the presence of high temperatures, or in the presence of juice components, therefore growth curves over a period of time are necessary to determine if the antimicrobial would remain effective over time. This may give some indication of the stability of the antimicrobial over the shelf-life of the juice. Determining which levels of antimicrobials are effective is important so that the lowest effective dose of antimicrobial may be used while obtaining a beverage that is not adversely affected by the antimicrobials added.

MATERIALS AND METHODS

Spore Stock Preparation

An *Alicyclobacillus acidoterrestris* (ATCC 49025) culture was streaked for isolation on Bacillus Agar (BAM; ATCC; Manassas, VA) and grown at 42°C for 72 hours. A portion of culture was removed and examined microscopically for spores under phase-contrast. If at least 80% spores were present, the BAM plate was flooded with five mls of sterile, distilled water and the colonies were scraped off using a sterile rod to create a cell suspension. The suspension was placed into a sterile test tube and heated at 80°C for ten min to inactivate vegetative cells. Spore stock cultures were centrifuged at 11,951 x g for ten min followed by a sterile distilled water wash, followed again by centrifugation and washing three times. Finally, the spore pellet was re-suspended in sterile, distilled water and stored at 4˚C for a maximum of two months. Spore viability was assessed weekly via plating to ensure consistent inoculum levels.

Antimicrobial Efficacy on *A. acidoterrestris* Spores in Apple and Tomato Juice:

Antimicrobial Preparation

The antimicrobials used for this study were: potassium sorbate (EM Science; Darmstad, Germany), sodium benzoate (ACROS Organics; New Jersey), dimethyl dicarbonate (Sigma Chemical Co.; Louis, MO), nisin (2.5% nisin; balance sodium chloride and denatured milk solids; Sigma Chemical Co.; Louis, MO), cinnamic acid (98+%; ACROS Organics; New Jersey), L-ascorbic acid (Chemical Co.; Louis, MO), sodium metabisulfite (ACROS Organics; New Jersey), and lysozyme (Inovapure™ 300; Canadian Inovatech; Abbotsford, B.C., Canada). Solutions of 1,000, 500, and 250 ppm (w/v) of sodium metabisulfite, sodium benzoate, and potassium sorbate were selected based upon legal limits in juices and beverages. Trans-cinnamic acid (97% cinnamic acid), dimethyl dicarbonate, and ascorbic acid were used at 500, 250, and 125 ppm (w/v) based on legal limits and literature research. Levels of 125, 75, and 25 ppm (w/v) of lysozyme and 5, 3, and 1 International Units/ml (IU/ml) of nisin were used in the juices based on preliminary and literature research. Unlike the other antimicrobials, nisin was prepared based on the enzymatic activity of an IU of pure nisin and the amount of pure nisin in the product (2.5% nisin). The amount of undissociated acid that is added to the juices was calculated for

sodium metabisulfite, sodium benzoate, potassium sorbate, and cinnamic acid for each juice based on the pH of the juice and the pKa of each antimicrobial (based on stoichiometry).

All of the antimicrobials were prepared as stock solutions with sterile distilled water and were filter sterilized with a 0.45µm filter with the exception of nisin. Nisin was prepared in sterile water without filtering because it cannot be filtered due to denatured milk proteins.

Inoculum Preparation

In this study only apple and tomato juice were used to determine antimicrobial efficacy because of the ability of *A. acidoterrestris* spores to survive and germinate in these juices. Four liters of juice were batch inoculated to achieve 1 x. 10^4 spores/ml of juice so that the spores could be easily measured to determine the effectiveness of the antimicrobials. Each juice (tomato and apple) was split into the separate 200 ml bottles, leaving a 50 ml headspace, for addition of each of the different antimicrobials to the juice. The juices were then incubated at 42°C.

Microbiological Examination

Every day for 5 consecutive days, a sample of the juice was spiral plated using a Spiral Biotech Autoplate 4000 spiral plater (Norwood, MA), in a 100 µl exponential method and a 250µl uniform plating method onto OSA. The OSA plates were incubated aerobically at 42°C for 48 hrs. For enrichment of *A. acidoterrestris*, 1 ml of the treated juice was inoculated into K medium (Walls and Chuyate, 2000). The K medium was incubated at 42°C for 24 hours and then streaked onto OSA to detect *A. acidoterrestris* presence or absence. Enrichment was used to detect *A. acidoterrestris* growth at low numbers that were not detected using spiral plating. Enrichment procedures were used initially until plate counts were achieved with enumeration and when viable populations began to decrease or stabilize. After the 5 consecutive days of sampling, the cell count numbers were compared to the positive and negative controls to determine the effectiveness of each of the antimicrobial treatment. Antimicrobial treatments that were not effective or allowed *A. acidoterrestris* spores to germinate and grow were discontinued. Antimicrobials that were effective for the first 5 days were then continually sampled every other day for the remaining 24 days. This study was replicated three times.

Juice Analysis

Juice soluble solids (Abbe Mark II Digital Refractometer; Reichert-Jung; Buffalo, NY) and pH (Accumet 10 pH meter; Fisher Scientific; Suwanee, GA) was measured for tomato and apple juices. In addition phenols were determined for tomato and apple juice using a spectral evaluation of phenolics as described below:

Phenolics were measured by adjusting the juice samples to pH 3.6 with 1N HCl and filtering through a 0.45µm filter. The juice was then diluted (1:200) in a buffer solution that was prepared by using 24 mls (200 proof) ethanol in 176 mls distilled water with 0.5 grams potassium bitartrate added. The blank was then adjusted to pH 3.60 with HCl or NaOH. All samples were incubated for five min, and a portion was transferred to a 1mm quartz cuvette. Absorbance of the juice was measured using a UV-VIS Spectronic Genesys spectrophotometer model 5 (Spectronic Instruments Inc.; Rochester, NY) at 280 nm with absorbance blanked to the buffer solution. Adjusted absorbance was then multiplied by 200 to account for the dilution factor to obtain an estimation of the total phenol concentration (expressed as AU) present in the juice (Zoecklein, 2003).

Sulfur dioxide degradation over time at 42ºC was analyzed to determine if a decrease of free and molecular free sulfur dioxide was occurring and the rate of decrease. To analyze the degradation of free sulfur dioxide in apple and tomato juice over a period of 30 days, an aeration/oxidation method was used at 0, 15, and 30 days according to Zoecklein et al., (1990). Based on pH and level of free sulfur dioxide, the amount of molecular free sulfur dioxide was determined (Zoecklein et. al., 1990).

The amount of insoluble solids present in the juice was determined by adjusting the pH to 1.6 with 1N HCl to precipitate any insoluble solids, and measuring the initial weight of 25 mls of the acidified juice. The tomato and apple juice samples were then centrifuged at 2,001 x g for 15 minutes. The supernatant was poured off and the sample was weighed again. Initial weight was subtracted from final weight to determine the total soluble solids percent. All juice analysis tests were performed in triplicate.

Statistical Analysis

Results were statistically analyzed to determine the effect of antimicrobial treatment and day within each juice on *A. acidoterrestris* populations (log CFU/ml) using a 2-way factorial design with repeated measures. Each treatment was replicated three times. All significant (α=0.05) main effects and interactions were analyzed using Tukey's HSD to separate treatment means. The data was analyzed using JMP statistical software (Statistical Analysis System, Cary, NC).

In addition, results were statistically analyzed to determine the affect of antimicrobial treatment, day, and juice on *A. acidoterrestris* population (log CFU/ml) using a 3-way factorial design with repeated measures. Each treatment was replicated three times. All significant (α=0.05) main effects and interactions were analyzed using Tukey's HSD to separate treatment means. Any interactions that were not significant were deleted from the model. The data was analyzed using JMP statistical software (Statistical Analysis System, Cary, NC).

RESULTS

A. acidoterrestris was inoculated into apple and tomato juices to result in a concentration of 4 log CFU/ml. Spores or vegetative cells that were detected by enumeration were reported as 0.5 log CFU/ml, and levels that were detected neither by enrichment nor plating were reported as non-detectable (ND). Populations that fell below the detection limit (0.6 log CFU/ml) decreased by a minimum of 3.4 log CFU/ml. It was necessary to determine if spore or vegetative cell reductions (inactivation) occurred and whether spore outgrowth was prevented (inhibition) so that spoilage due to germination and vegetative cell growth would not occur over a period of 29 days.

Apple Juice

Table 1 shows differences in *A. acidoterrestris* populations in the presence of the different antimicrobial treatments. In respect to *A. acidoterrestris* populations in apple juice, all 3 levels of each antimicrobial was not significantly different from each other, with the exception that cinnamic acid - 500 ppm was significantly less effective than cinnamic acid - 250 and 125 ppm ($p<0.05$). In addition, nisin - 1 IU/ml was significantly less effective than 3 IU/ml and 5 IU/ml ($p<0.05$).

In apple juice, *A. acidoterrestris* populations in the presence of dimethyl dicarbonate - 500 ppm, sodium metabisulfite - all levels, potassium sorbate - all levels, cinnamic acid - all levels, nisin - all levels, and lysozyme - all levels were significantly different from the control *A. acidoterrestris* populations ($p<0.05$). Nisin - 3 IU/ml and 5 IU/ml and lysozyme - all levels, which were the most effective antimicrobials, were significantly different from the other treatments ($p<0.05$), but not significantly different from each other ($p>0.05$). Ascorbic acid - all levels, sodium benzoate - all levels, dimethyl dicarbonate - all levels, and sodium metabisulfite - all levels were not significantly different from each other ($p<0.05$). Dimethyl dicarbonate - 500 ppm, sodium metabisulfite - 1,000 ppm, cinnamic acid - 500 ppm are similar ($p>0.05$). Cinnamic acid - 125 ppm and nisin - 1 IU/ml were also not significantly different from each other ($p>0.05$). Overall, antimicrobial treatments were highly significant ($p<0.0001$). There was no significant difference between juices ($p= 0.2867$). However, the interaction between antimicrobial treatment and juice was highly significant ($p<0.0001$).

A. acidoterrestris populations in apple juice began at 5.1 log CFU/ml at day 0 and stabilized in a range from 4.1 to 5.6 log CFU/ml over the 29 days, with a final population of 5.1 log CFU/ml at day 29. Overall there was no net increase in populations over the 29-day period. On average, when antimicrobials were applied in apple juice, they were effective for reducing *A. acidoterrestris* in the following order: lysozyme > nisin > cinnamic acid > potassium sorbate > sodium metabisulfite > dimethyl dicarbonate >sodium benzoate > and ascorbic acid.

Figure 1 shows the effect of sodium metabisulfite on *A. acidoterrestris* spores in apple juice. All three levels decreased *A. acidoterrestris* initially from 4.4 - 4.5 log CFU/ml to 2.6 - 3.4 log CFU/ml; however, the population began to rise from day 19 to 29, with the exception of sodium metabisulfite - 1,000 ppm. At day 29, A. *acidoterrestris* populations ranged from 5.1 to 5.3 log CFU/ml, therefore the sodium metabisulfite allowed spores to germinate and allowed growth of vegetative cells. Benzoates caused no significant log reduction of *A. acidoterrestris* after 5 days and were discontinued (Figure 2).

Dimethyl dicarbonate - 125 ppm and 250 ppm caused no significant reduction in *A. acidoterrestris* populations after 5 days. As a result, these two ineffective levels were discontinued (Figure 3). The 500 ppm level of dimethyl dicarbonate caused an initial decrease from 4.4 to 2.3 log CFU/ml *A. acidoterrestris*, but *A. acidoterrestris* populations increased to a level of 4.6 by day 25. At day 29, *A. acidoterrestris* had decreased to 3.2 log CFU/ml.

Therefore, dimethyl dicarbonate - 500 ppm produced an overall reduction of 1.9 log CFU/ml, however the increase of populations at day 25 suggests that it may allow the spores to germinate and cause spoilage.

Ascorbic acid was ineffective and did not prevent spores from germinating, and populations rose from log 4.4 - 4.5 log CFU/ml to 5.0 - 5.3 log CFU/ml over a period of 29 days (Figure 4). Figure 5 shows the effect of potassium sorbate on *A. acidoterrestris* spores. Potassium sorbate - all levels had a gradual decrease in population from 4.2 - 4.4 log CFU/ml to 2.6-2.8 log CFU/ml, for an average reduction of 2.4 log CFU/ml, with no spore outgrowth.

All three levels of cinnamic acid caused *A. acidoterrestris* levels to decrease, however, cinnamic acid - 125 ppm and 250 ppm caused more decrease than cinnamic acid - 500 ppm (Figure 6). For cinnamic acid, there were log reductions of 3.1, 2.6, and 1.4 CFU/ml *A. acidoterrestris* when 125 ppm, 250 ppm, and 500 ppm were used, respectively. The average reduction after 29 days for potassium sorbate - 1,000 ppm and 500 ppm was 2.3 log CFU/ml *A. acidoterrestris*, while the log reduction for potassium sorbate - 250 ppm was 2.5 log CFU/ml. In addition, at all three levels of potassium sorbate, no significant spore outgrowth occurred during 29 days.

A. acidoterrestris populations in the presence of nisin - 1 IU/ml initially decreased from 5.1 log CFU/ml to non-detectable levels. However at day 7, populations began to increase (Figure 7). There were a lot of large increases and decreases in growth of *A. acidoterrestris* populations combined with nisin - 1 IU/ml. This variation was suspected to be from inhibition of nisin on *A. acidoterrestris* when plated on OSA. After 29 days, nisin - 1 IU/ml caused a reduction of 2.4 log CFU/ml *A. acidoterrestris.* Nisin - 3 IU/ml caused A. *acidoterrestris* levels to decrease to below detectable limits for 21 days. However growth occurred at day 23 and days 27-29. Overall, nisin - 3 IU/ml caused a 4.2 log CFU/ml reduction of *A. acidoterrestris* after 29 days. Nisin - 5 IU/ml decreased *A. acidoterrestris* populations to non-detectable levels at day 0 with no outgrowth present for 29 days, and resulted in a 5.1 log CFU/ml reduction in *A. acidoterrestris* populations.

Figure 8 shows the effect of lysozyme on *A. acidoterrestris* spores in apple juice. When lysozyme - 25 and 75 ppm was used, populations decreased by an average of 4.0 and 3.7 log CFU/ml, respectively, at day 0. Populations then decreased to non-detectable levels (by plating or enrichment) for the remaining 29 days with the exception of day four for both levels and day

nine for 75 ppm lysozyme. Lysozyme - 125 ppm caused complete reduction of spores with no outgrowth. Lysozyme - all three levels caused a 5.6 log CFU/ml reduction of *A. acidoterrestris* spores over a period of 29 days.

Overall, *A. acidoterrestris* population reductions were caused by the following antimicrobials (magnitude of reduction in log CFU/ml) in apple juice: Lysozyme - all levels (5.1), nisin - 5 IU/ml (5.1), nisin - 3 IU/ml (4.2), cinnamic acid - 125 ppm (3.1), cinnamic acid - 250 ppm (2.6), potassium sorbate - 250ppm (2.5), nisin - 1 IU/ml (2.4), potassium sorbate - 1,000 ppm (2.3), potassium sorbate - 500 ppm (2.3), dimethyl dicarbonate - 500 ppm (1.9), cinnamic acid - 500 ppm (1.4). No reduction in *A. acidoterrestris* population was seen for the following treatments: sodium metabisulfite - all levels, ascorbic acid - all levels, dimethyl dicarbonate -125 ppm and 250 ppm, and sodium benzoate - all levels.

Tomato Juice

For tomato juice, all levels of the same antimicrobial were not significantly different with the exception that nisin - 1 IU/ml was significantly less effective than nisin – 3 and 5 IU/ml ($p>0.05$)(Table 1). Ascorbic acid - 125 ppm and dimethyl dicarbonate - 125ppm were not significantly different from the control when comparing mean populations over the 29 day period($p>0.05$). Ascorbic acid - all levels, sodium benzoate - all levels, dimethyl dicarbonate - all levels, and sodium metabisulfite - all levels were not significantly different from each other ($p>0.05$). Sodium metabisulfite - 1,000 ppm, potassium sorbate - 250 ppm, and potassium sorbate -1,000 ppm were also not significantly different from each other ($p>0.05$). Potassium sorbate - all levels and cinnamic acid - all levels were not significantly different from each other ($p>0.05$). Nisin - 1 IU/ml and lysozyme - 25 ppm were also not significant from each other ($p>0.05$). Nisin - $\geq$ 3 IU/ml and lysozyme - all levels were not significantly different from each other ($p>0.05$), but are significantly better than the other antimicrobials ($p<0.05$).

A. acidoterrestris populations in tomato juice began at 4.4 log CFU/ml at day 0 and remained steady. At day 19, the populations began to increase and reached a final level of 6.2 log CFU/ml. Therefore, *A. acidoterrestris* populations in tomato juice had a net increase of 1.8 log CFU/ml over the 29-day period, demonstrating that *A. acidoterrestris* spores were able to germinate and grow.

On average, antimicrobials in apple juice were effective in reducing *A. acidoterrestris* populations in the following order: lysozyme > nisin > cinnamic acid > potassium sorbate > sodium metabisulfite > sodium benzoate > dimethyl dicarbonate > and ascorbic acid. Figure 9 shows the effect of sodium metabisulfite on *A. acidoterrestris* spores in tomato juice. There was an initial reduction of *A. acidoterrestris* but after 3 days there were large and variable increases and decreases for the next 26 days, where all three levels stabilized at populations near each other. Log CFU/ml populations of *A. acidoterrestris* in the presence of sodium metabisulfite - all levels ranged from 4.6 to 5.0, which is a higher population than the control populations. Therefore, it is apparent that spore outgrowth occurred. Benzoates were not effective in reducing *A. acidoterrestris* survival or growth at five days so they were discontinued (Figure 10).

When dimethyl dicarbonate was applied to *A. acidoterrestris* spores, only dimethyl dicarbonate - 500 ppm was effective by 5 days in reducing *A. acidoterrestris*, so the lower levels were discontinued for the duration of the study (Figure 11). Dimethyl dicarbonate - 500 ppm reduced the spores to 4.2 log CFU/ml at day 5 and stabilized at 4.7 log CFU/ml at day 29, which was also higher than control populations. Therefore, dimethyl dicarbonate allowed the spores to germinate and did not prevent vegetative cell growth.

A. acidoterrestris in the presence of ascorbic acid was nearly similar to control populations with a slight increase from 4.4 - 4.5 log CFU/ml to 5.0 - 5.5 log CFU/ml (Figure 12). Although there was only slight spore outgrowth, spores were not inactivated. Figure 13 shows the effects of potassium sorbate on *A. acidoterrestris* spores. Levels of potassium sorbate - 250 and 500 ppm steadily decreased *A. acidoterrestris* with an increased rate of reduction at day 4 and 25. The reduction for potassium sorbate - 500 and 1,000 ppm on *A. acidoterrestris* was 1.6 and 2.6 log CFU/ml, respectively. The 250-ppm level decreased populations until day 13 when a large decline was observed. At day 21, *A. acidoterrestris* was undetectable by spiral plating, however, enrichment revealed that the organism was present. Therefore, *A. acidoterrestris* cells in this sample may have sporulated. By day 23, the *A. acidoterrestris* began to increase to a level of 2.8 log CFU/ml to achieve a 1.6 log CFU/ml reduction at day 29.

All three levels of cinnamic acid caused a steady decrease of *A. acidoterrestris* over the 29 day period to achieve a 2.1 - 2.5 log CFU/ml reduction (Figure 14). Nisin - 1 IU/ml caused an initial decrease of 1.7 log CFU/ml *A. acidoterrestris* at day 0 but populations exhibited dramatic increases and decreases until day 25. These increases and decreases of *A.*

acidoterrestris may be attributed to either sporulation and then germination or nisin inhibition of cells on the plating medium (Figure 15). After day 29, there was a 4.1 log reduction of spores and vegetative cells. Nisin - 3 and 5 IU/ml nisin reduced *A. acidoterrestris* to undetectable levels by day 2 and remained undetectable for 29 days to achieve a 4.4 log CFU/ml reduction.

Figure 16 shows the effect of lysozyme on *A. acidoterrestris* spores. When lysozyme - 125 ppm was used, there was an initial 3.2 log CFU/ml reduction of spores at day 0. Over the 29-day period growth ranged from non-detectable to log 1.3 log CFU/ml to achieve a 3.8 log CFU/ml reduction in *A. acidoterrestris*. *A. acidoterrestris* populations combined with levels of lysozyme - 25 and 75 ppm were reduced to non-detectable limits on day 0 and remained undetectable for the remaining 29 days to achieve a 4.4 log reduction.

Overall, *A. acidoterrestris* reductions were caused by the following antimicrobials (magnitude of reduction in log CFU/ml) in tomato juice: Nisin - all levels (4.4), lysozyme - 75 and 125 ppm (4.4), lysozyme - 25 ppm (3.8), potassium sorbate - 500 ppm (2.6), cinnamic acid - 500 ppm (2.5), cinnamic acid - 250 ppm (2.4), cinnamic acid - 125 ppm (2.1), potassium sorbate - 1,000 ppm (1.9), and potassium sorbate - 250 ppm (1.6). In tomato juice, *A. acidoterrestris* was not reduced when juices were treated with dimethyl dicarbonate - all levels, sodium benzoate - all levels, sodium metabisulfite - all levels, and ascorbic acid - at all levels.

The amount of undissociated acid added when 250 ppm to 1,000 ppm sodium metabisulfite was added to juices ranged from 1.8 to 7.4 ppm molecular free sulfur dioxide in apple juice and from 0.4 to 1.5 ppm in tomato juice (Table 2). When 1,000, 500, and 250 ppm sodium benzoate was added in apple juice, 704, 352, and 176 ppm benzoic acid was present, respectively. In tomato juice, when 1,000, 500, and 250 ppm sodium benzoate was added to juices, 414, 207, and 104 ppm benzoic acid was present in tomato juice, respectively. When potassium sorbate was added at levels of 1,000, 500, and 250 ppm, the amount of sorbic acid ranged from 177 to 707 ppm in apple juice and from 145 to 579 ppm in tomato juice. Cinnamic acid levels ranged from 113 to 451 ppm in apple juice and 80 to 320 ppm in tomato juice, when 500, 250, and 125 ppm of the trans-cinnamic acid salt was added.

The pH of tomato juice (pH 4.21) was somewhat higher and the soluble solids content (5.3° Brix) was about 2 times lower in comparison to apple juice (pH 3.5; 11.4° Brix). In addition the insoluble solids for tomato juice were 44.6 % while in apple juice they were 0%. In

addition, tomato juice also had about two times the amount of total phenols (24.5 ppm) compared to apple (10.4 ppm) (Table 3).

Initially, there was approximately 2.6 times higher free sulfur dioxide in apple juice than tomato juice. However the apple juice free sulfur dioxide decreased much more at 15 and 30 days compared to apple. Apple juice had 436, 43, and 12.1 ppm free sulfur dioxide at 0, 15, and 30 days, respectively, while tomato had 166, 159, and 60 ppm free sulfur dioxide at 0, 15, and 30 days, respectively (Table 4).

DISCUSSION

Application of most of the antimicrobials tested were effective in reducing *A. acidoterrestris* in apple and tomato juice. Based on reduction levels and the inhibition of spore outgrowth, lysozyme - all levels, nisin - ≥ 3IU/ml, and cinnamic acid - 125 ppm may be used effectively in apple juice to inactivate or inhibit log 4 -5 spores/ml for at least 29 days at optimum growth temperatures without allowing spore outgrowth to occur. In tomato juice, cinnamic acid - ≥250 ppm, potassium sorbate - 500 ppm, lysozyme - ≥ 25ppm, and nisin - ≥ 3 IU/ml could be used effectively for 29 days at 42ºC.

Juice components may react with antimicrobials (Davidson, 1990; Davidson, 1993b, Jay, 2000; Sofos, 1989). Although, the juice components of total phenols, soluble solids, insoluble solids and pH were different between apple juice and tomato juice, in this study there was not relationship between juice components and antimicrobial efficacy.

In this project, the antimicrobial type was more significant than either juice type or level of antimicrobial. This may be due to the overall mechanism of each antimicrobial and how effective that mechanism is in reducing *A. acidoterrestris* or if juices in general affected the antimicrobial efficiency or residual amount present during the 29 days.

Lysozyme

Lysozyme caused an average reduction of 5.1 log CFU/ml *A. acidoterrestris* in apple juice and a 4.4 log CFU/ml reduction in tomato juice. This may be a result of the cellular target of lysozyme, on the peptidoglycan in the Gram-positive cell wall (Davidson, 1993b). Lysozyme causes the hydrolysis of β-1,4 glycoside linkages in the NAG and NAM of the bacterial wall and leads to lysis (Task Force, 1998; Worthington Biochemical Inc.). This may cause death of the *A. acidoterrestris* cells after germinating to the vegetative cell form. However, this does not

explain the high amount of reduction on Day 0 when *A. acidoterrestris* spores were added. Although there has been little or no work on the effectiveness of lysozyme to inactivate *A. acidoterrestris* spores, these results suggest that lysozyme may in fact have an effect on spore viability.

In citrate buffer (pH 4.0), 10 ppm lysozyme caused a decrease in thermal resistance of the *A. acidoterrestris* and reduced spores by 70 % compared to heating alone (Yamakazi, 1997). In this study, the concentration of lysozyme that reduced spores was twice as high as the Yamakazi study. Although, levels lower than 25 ppm were not tested, it is likely that levels higher than 10 ppm would be needed in juice as compared to buffered media. Juice components may bind lysozyme so that less residual lysozyme would be available to act upon the microorganism (Lagarde, 1997; Task Force, 1998). However, no rapid degradation or loss of activity in both tomato juice and apple juice was observed over the 30 day storage period. In addition, Yamakazi (1996) stated that lysozyme should be added in addition to other antimicrobial agents to properly prevent spoilage of *A. acidoterrestris* in beverages because of its heat lability at pasteurization temperatures. However, 25 ppm or greater lysozyme was effective for preventing both spore outgrowth and vegetative cell growth to spoilage levels in both juices for at least 29 days.

Lysozyme may be a suitable choice for shelf-stable juices because it can resist low pH and is most effective in homogenized foods, such as juices. In addition, lysozyme may remain active in potentially high temperatures of the warehouse over the summer during storage without a reduction in its antimicrobial efficacy (Lagarde, 1997; Task Force, 1998). According to Lagarde (1997) and Task Force (1998), lysozyme remains active at temperatures up to 80˚C at a pH of 5.0, and can withstand boiling at acidic pH with no loss of activity. However, Yamakazi (2000), stated that lysozyme is heat labile and would not cause an antibacterial effect after pasteurization of beverages and canned tomatoes. Therefore**,** the addition of lysozyme should probably occur after pasteurization to ensure that a residual amount of lysozyme is present in case the effect on spores is sporostatic instead of sporicidal. One drawback to lysozyme use in clear juice could be cloudiness associated with the addition of the antimicrobial. In apple juice, cloudiness was observed at all three levels in apple juice although the results were less dramatic at 25 ppm compared to the higher levels used. However, no effect of cloudiness was seen in tomato juice. Therefore the prevention of cloudiness in clear juices, such as apple and white grape juice need to be addressed before lysozyme could be used commercially.

Nisin

Nisin caused a 5.1, 4.2, and 2.4 log CFU/ml reduction of *A. acidoterrestris* in apple juice at 5, 3, and 1 IU/ml, respectively and all three levels caused a 4.4 log CFU/ml reduction in tomato juice, by day 29. Similar to lysozyme, nisin may target bacteria by affecting Gram-positive cell walls. In vegetative bacterial cell walls, it acts as a surfactant absorbed strongly on the plasma membrane of sensitive cells, where it destabilizes the cellular membrane, binds vital sulfhydryl groups, and inactivates enzymes such as Coenzyme A (Daeschel, 1999; Ray, 1992; Task Force, 1998). Cell death following adsorption of nisin is associated with the release of cytoplasmic materials, leakage of ATP, and in some strains, cell lysis (Delves-Broughton, 1990; Hoover and Steenson, 1993; Ray, 1992). On species of bacteria with small spores (such as *Alicyclobacillus*)**,** nisin has a mechanism of inhibiting pre-emergent swelling of the spore to prevent vegetative cell formation and spore outgrowth (Delves-Broughton, 1990; Komitopoulou, 1999; Prittjarvi, 2001). According to Delves-Broughton (1990), the action of nisin on spores is sporostatic instead of sporicidal, whereas other researchers report that spores are killed by nisin. In this study, it appears that 1 IU/ml of nisin may be degrading to ineffective levels over time because of the ability of *A. acidoterrestris* to grow to detectable levels. In addition, some inhibitory action was observed on the OSA, therefore the population numbers may not be a completely accurate representation of what is occurring in the juice when nisin is present. However, it is apparent that nisin is effective against *A. acidoterrestris* spores. Therefore, unless another method of plating is used, evaluating the effectiveness of nisin on *A. acidoterrestris* on a qualitative basis may be more appropriate compared to a quantitative evaluation. However, no growth, even by enrichment, was observed with nisin - 3 IU/ml and 5 IU/ml in apple or tomato juice, suggesting that sporicidal or bactericidal effects on *A. acidoterrestris* may exist at these levels.

In addition, according to Yamakazi (1997), total phenols in the juice may be acting synergistically with nisin on *A. acidoterrestris* to prevent growth. However, even though tomato juice has approximately twice the amount of total phenols, there was no additional decrease in *A. acidoterrestris* populations in tomato juice compared to apple juice. Although, the amount of total phenols in both apple juice and tomato juice may be enough to inhibit the organism or other factors besides total phenols may be present to act synergistically with the organism.

According to Yamakazi (2000), vegetative cells of *A. acidoterrestris* were inhibited by 1-100 IU/ml and 0.78 - 100 IU/ml was needed to inactivate spores. The current study supports Yamakazi's findings since nisin - ≥ 3 IU/ml in apple juice and nisin - ≥ 1 IU/ml in tomato juice was effective in reducing *A. acidoterrestris* spores. Komitopoulou (1999) concluded that at 44ºC, the Minimum Inhibitory Concentration (MIC) for apple and orange juice was 100 IU/ml and at normal processing conditions only 5 IU/ml was effective. In this study, nisin - 3 and 5 IU/ml inhibited *A. acidoterrestris* spores for 29 days at 42ºC. These results suggest that certain factors in the experiment may have affected the results such as a synergistic effect between juices and antimicrobial. Differences in juice or in nisin preparation may have decreased the MIC of the *A. acidoterrestris* strain tested in this study, or the strain used in the Komitopoulou study may have been more resistant to antimicrobials.

Overall, nisin at low levels would be an effective control method in apple and tomato juice and potentially in a wide variety of juices. In addition, nisin may be added to the juice before pasteurization because it is heat stable even at low pH (Daeschel, 1993; Delves-Broughton, 1990). This would also allow a residual amount of nisin to continue to inhibit outgrowth and spoilage if these levels are in fact sporostatic rather than sporicidal (Komitopoulou, 1999).

Cinnamic Acid

Cinnamic acid was shown to have a 3.1, 2.6, and 1.4 log CFU/ml reduction on *A. acidoterrestris* when 125, 250, and 500 ppm were used, respectively in apple juice with no spore outgrowth. When spore-formers, such as *A. acidoterrestris,* are exposed to high amounts of heat, pressure, or harsh environment, the microorganism tends to sporulate very quickly (Jay, 2000). However, if small amounts of heat, pressure, or preservatives are added, the spore would germinate and therefore could be inactivated by continued addition of preservative, heat or pressure. Therefore the higher levels of cinnamic acid added in apple juice may have been less effective because they caused the microorganisms to sporulated instead of gradually being inactivated by the antimicrobial. Therefore, the higher level may have caused the microorganism to sporulate or remain in the spore state and was not inhibited since cinnamic acid's mechanism of action is on vegetative cells. However, small amounts of cinnamic acid may have caused the microorganism to germinate and be inactivated by cinnamic acid, since vegetative cells are more

susceptible to inhibition compared to spores. This may have occurred with the lower level of cinnamic acid, although it is unknown. In addition, solubility issues with the cinnamic acid may have occurred with the higher levels of cinnamic acid in the apple juice.

In tomato juice, cinnamic acid had log reductions of 2.1-2.5 log CFU/ml with no spore outgrowth in tomato juice when 125 to 500 ppm cinnamic acid was applied. Cinnamic acid, like other organic acids, inactivates bacteria by moving across the bacterial membrane and dissociating within the cytoplasm of the cell, therefore, lowering the internal pH and inhibiting enzymes (Anslow, 2000; Cirigliano, 2000a). However, cinnamic acid also has an unsaturated side chain that prevents the cinnamic acid from being pumped out of the cell. After the spores germinated, the cinnamic acid may have began to inhibit or injure the vegetative cells, therefore, reductions in growth rate may have occurred. This mechanism would require a residual amount of cinnamic to continue to inhibit the microorganism throughout storage. The increased reduction of *A. acidoterrestris* due to cinnamic acid, compared to benzoic and sorbic acid, with the exception of cinnamic acid - 500 ppm in apple juice, may be related to the side chain of cinnamic acid. The ω-alicyclic compounds stabilize the cytoplasmic pH by continually pumping out protons across the membrane to maintain neutral pH (Matzke, 1997). However, the side chain may not allow the antimicrobial to be pumped out. In addition, it has been reported that polyphenols also in juice may prevent *A. acidoterrestris* growth (Yamakazi, 1997). Therefore, cinnamic acid may have a secondary effect on *A. acidoterrestris* since it is a phenolic compound.

According to Anslow (2000), acido-thermophilic spore formers have been inhibited by 25 to 600 ppm cinnamic acid in teas. The current study in juices supports the data in teas. Cinnamic acid initially precipitated when added at ambient temperatures, but when juices were stored at 42˚C it dissolved back into solution. However to avoid problems with precipitation during ambient storage, a solubility step may be needed. It was also reported that cinnamic acid may be effective as an antimicrobial at ambient and refrigerated temperature, but its effect at higher temperatures is unknown. In this study, high temperature storage (42˚C) did not affect the efficiency of the antimicrobial and may have acted synergistically with the antimicrobial.

In addition is has been stated that above 31 ppm the flavor and odor impact of cinnamic acid may become undesirable (Anslow, 2000). The effect of cinnamic acid on odor of the juice was observed in both juices. The odor of cinnamic acid, characterized as " slightly spicy" and "floral" (Anslow, 2000), was observed by this author at all three levels, although at 125 ppm, the

odor was less pronounced compared to 250 and 500 ppm. Although such odors may be acceptable in apple juice they would not be desirable in tomato juice. Therefore, even though cinnamic acid is effective at preventing *A. acidoterrestris* in tomato juice, its sensory aspects may negate its use in tomato juice unless altered.

Potassium Sorbate

In apple juice, potassium sorbate caused a reduction of 2.5 log CFU/ml in *A. acidoterrestris* populations when potassium sorbate - 250 ppm was used. Reductions of 2.3 *A. acidoterrestris* was obtained when potassium sorbate - 500, and 1,000 ppm were used. In tomato juice, a 2.6 log CFU/ml reduction of *A. acidoterrestris* populations was obtained with potassium sorbate - 500 ppm, and reductions of 1.9 and 1.6 log CFU/ml in population were achieved with potassium sorbate - 1,000 and potassium sorbate - 250 ppm, respectively. There was no spore outgrowth of *A. acidoterrestris* in the presence of potassium sorbate, with the exception of potassium sorbate - 250 ppm in tomato juice. Potassium sorbate acts as a lipophilic weak acid that may lower the internal pH of the microorganisms. Similar to cinnamic acid, this would require *A. acidoterrestris* to germinate before it could be inhibited. However, data has suggested that sorbate acts as a competitive and reversible inhibitor of amino acid-induced germination. At least one study has suggested that sorbate inhibits spores that have been triggered to germinate or after germinant binding (Sofos and Busta, 1993). In general, it was observed that the trend of inhibition for potassium sorbate was similar to cinnamic acid but with a lower amount of reduction. This is suspected because the antimicrobials have a similar mechanism, however cinnamic acid may have caused more reduction because it more effectively reduces the internal pH through it use of the unsaturated side chain, which potassium sorbate does not contain. Therefore, potassium sorbate may have inhibited both vegetative cells and spores, although the overall inhibition was less than cinnamic acid.

It was shown that the potassium sorbate reduction in *A. acidoterrestris* populations was much higher compared to sodium benzoate. This could be related to potassium sorbate's higher pKa value. In apple juice (pH 3.5), similar levels of potassium sorbate (737 ppm undissociated acid) and sodium benzoate (740 ppm undissociated acid) were present when 1,000 ppm of potassium sorbate and sodium benzoate were used. However, in tomato juice (pH 4.21), sorbic acid was at 579 ppm compared to 414 ppm undissociated acid of benzoic acid, when 1,000 ppm

of each salt was compared. Therefore the amount of undissociated acid of potassium sorbate may have been high enough to cause greater reductions compared to sodium benzoate. In addition, potassium sorbate has shown efficacy against spores, whereas sodium benzoate has not.

According to Pettipher (2000), approximately 300 ppm potassium sorbate reduced vegetative cells by approximately 2.7 log CFU/ml in 10 days and reduced spores by 2.7 log CFU/ml in 3 months. The results in this study are similar, although in tomato juice *A. acidoterrestris* exhibited somewhat lower levels of reduction.

In addition to the increased antimicrobial inhibition, it has been reported that sorbate at 0.025% to 0.1% is preferred to the use of benzoates due to flavor (Davison and Juneja, 1990). Although it does not appear that spore outgrowth occurred, with the exception of 500 ppm potassium sorbate in tomato juice, juice components of salt, sugar, and soluble food components may reduce the concentration of sorbic acid available (Davison and Juneja, 1990). Therefore, a residual amount of this acid needs to be maintained to prevent spore outgrowth and spoilage since the inhibition caused by potassium sorbate is bacteriostatic. In addition, since potassium sorbate solubilizes at 60˚C, it should be added after pasteurization to avoid loss of the antimicrobial efficiency (Davidson and Juneja, 1990; Sofos, 1989). Potassium sorbate may be more effectively used when the concentration of spores are below 4 log spores/ml.

Sodium Benzoate

Sodium benzoate caused no overall reduction in *A. acidoterrestris* populations after 5 days. Since sodium benzoate acts as a lipophilic weak acid, and reduces the internal pH to inhibit microorganisms, the microorganism would need to germinate before the cell could be inhibited. Therefore, inhibition may have taken longer than 5 days before a decrease of vegetative cells could be observed. Research by Pettipher (2000) revealed that benzoic acid at 150 ppm was shown to decrease vegetative cells at 5.8×10^2 CFU/ml to <100 cells/ml in ten days and reduced 6.8×10^2 spores/ml to 1.4×10^2 spores/ml in three months in apple juice and orange juice. In this study, approximately 740 and 414 ppm undissociated acid was present in apple and tomato juice before binding to juice components when 1,000 ppm sodium benzoate was added, respectively. This research somewhat coincides with Pettipher's study, since there was only a 0.68 log CFU/ml reduction of *A. acidoterrestris* spores in three months using 150 ppm benzoic acid. However, in this study a concentration approximately 3 to 5 times higher than the concentration

used in Pettipher's study did not provide a significant reduction by 5 days. Therefore, a longer observation period may have been needed to see a reduction. However, on a practical standpoint, benzoic acid would not be effective in reducing *A. acidoterrestris* spores in acidic beverages tested on a commercial basis.

Sodium Metabisulfite

Sodium metabisulfite caused no overall reduction in *A. acidoterrestris* populations. Although there was an initial reduction, spore outgrowth occurred during the 29-day period. Spore germination may have occurred due to its mechanism of action. Sodium metabisulfite reacts mostly with sulfide linkages to interfere with enzymes of the bacteria or may cause DNA breakdown (Furia, 1986; Wedzicha, 1984). It has also been reported that sulfur dioxide may also reduce the amount of oxygen below the limit that aerobic bacteria may grow. According to Splittstoesser (1998) sulfur dioxide did not sensitize *A. acidoterrestris* spores to heat. However, Walls and Chuyate (2000) reported that A. *acidoterrestris* was unable to growth in grape juice that contained metabisulfite but was able to grow in grape juice that did not contain metabisulfites. However, the authors of the latter study did not state what concentration of sodium metabisulfite was present in the juice. This study showed that *A. acidoterrestris* was able to survive over a 29 day period at sodium metabisulfite - 1,000, 500, and 250 ppm.

One reason that sodium metabisulfite was ineffective in preventing *A. acidoterrestris* could be that the main antimicrobial portion of sulfur dioxide is the molecular free portion. On day 0, the amount of molecular free sulfur dioxide (undissociated acid) present in 1,000 ppm sodium metabisulfite before binding was 7.4 ppm in apple and 1.5 ppm in tomato juice, based on stoichiometry calculations. When molecular free sulfur dioxide, the antimicrobial portion of the compound, was analyzed to determine the amount present after binding at day 0 and the amount of molecular free sulfur dioxide that was degrading over time, more than 0.8 ppm was present at day 0, but by day 30, it had decreased to < 0.5 ppm. The degradation of the molecular free sulfur dioxide may have allowed the spores of *A. acidoterrestris* to outgrow and allow the vegetative cells to cause spoilage. In addition, approximately 10 ppm sulfur dioxide was lethal to *Leuconostoc*, however *Lactobacillus* and *Pediococcus* were shown to be more tolerant to sulfur dioxide (Zoecklein et al., 1995). Since *Lactobacillus* is more s similar to *A. acidoterrestris*, more than 10 ppm free molecular sulfur dioxide may need to be present to inhibit this microorganism.

In wine, a 10,000-fold reduction of lactic acid bacteria and other spoilage microbes was achieved when 0.8 ppm molecular free sulfur dioxide was present (Zoecklein et al., 1995). However, concentrations above 0.8 ppm were needed to inhibit *A. acidoterrestris* for the duration of 29 days.

Dimethyl dicarbonate

Dimethyl dicarbonate did not cause any significant reduction of *A. acidoterrestris* in apple or tomato juice at 42˚C after 30 days. Dimethyl dicarbonate reacts with enzymes to inactivate them after hydrolysis of the compound (Jay, 2000; Ough, 1983). It has been reported that 20-30 ppm dimethyl dicarbonate was effective in reducing *Lactobacillus* and 250 ppm dimethyl dicarbonate reduced levels of aerobic plate counts in tomato juice. However, levels of 500 ppm did not significantly reduce the levels of *A. acidoterrestris* by day 29 in this study. Dimethyl dicarbonate may have been ineffective due to its bactericidal mechanism, or it may have not provided enough of a residual to prevent *A. acidoterrestris* spore outgrowth. In addition, certain juice components such as sugars, water, and phenols may have caused it to hydrolyze before inhibition of the spores could occur. Temperature of storage may have also had a negative effect on this antimicrobial. According to Ough, (1983), higher temperatures cause increased hydrolysis. DMDC in water at 40˚C has a half-life of approximately 1 - 2 minutes compared to a half-life of approximately 20 minutes at 20˚C (Ough, 1983). Therefore, the DMDC hydrolyzes very quickly and may be ineffective after the hydrolysis, which would occur rapidly at 42˚C. Although the initial effect of dimethyl dicarbonate may cause a reduction in vegetative cells, overall, dimethyl dicarbonate would not be effective in preventing *A. acidoterrestris* during high storage temperatures over time against spores.

Ascorbic Acid

Ascorbic acid caused no significant reduction in *A. acidoterrestris* populations. Ascorbic acid reduces bacteria levels by lowering the oxygen availability (Bauernfield, 1982). Cerny et al., (2000) stated that 150 ppm ascorbic acid reduced the level of *A. acidoterrestris* due to decreased redox potential and available oxygen, but 100 ppm ascorbic acid promoted growth of the organism. It was not stated whether the ascorbic acid reduced the spores or vegetative cells of *A. acidoterrestris*, although it is suspected that vegetative cells were inhibited. In this study, levels as high as 500 ppm ascorbic acid in combination with 304 and 340 ppm ascorbic acid

already present in apple juice and tomato juice, respectively, did not reduce *A. acidoterrestris* spores. It is suspected that the spores remained stable because the mechanism of action for ascorbic acid would not be effective on spores, because oxygen is not a limiting factor in the spore state of *A. acidoterrestris*.

This study suggests that nisin and lysozyme may be effectively used in apple and tomato juice at levels ≥ 3 IU/ml and ≥125 ppm, respectively even when the storage temperatures reach 42ºC for at least 29 days. Cinnamic acid - ≥ 125 ppm, nisin - 1 IU/ml, and potassium sorbate - ≥ 250 ppm may be used to prevent spoilage when ≤ 4-5 log spores/ml are present in the juice products. . Therefore, under the conditions of this study, these antimicrobial treatments may prevent *A. acidoterrestris* spoilage even when stored at optimum growth temperatures. However, labeling issues for cinnamic acid, lysozyme, and nisin need to be addressed. In addition, the cloudiness caused by lysozyme in clear juices and the odor impact of cinnamic acid needs to be addressed before commercial use. Overall, there has been little research on the inhibition and inactivation of *A. acidoterrestris* by these antimicrobials, so different juices, different antimicrobial levels, different storage temperatures, smaller load of spores or vegetative cells, and combinations of antimicrobials may need to be explored.

Overall, there has been little research on the inhibition and inactivation of *A. acidoterrestris* by these antimicrobials, so different juices, different antimicrobial levels, longer incubations times, different storage temperatures, smaller load of spores or vegetative cells, and combinations of antimicrobials may need to be explored.

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TABLE 1: Mean *Alicyclobacillus acidoterrestris* populations present in juices after addition of different antimicrobials (n = 3,456).

	Bacterial Populations Present in Juice [a] (Log CFU/ml ±SD)[b]	
Treatment	Apple Juice	Tomato Juice
Control	4.91±0.65 A	5.43±0.75 A
Ascorbic acid - 125 ppm	4.47±0.90 AB	4.85±0.67 AB
Ascorbic acid - 250 ppm	4.35±0.84 ABC	4.71±0.64 BC
Ascorbic acid - 500 ppm	4.07±0.94 BCD	4.53±0.63 BC
Sodium benzoate - 250 ppm	4.34±0.35 ABCD	4.28±0.33 BCD
Sodium benzoate - 500 ppm	4.43±0.28 ABC	4.44±0.25 BCD
Sodium benzoate - 1,000 ppm	4.34±0.22 ABCD	4.53±0.21 BC
Dimethyl dicarbonate - 125 ppm	4.43±0.55 ABC	4.72±0.42 ABC
Dimethyl dicarbonate - 250 ppm	4.42±0.45 ABC	4.56±0.45 BC
Dimethyl dicarbonate - 500 pm	3.65±1.14 CDEF	4.27±0.69 BCD
Sodium metabisulfite - 250 ppm	4.17±1.28 BCD	4.19±1.16 CD
Sodium metabisulfite - 500 ppm	3.91±1.42 BCDE	3.96±1.17 CD
Sodium metabisulfite - 1,000 ppm	3.47±1.56 DEFG	3.63±1.50 DE
Potassium sorbate - 250 ppm	3.02±0.78 FGH	3.65±1.14 EF
Potassium sorbate - 500 ppm	3.12±0.69 FGH	3.08±1.00 EF
Potassium sorbate - 1,000 ppm	3.31±0.58 EFG	2.86±1.44 F
Cinnamic acid - 125 ppm	2.53±0.84 HI	3.01±0.71 F
Cinnamic acid - 250 ppm	2.84±0.70 GHI	2.88±0.72 F
Cinnamic acid - 500 ppm	3.67±0.50 CDEF	3.01±0.89 F
Nisin - 1 IU/ml	2.31±1.75 I	0.78±1.23 G
Nisin - 3 IU/ml	0.32±0.79 J	0.04±0.20 H
Nisin - 5 IU/ml	0.07±0.33 J	0.04±0.21 H
Lysozyme - 25 ppm	0.28±1.13 J	0.55±1.12 GH
Lysozyme - 75 ppm	0.12±0.63 J	0.00±0.00 H
Lysozyme - 125 ppm	0.00±0.00 J	0.00±0.00 H

[a] Bacterial population values are means of populations present in juice averaged over the 29 day period with 3 reps.
[b] Mean values with different letters in the same column are significantly different ($p<0.05$). There were no significant differences between juices for each treatment ($p>0.05$).

TABLE 2: Undissociated acid present in apple and tomato juice at day 0 before binding based on stoichiometry calculations (n = 12)

		Amount of Undissociated Acid Present (ppm)	
Antimicrobial	Amount of Antimicrobial Salt added (ppm)	Apple	Tomato
Sodium metabisulfite	1,000	7.4	1.5
	500	3.7	0.7
	250	1.8	0.4
Sodium benzoate	1,000	704	414
	500	352	207
	250	176	104
Potassium sorbate	1,000	707	579
	500	353	290
	250	177	145
Trans-cinnamic acid	500	451	320
	250	225	160
	125	113	80

TABLE 3: Brix, pH, total phenols, and insoluble solids content in apple and tomato juices (n = 30).

Test	Apple Juice	Tomato Juice
pH	3.50	4.21
Brix	11.4º Brix	5.3º Brix
Insoluble Solids	0%	44.6%
Vitamin C	340 ppm	304 ppm
Total phenols	10.4ppm	24.5 ppm

TABLE 4: Free and molecular free sulfur dioxide in apple and tomato juice at days 0, 15, and 30 (n = 18).

Juice	Day	Free Sulfur Dioxide	Molecular Free Sulfur Dioxide
Apple	0	436±27 ppm	>0.8 ppm
	15	43±15 ppm	0.8 ppm
	30	12±15 ppm	<0.5 ppm
Tomato	0	166±17 ppm	>0.8 ppm
	15	159±31 ppm	>0.8 ppm
	30	60±6 ppm	<0.5 ppm

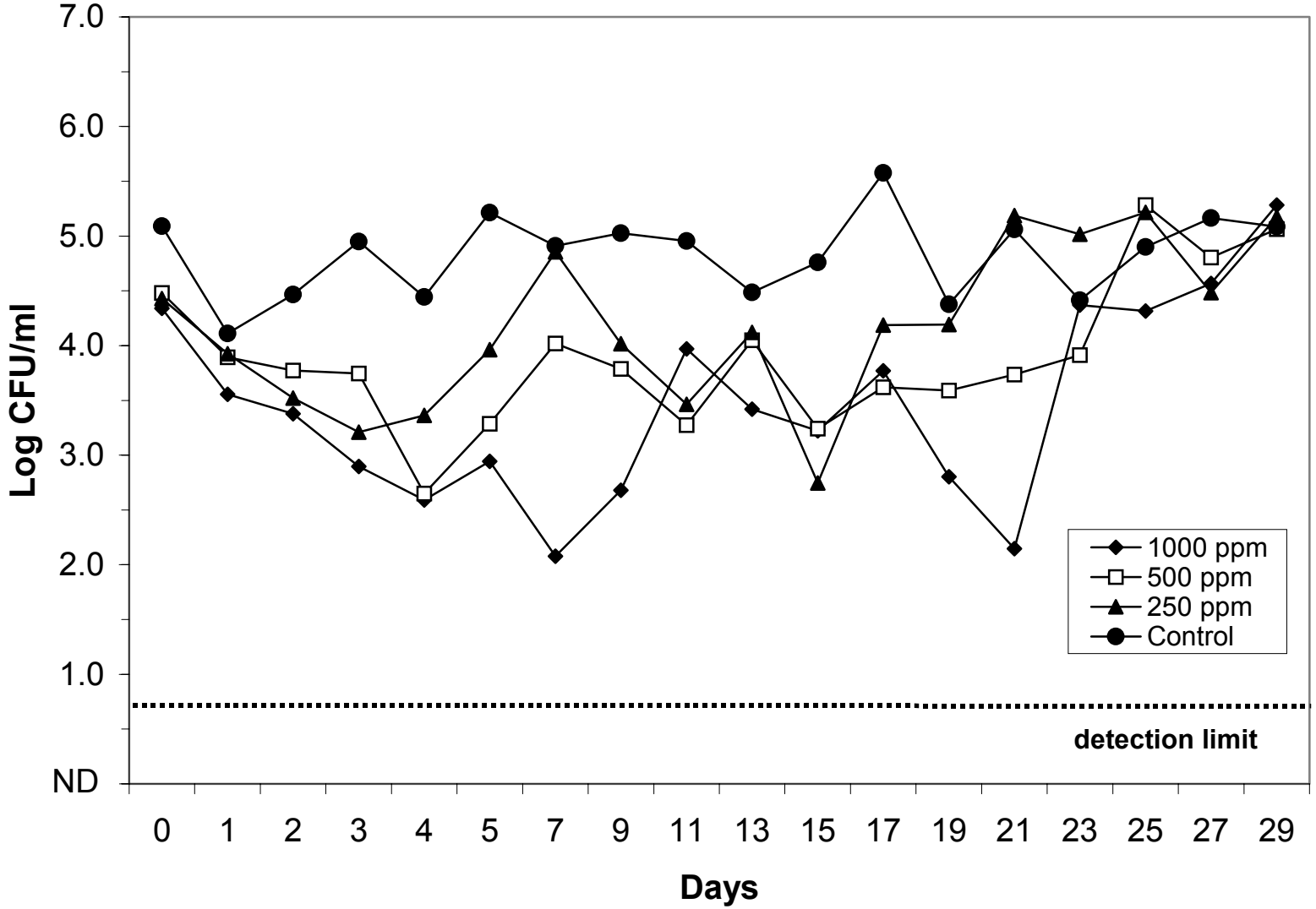

FIGURE 1: Effect of sodium metabisulfite on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 432). ND = not detected by spiral plating or enrichment.

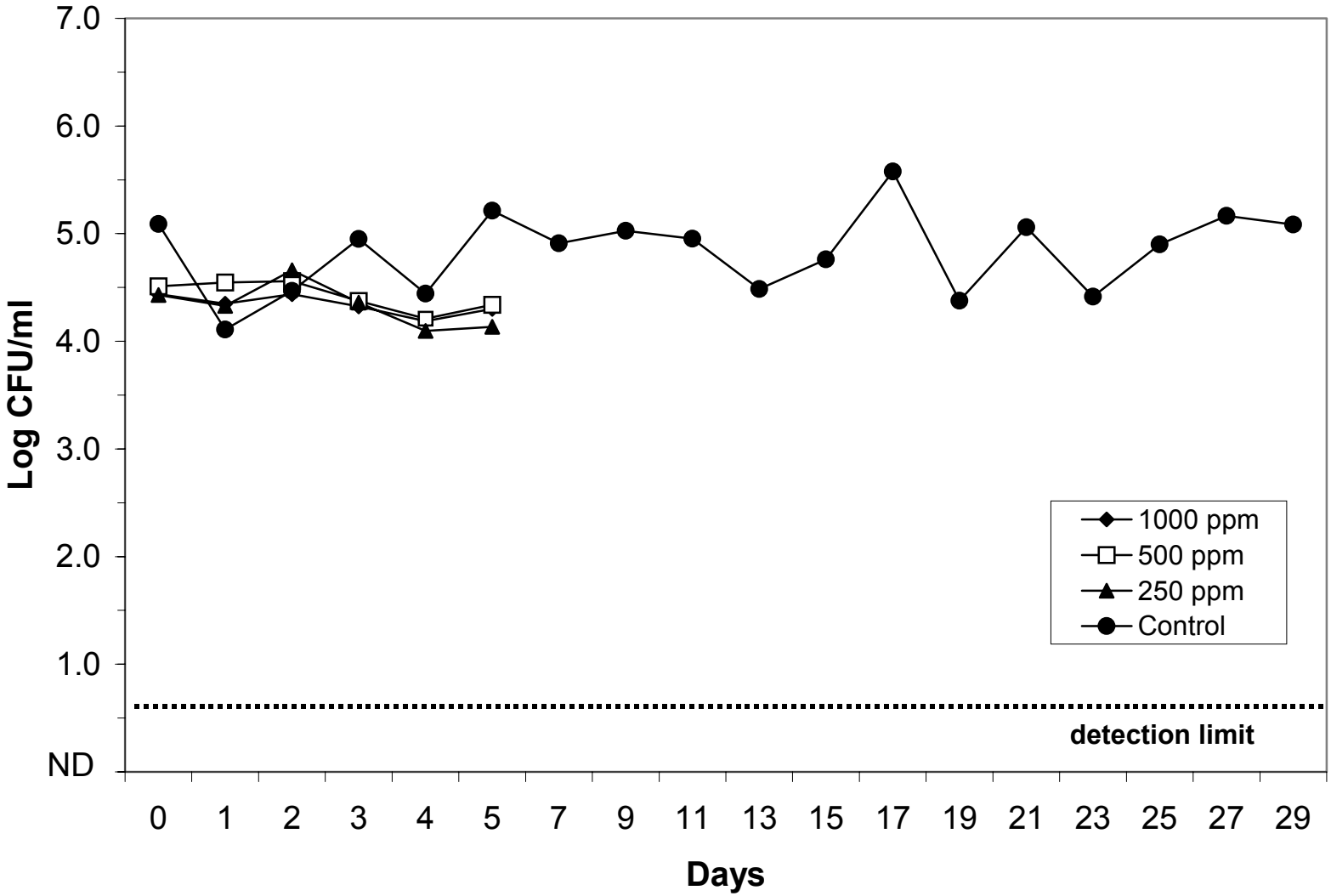

FIGURE 2: Effect of sodium benzoate on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 180). ND = not detected by spiral plating or enrichment.

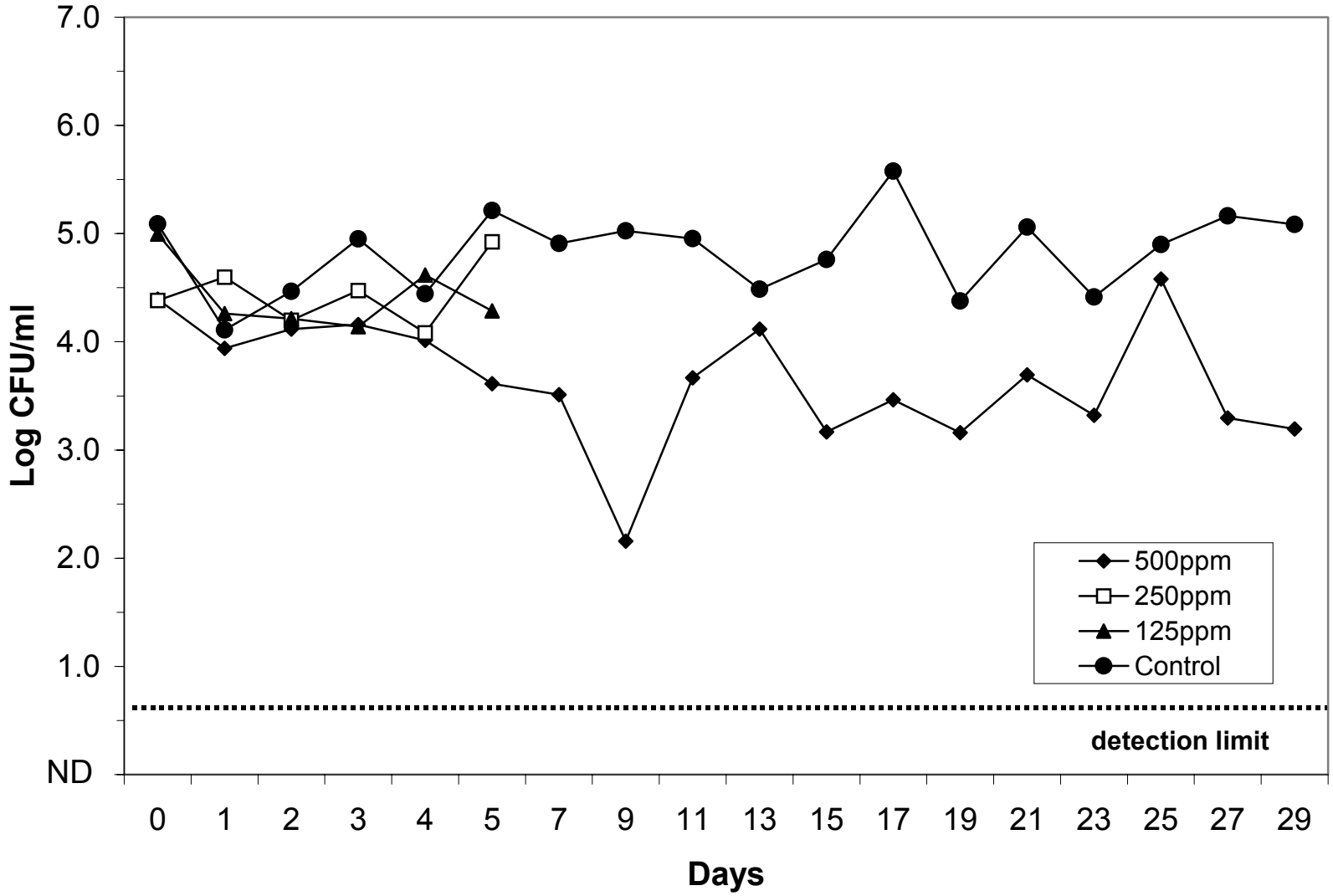

FIGURE 3: Effect of dimethyl dicarbonate on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 252). ND = not detected by spiral plating or enrichment.

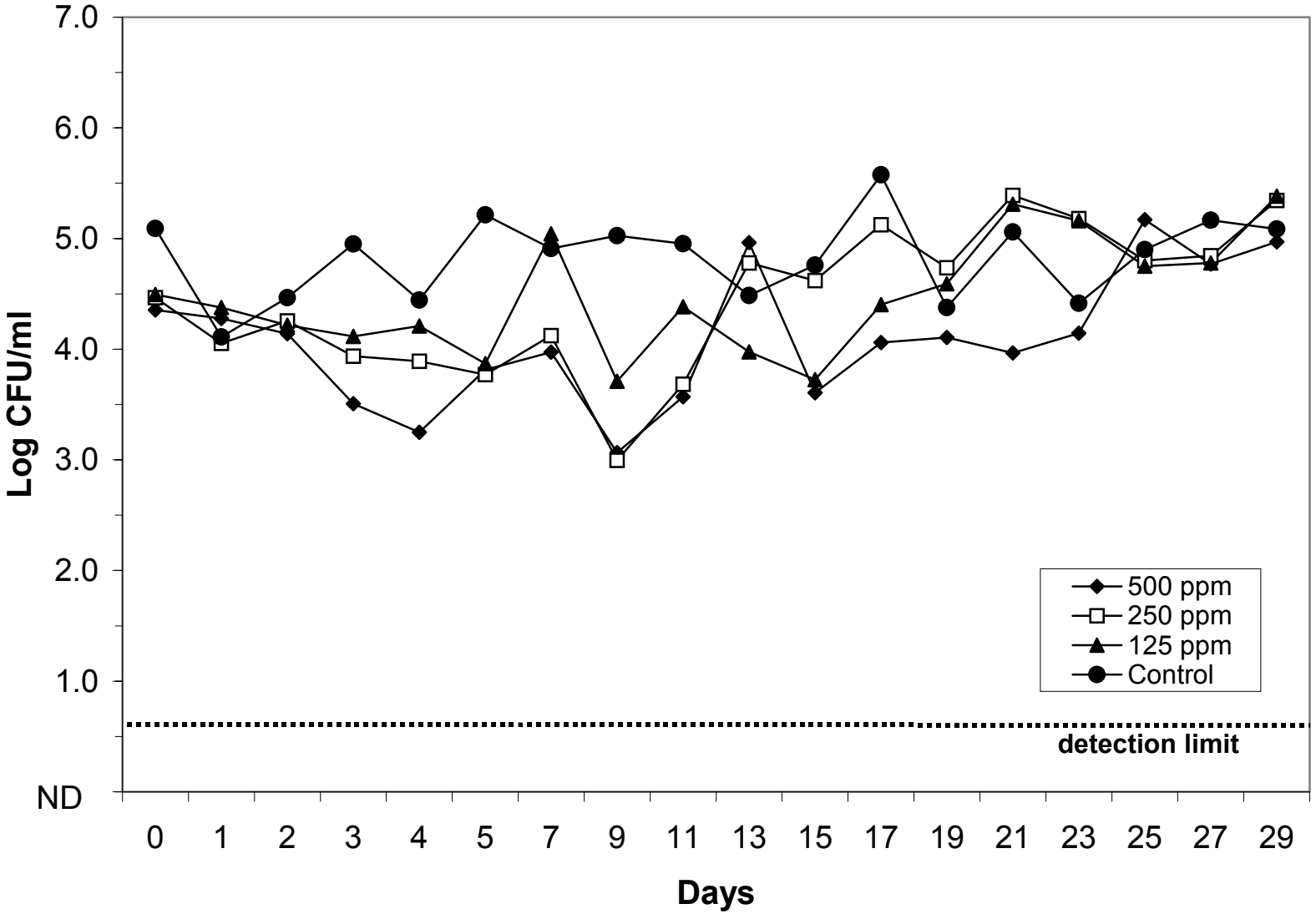

FIGURE 4: Effect of ascorbic acid on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 432). ND = not detected by spiral plating or enrichment.

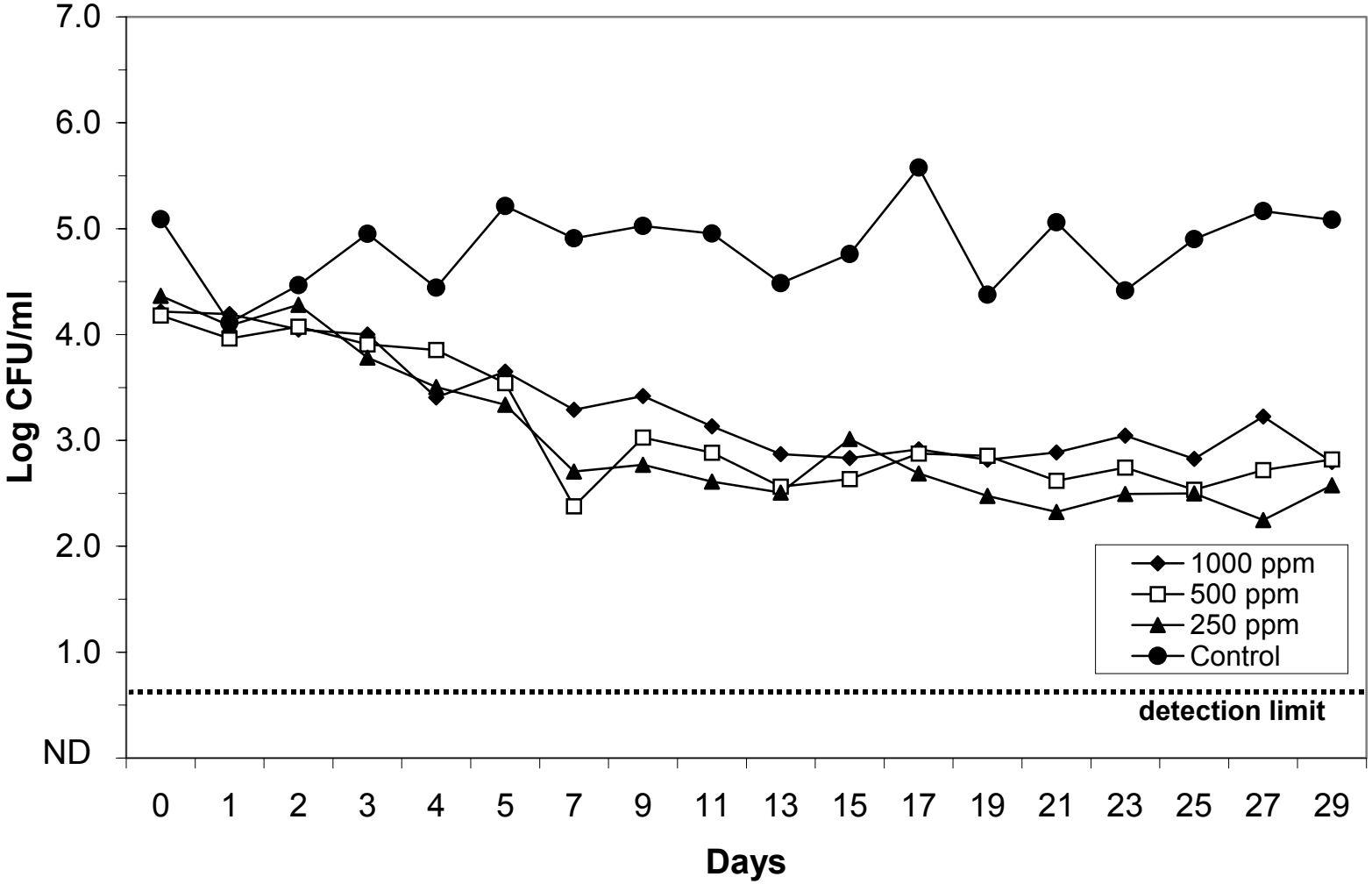

FIGURE 5: Effect of potassium sorbate on the germination and survival of *A. acidoterrestris* spores in apple juice (n =432). ND = not detected by spiral plating or enrichment.

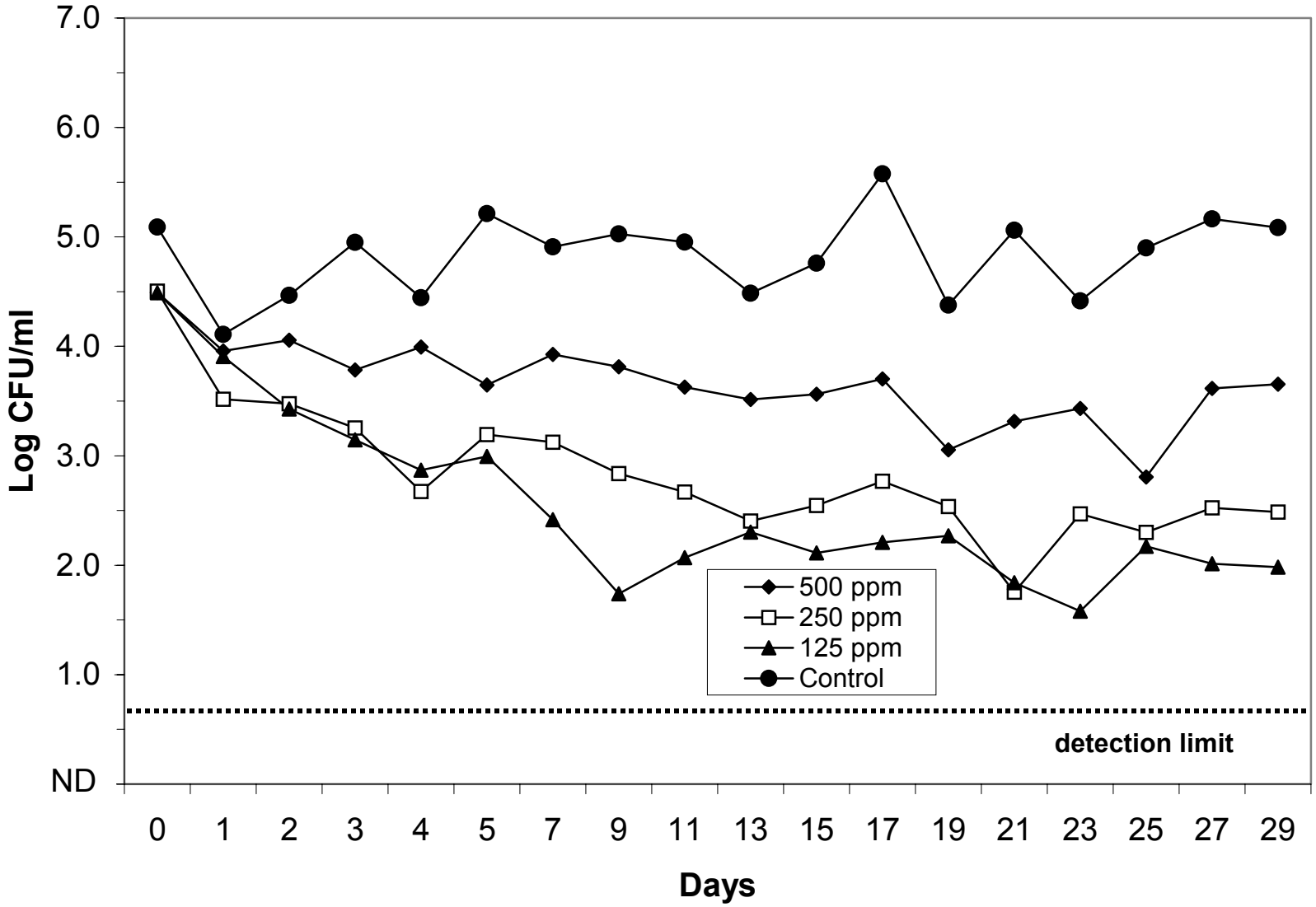

FIGURE 6: Effect of cinnamic acid on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 432). ND = not detected by spiral plating or enrichment.

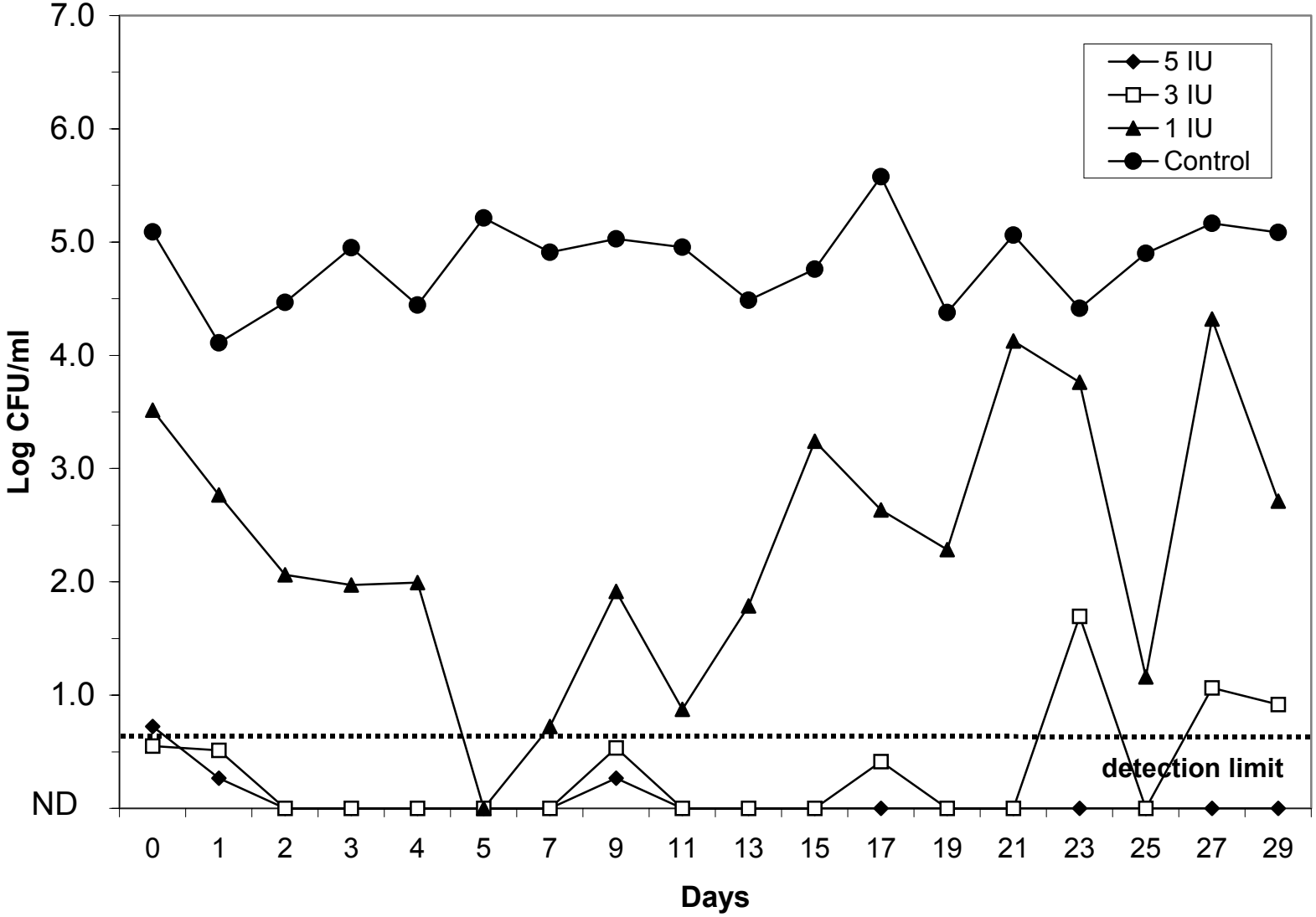

FIGURE 7: Effect of nisin on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 432). ND = not detected by spiral plating or enrichment.

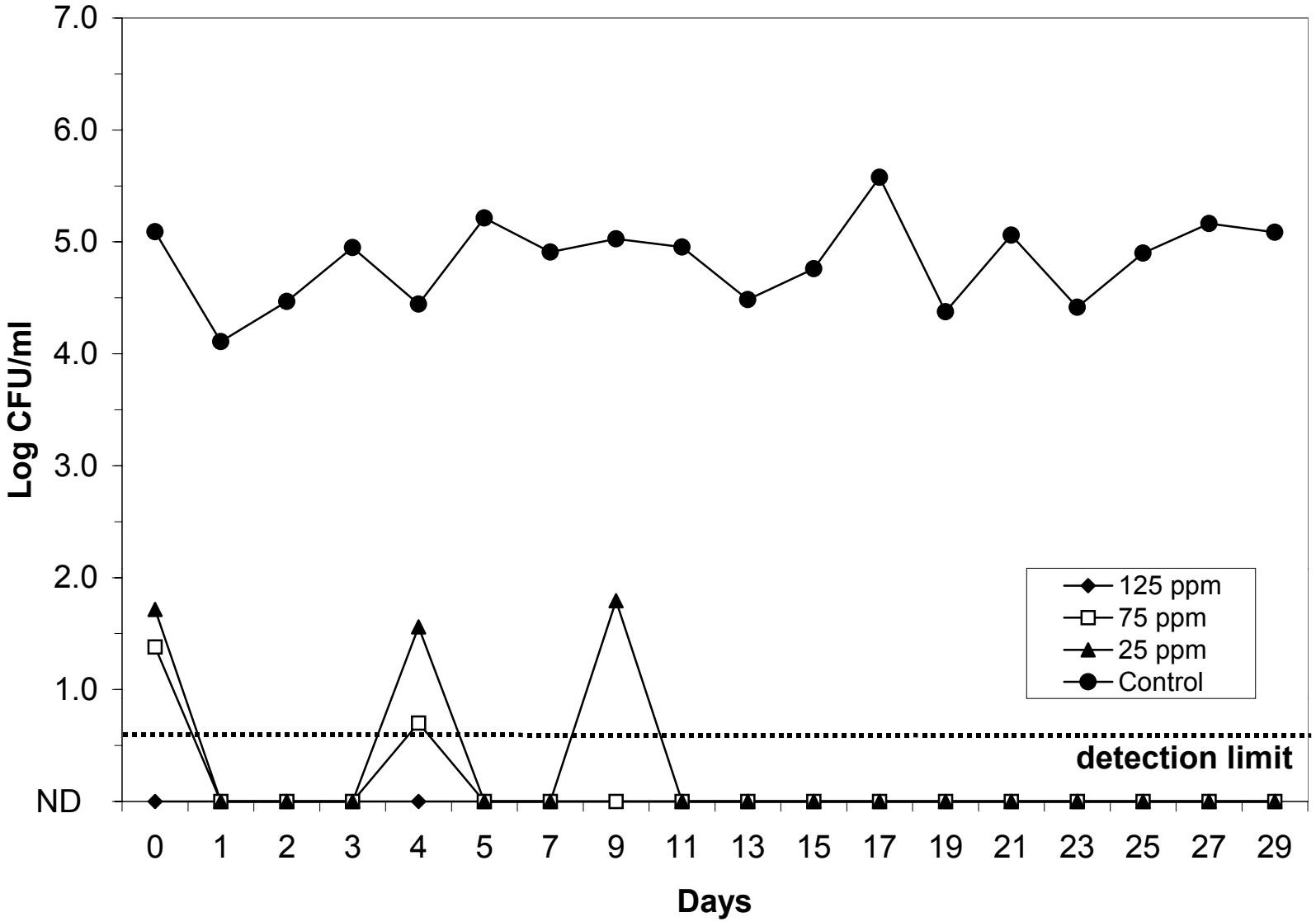

FIGURE 8: Effect of lysozyme on the germination and survival of *A. acidoterrestris* spores in apple juice (n = 432). ND = not detected by spiral plating or enrichment.

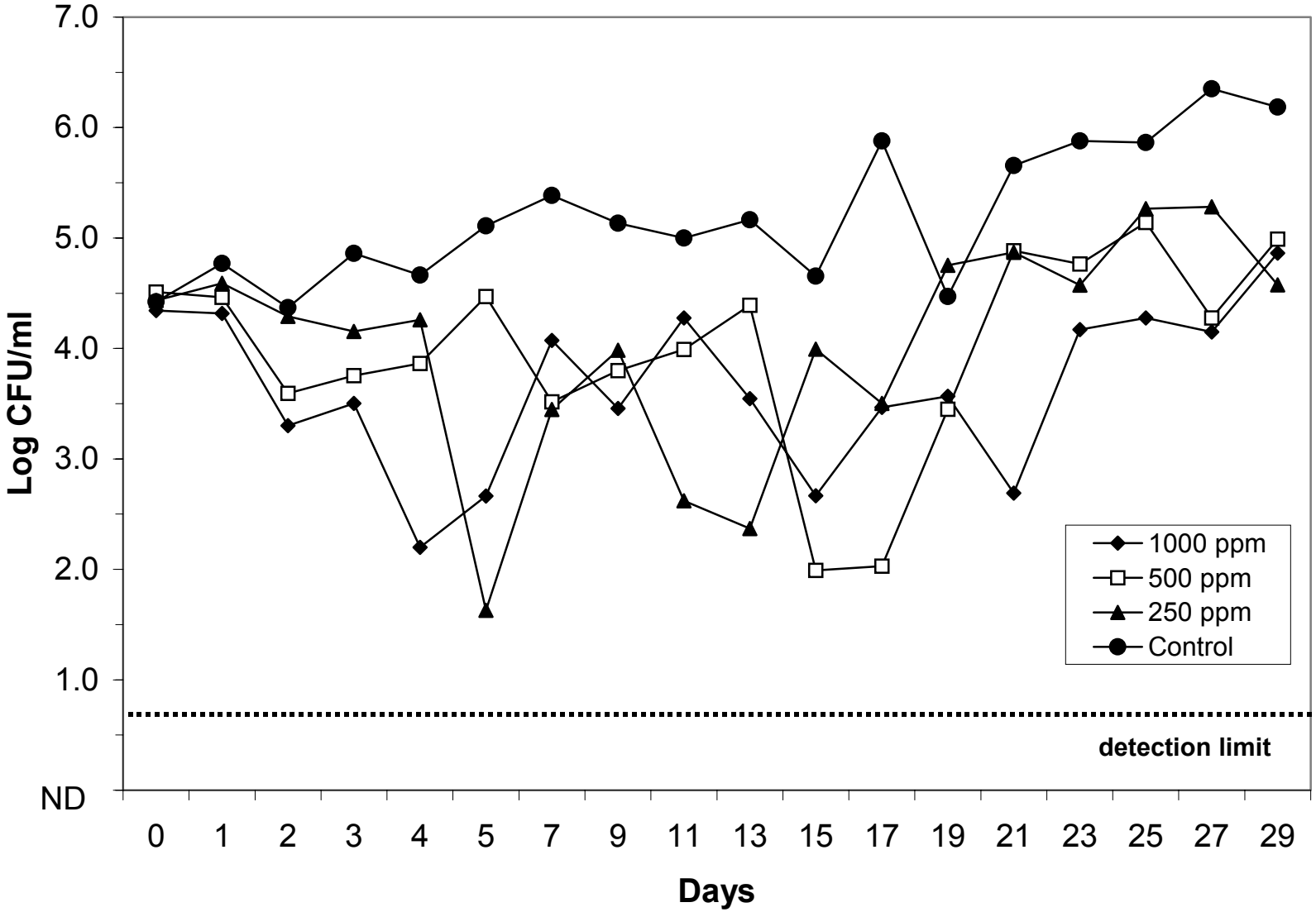

FIGURE 9: Effect of sodium metabisulfite on the germination and survival of *A. acidoterrestris* spores in tomato juice (n =432). ND = not detected by spiral plating or enrichment.

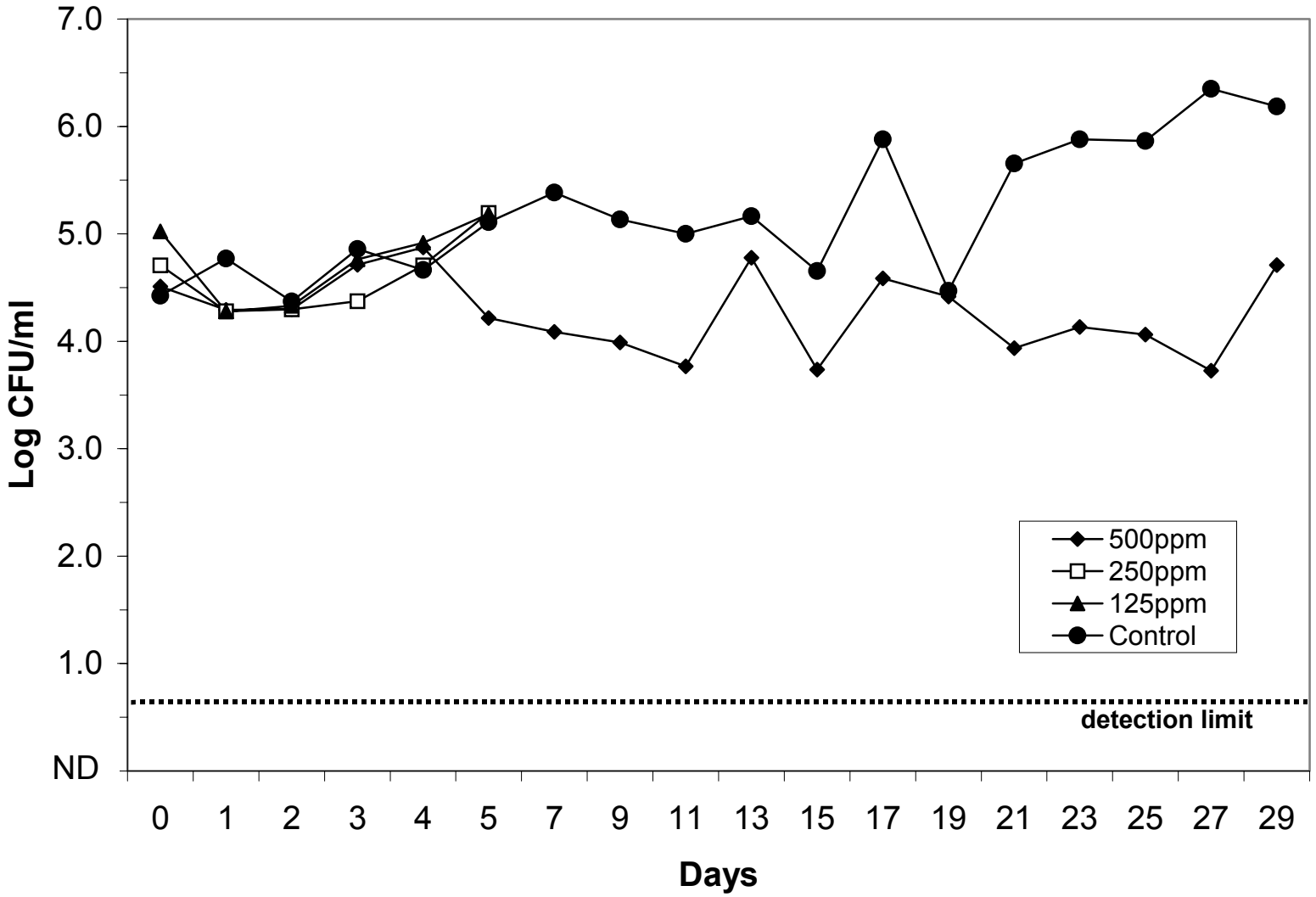

FIGURE 10: Effect of dimethyl dicarbonate on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 252). ND = not detected by spiral plating or enrichment.

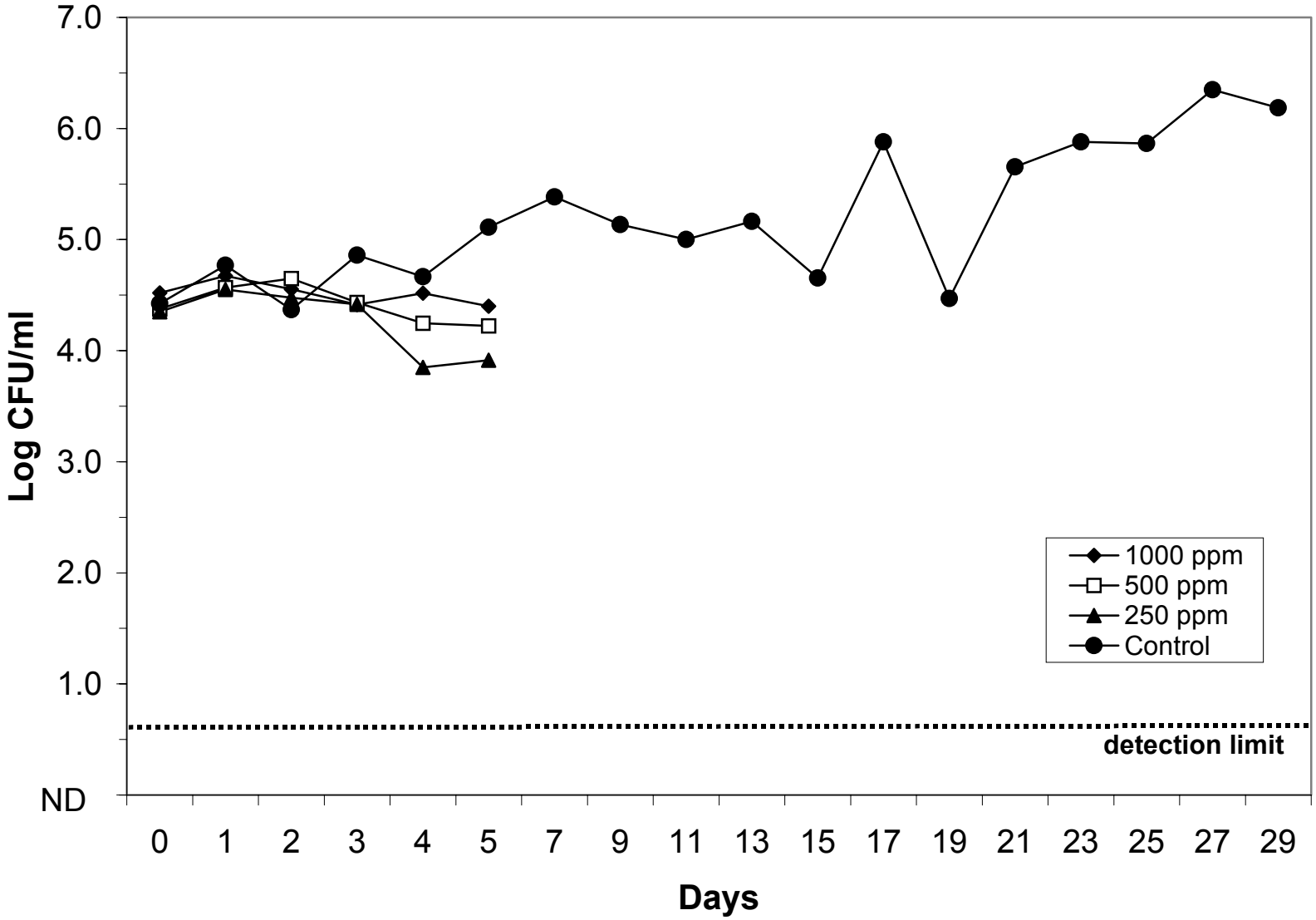

FIGURE 11: Effect of sodium benzoate on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 180). ND = not detected by spiral plating or enrichment.

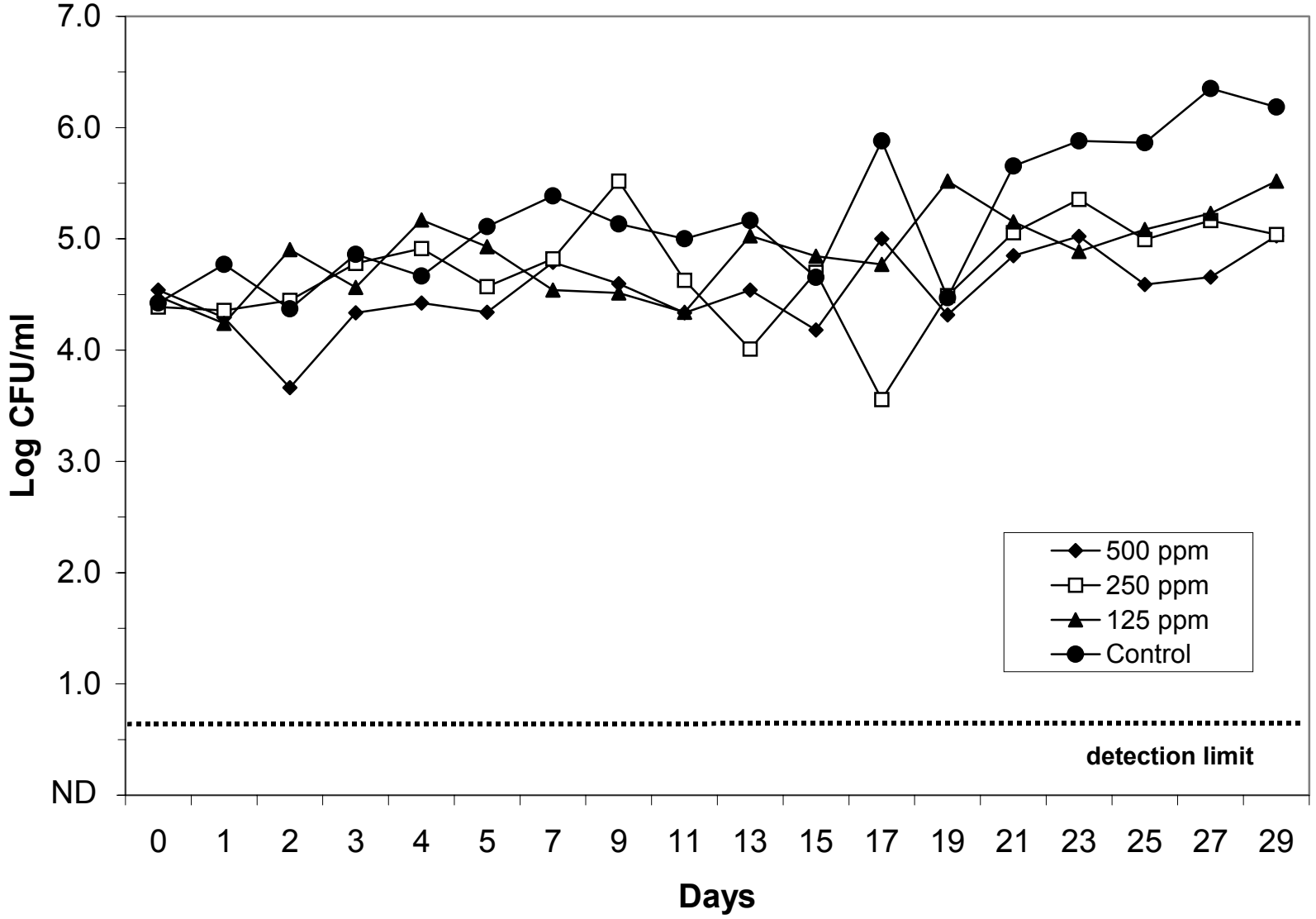

FIGURE 12: Effect of ascorbic acid on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 432). ND = not detected by spiral plating or enrichment.

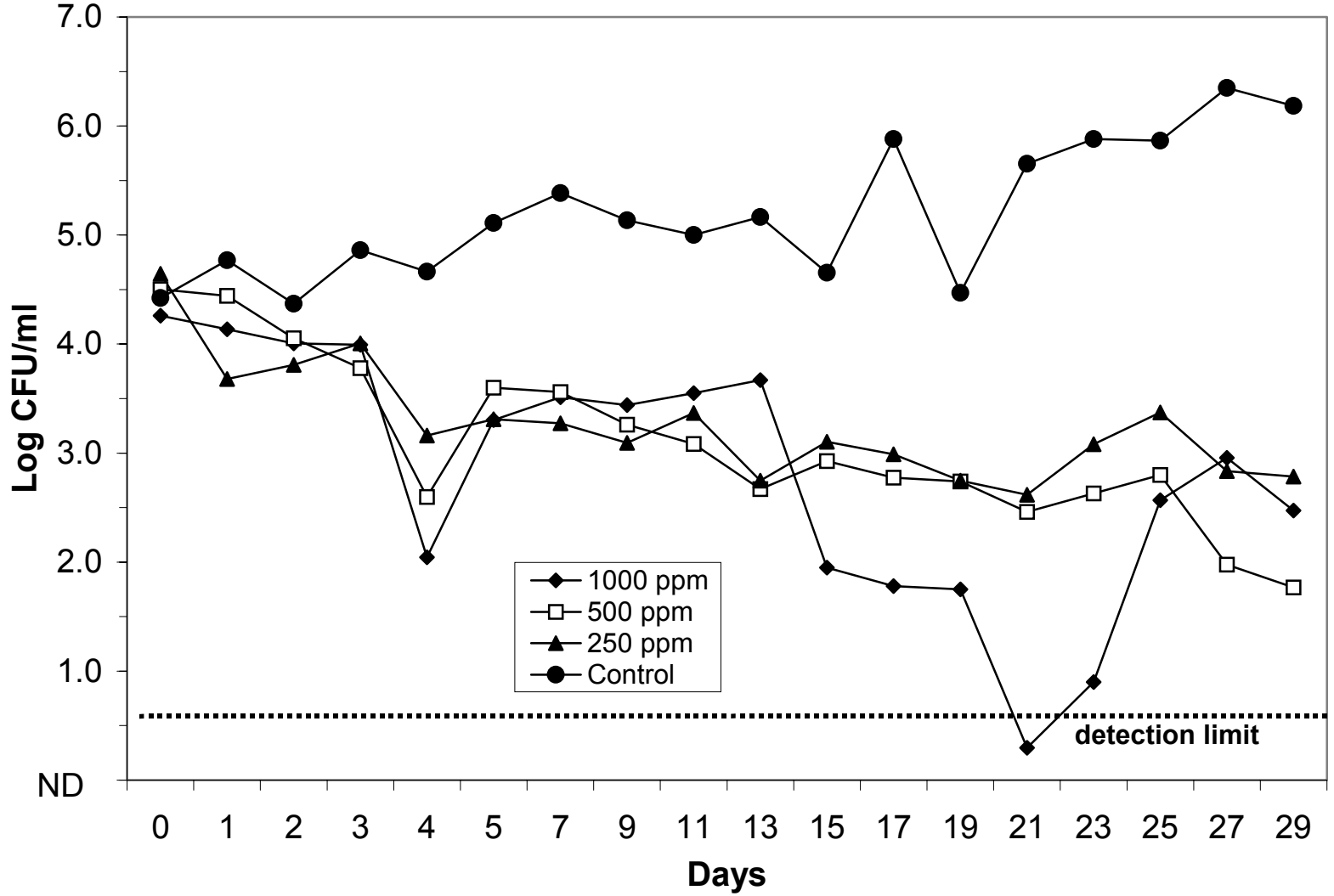

FIGURE 13: Effect of potassium sorbate on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 432). ND = not detected by spiral plating or enrichment.

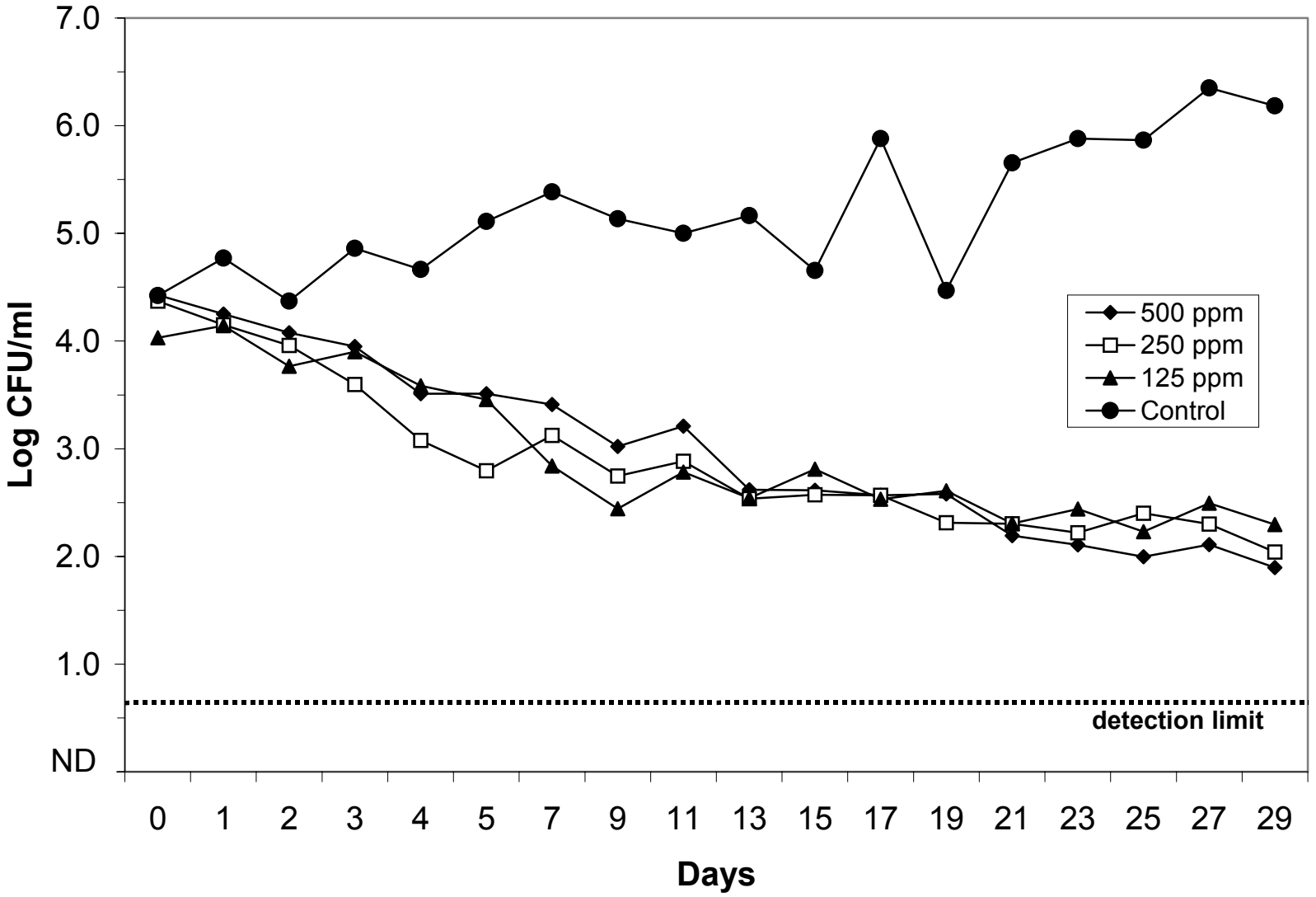

FIGURE 14: Effect of cinnamic acid on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 432). ND = not detected by spiral plating or enrichment.

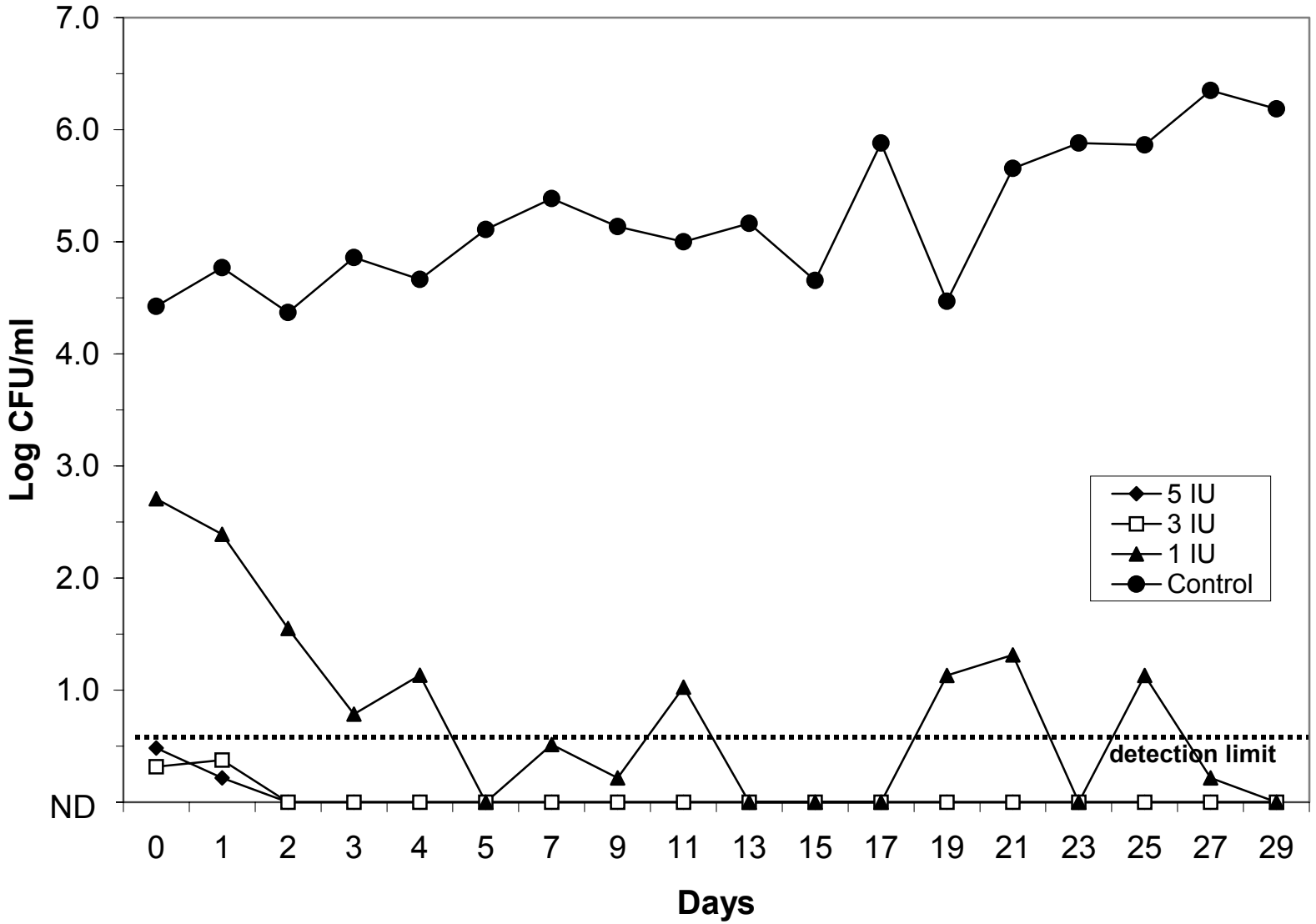

FIGURE 15: Effect of nisin on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 432). ND = not detected by spiral plating or enrichment.

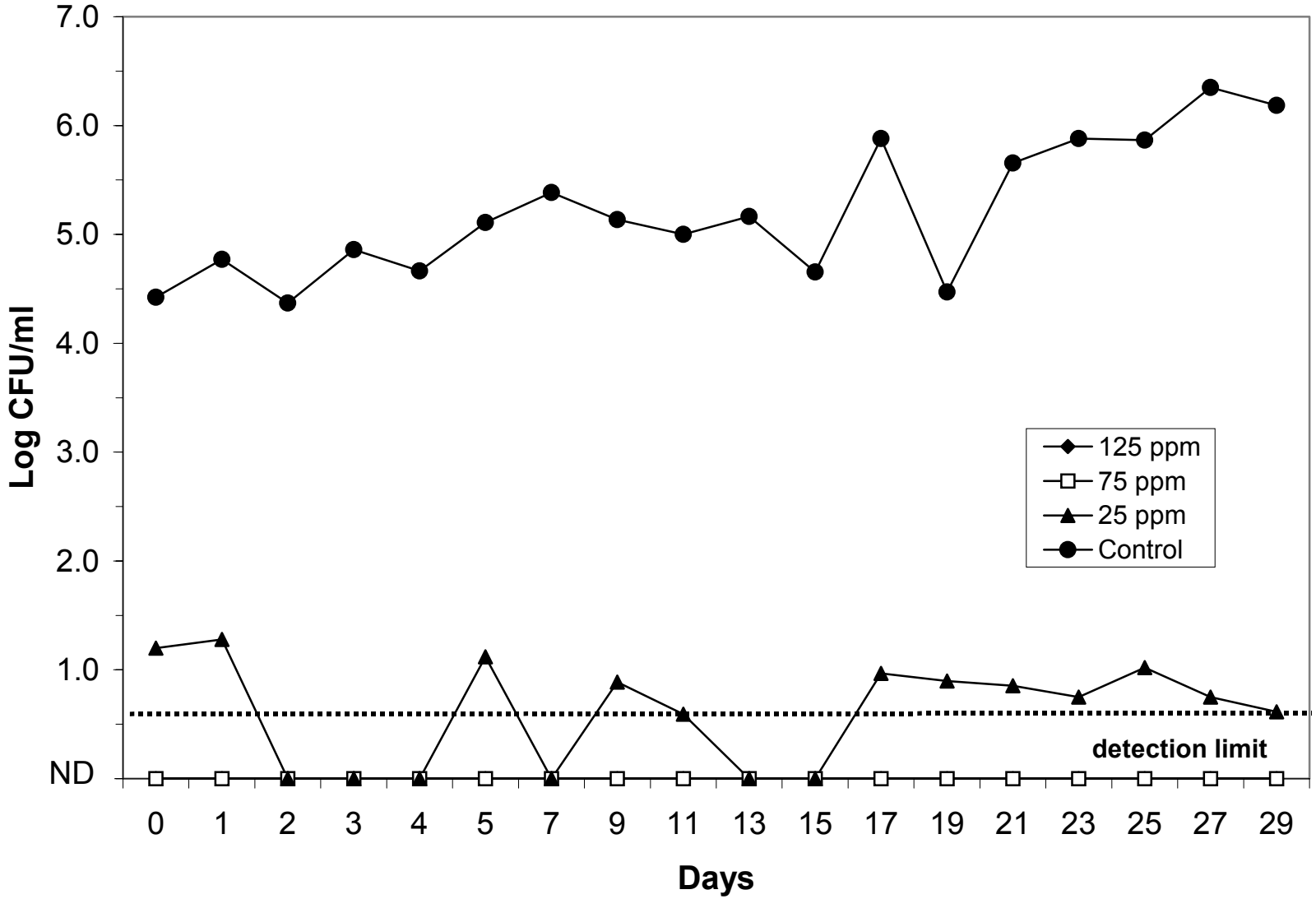

FIGURE 16: Effect of lysozyme on the germination and survival of *A. acidoterrestris* spores in tomato juice (n = 432). ND = not detected by spiral plating or enrichment.

SUMMARY

Recently, fresh juices, pasteurized shelf stable juices and juice blends have been recognized in several spoilage incidences. It was concluded that the microorganism responsible, *Alicyclobacillus acidoterrestris*, is able to withstand the pasteurization and hot-fill-hold process applied to juices and the acidic environment that usually prevents other spoilage organisms from growing (Eguchi, 1999). This organism causes off-odors and off-flavors in the juices that is undetectable to producers until consumer complaints are received (Walls and Chuyate, 2000).

This microorganism has been found throughout many stages of processing: from the incoming fruit to water used to finish the beverages, suggesting that one ingredient or aspect of processing is not the only cause of spoilage, thus making removal more difficult (Eguchi, 1999). The reduction of *A. acidoterrestris* has been a demanding challenge because the organism is ubiquitous, has the ability to survive high temperatures for a period of time, resists thermal condensation, and is highly resistant to many cleansers, sanitizers, and other prevention methods (Parrish, 2000). Therefore, processing changes may not inhibit this microbe in all products and instances without causing excessive energy costs or decreased organoleptic qualities in the juices.

A survey of juice producers showed that 35% of respondents have had at least one spoilage incidence characteristic of *A. acidoterrestris* spoilage in a five-year span. The spoilage caused loss on average of more than half of their product (Walls and Chuyate, 2000). In addition, *A. acidoterrestris* spoilage is unable to be recognized under visible inspection procedures so producers are unaware of the problem until it reaches the costumers. Spoilage by *A. acidoterrestris* may relate to scrapping costs, customer dissatisfaction, loss of brand quality, and cost of removing organism from production environments (Borlinghaus, 1997; Eguchi, 1999; Jensen, 1999; Walls and Chuyate, 2000). Therefore, a successful intervention needs to be applied to prevent *A. acidoterrestris* spoilage in juices, juice blends, and beverages containing juice.

Fruit juice producers are looking for a control method for *A. acidoterrestris* while not drastically affecting the quality of the juice or beverage. The use of antimicrobial treatments may provide juice and beverage producers a potential control method for this spoilage organism in their products.

The objectives of this study were 1) determine the optimum medium and diluent combination for enumerating *A. acidoterrestris* spores and vegetative cells. (2) determine the growth rate and characteristics of *A. acidoterrestris* spores and vegetative cells in apple, white grape, orange, and tomato juices, (3) determine most effective antimicrobials and the optimum level of antimicrobial to prevent spoilage and spore outgrowth of *A. acidoterrestris* in apple and tomato juice over a 29 day time period at 42ºC.

The results of the first objective showed that a detection level of 3.73, 3.27, and 3.26 log CFU/ml was achieved when the *A. acidoterrestris* spore culture was enumerated using peptone, peptone pH 4.0, and Bacillus Broth on Orange Serum Agar, respectively. On K Agar, values of 3.59, 3.74, and 3.59 log CFU/ml were detected using peptone pH 4.0, peptone, and Bacillus Broth, respectively. The enumeration of *A. acidoterrestris* spores was the most effective when using K agar combined with peptone, or Orange Serum Agar combine with peptone pH 4.0 as the diluent. Enumeration of *A. acidoterrestris* spores/vegetative cells, showed that 6.18, 6.03, and 6.09 log CFU/ml was recovered using peptone pH 4.0, peptone, and Bacillus Broth on K Agar, respectively. On Orange Serum Agar, peptone pH 4.0 had the highest detection at 6.35 log CFU/ml. However, 6.25 and 6.18 log CFU/ml was found using peptone and Bacillus Broth on Orange Serum Agar, respectively. On Bacillus Medium, no spore or vegetative cells were observed above detection limits for either spores or spore combined with vegetative cells. Therefore, OSA combined with peptone pH 4.0 or K Agar combined with peptone was the most effective at enumerating spores and vegetative cells of *A. acidoterrestris*.

The results for the second objective showed the growth of spore/vegetative cells in juice followed the order: tomato = apple > grape = orange juice. *A. acidoterrestris* populations in orange and grape juices decreased to below the detection limit after two days. *A. acidoterrestris* populations in apple juice increased to approximately 5 log CFU/ml at day 6 and then stabilized at 4.5 log CFU/ml for the remainder of the 30 day sampling period. Populations in tomato juice peaked at 5.5 log CFU/ml at day 10 and remained at approximately log 4.6, until an increase to 5.55 log CFU/ml was observed at day 26-29.

In the spore study, the growth rate in juices followed the order: apple = tomato > orange = grape. *A. acidoterrestris* spores in grape juice decreased below the detection level after 2 days, and orange juice populations fell below the detection limit after 4 days. In both apple and tomato juices, the *A. acidoterrestris* populations increased to approximately 5.2 log CFU/ml at day 8 and

then decreased to 4.1 log CFU/ml over a 8 day period. For the remaining 14 days, *A. acidoterrestris* increased to approximately 4.6 log CFU/ml. Overall, *A. acidoterrestris* grew better in tomato and apple juices than in orange and grape juices. Spore germination and cell growth in tomato juice had an increase of spore germination and vegetative cell growth, whereas apple juice increased slightly and then stabilized over a period of time. In orange and grape juices, sustained growth of *A. acidoterrestris* was not observed after 2-6 days under conditions tested.

This study helps to show that even a short time of 2-8 days and low level of spores or vegetative cells at an optimum temperature may provide adequate growth for taint formation in certain juices. However certain juices, such as orange and white grape may need other factors present to sustain growth or may be able to only support growth of certain strains of the organism under ideal conditions. Therefore, certain juices may have a protective barrier against spoilage while other juices are highly susceptible to spoilage.

In the third objective, juice and antimicrobial level did not significantly affect the antimicrobial efficacy, however, antimicrobial type did significantly reduce levels of *A. acidoterrestris* populations. In apple juice, *A. acidoterrestris* population reductions were caused by the following antimicrobials (reduction in log CFU/ml): lysozyme - all levels (5.1), nisin - 5 IU/ml (5.1), nisin - 3 IU/ml (4.2), cinnamic acid - 125 ppm (3.1), cinnamic acid - 250 ppm (2.6), potassium sorbate - 250ppm (2.5), nisin - 1 IU/ml (2.4), potassium sorbate - 1,000 ppm (2.3), potassium sorbate - 500 ppm (2.3), dimethyl dicarbonate - 500 ppm (1.9), cinnamic acid - 500 ppm (1.4). No reduction in *A. acidoterrestris* population was seen for sodium metabisulfite - all levels, ascorbic acid - all levels, dimethyl dicarbonate -125 ppm and 250 ppm, and sodium benzoate - all levels. In tomato juice, *A. acidoterrestris* log reductions were caused by the following antimicrobials (reduction log CFU/ml): nisin - all levels (4.4), lysozyme - 125 ppm and 75 ppm (4.4), lysozyme - 25 ppm (3.8), potassium sorbate - 500 ppm (2.6), cinnamic acid - 500 ppm (2.5), cinnamic acid - 250 ppm (2.3), cinnamic acid - 125 ppm (2.1), potassium sorbate - 1,000 ppm (1.9), and potassium sorbate - 250 ppm (1.6). In tomato juice, *A. acidoterrestris* was not reduced when juices were treated with dimethyl dicarbonate - all levels, sodium benzoate - all levels, sodium metabisulfite - all levels, and ascorbic acid - at all levels.

Antimicrobial treatments of nisin - $\geq$ 3 IU/ml and lysozyme - $\geq$ 25 ppm reduced *A. acidoterrestris* populations and prevented spore germination throughout the duration of the

study. Cinnamic acid - ≥ 125 ppm, nisin - 1 IU/ml, and potassium sorbate - ≥ 250 ppm may be used to prevent spoilage when ≤ 4-5 log spores/ml are present in the juice products. Therefore, under the conditions of this study, these antimicrobial treatments may prevent *A. acidoterrestris* spoilage even when stored at optimum growth temperatures. However, labeling issues for cinnamic acid, lysozyme, and nisin need to be addressed. In addition, the cloudiness caused by lysozyme in clear juices and the odor impact of cinnamic acid needs to be addressed before commercial use. Overall, there has been little research on the inhibition and inactivation of *A. acidoterrestris* by these antimicrobials, so different juices, different antimicrobial levels, different storage temperatures, smaller load of spores or vegetative cells, and combinations of antimicrobials may need to be explored.

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APPENDIX 1: Effect of antimicrobial treatments for the control of *Alicyclobacillus acidoterrestris* in juices: experiment design layout

Juice	Tomato or Apple Juice																							
Anti-microbial	Lysozyme			Ascorbic acid			Sodium benzoate			Potassium sorbate			Cinnamic acid			Dimethyl dicarbonate			Nisin			Sodium metabisulfite		
Level	125 ppm	75 ppm	25 ppm	500 ppm	250 ppm	125 ppm	1000 ppm	500 ppm	250 ppm	1000 ppm	500 ppm	250 ppm	500 ppm	250 ppm	125 ppm	500 ppm	250 ppm	125 ppm	5 IU/ml	3 IU/ml	1 IU/ml	1000 ppm	500 ppm	250 ppm
	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0	0
	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1	1
	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2	2
	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3	3
	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4	4
	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5	5
	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7	7
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	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27	27
Day	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29	29
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	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	3	3	3	3	3	3	3	3	3	3	3	3

Curriculum Vitae

Angela D. Hartman

Angela Hartman was born in Roanoke, VA, where she attended Patrick Henry High School. She received her Bachelor's degree in Food Science and Technology from Virginia Tech. After an internship at Pepsi Co., she decided to continue her education towards a Mater's degree. Angela has been working on her graduate degree in Food Science at Virginia Tech in the area of Food Microbiology since August 2001. She is currently a member of International Association of Food Protection, Institute of Food Technologists, and the Food Science Club. Future plans are to continue her education in Food Science and Technology in the PhD program in the area of Food Microbiology and Food Safety.