INTERVENTION METHODS TO IMPROVE THE

MICROBIOLOGICAL SAFETY AND QUALITY OF JUICES

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INTERVENTION METHODS TO IMPROVE THE

MICROBIOLOGICAL SAFETY AND QUALITY OF JUICES

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Juices and beverages have increased in production and consumption in the U.S. over the last several decades due to health benefits and convenience. However, *Alicyclobacillus*, a significant spoilage bacterium in the juices and beverages, and patulin, a heat stable mycotoxin commonly associated with apple juice and cider, are critical concerns for juice and beverage industry.

Non-thermal treatments and techniques have been investigated as potential methods to control patulin and *Alicyclobacillus* in juices.

Ultraviolet radiation (UV) was used to control patulin in apple juice from concentrate (UV exposure from 14.2 to 99.4 mJ/cm^2) and kinetic degradation models between apple cider and apple juice from concentrate were compared. UV radiation was shown to effectively reduce patulin with minimal changes in physicochemical properties and sensory characteristics of apple juice from concentrate. Patulin reduction by UV radiation in apple juice from concentrate followed first-order kinetics.

Dimethyl dicarbonate (DMDC) with 0, 50, 125, and 250 ppm, and papain and bromelain (0, 100, and 1000 ppm) were used to investigate the inhibitory effect of

vegetative cells and spores of *Alicyclobacillus acidoterrestris* strains VF, WAC, and SAC in potato dextrose broth (PDB), apple juice, and orange juice. DMDC showed a significant reduction in vegetative cells and spores of *Alicyclobacillus* in all samples, with no changes in juice quality, whereas, papain and bromelain showed antimicrobial activity only against vegetative cells of all strains with all samples, and minimal alterations in juice quality and sensory attributes.

Various media and potential bacteriocin-producing lactic acid bacteria (LAB) were evaluated as alternative biopreservation methods to control *Alicyclobacillus*. Potato dextrose agar pH 3.5 (PDA) was the only medium that allowed the growth of *Alicyclobacillus* whereas sodium citrate and dipotassium phosphate (APT ingredients) inhibited *Alicyclobacillus* species. No potential bacteriocin-producing LAB were isolated from kimchi and fermented apple slurries.

Non-thermal treatments including non-thermal processing and natural antimicrobial compounds were investigated as alternative treatments to enhance the microbiological safety and quality of juices. The physicochemical and sensory properties of these alternative methods need to be preserved which assists in promoting the juice and beverage market through enhancing consumer's trust by ensuring consistently wholesome and safe products.

BIOGRAPHICAL SKETCH

Kitipong Assatarakul was born and raised in Bangkok, Thailand with his one older brother, Wittawat Assatarakul, and one younger sister, Titikarn Assatarakul.

He received B.Sc. in Food Technology in 2005 from Chulalongkorn University. After his undergraduate studies, he continued his graduate studies at the same University and completed his M.Sc. in 2007. During his graduate studies at Chulalongkorn University, he worked as a teaching and research assistant that was funded by The Thailand Research Fund for his graduate studies. At that time, he realized his aspiration to work as a lecturer at Chulalongkorn University so he decided to apply for a Government scholarship in the field of Food Science and Technology with Food Microbiology concentration to pursue his doctoral degree in the United States. He was the recipient of the Science and Technology Scholarship awarded by the Royal Thai government in 2007 and decided to come to Cornell University to pursue his Ph.D. degree with Dr. Randy W. Worobo, an Associate Professor of Food Microbiology.

After completing his Ph.D. program at Cornell, he will return to Thailand and work for Royal Thai government as a lecturer in the Department of Food Technology, Faculty of Science, at Chulalongkorn University.

To my beloved family,

my supportive friends,

and my motherland, Thailand

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opportunity to earn my precious experiences in the U.S.

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CHAPTER 1

JUSTIFICATION AND INTRODUCTION

The US Department of Agriculture and the US Department of Health and Human Services launched a recommendation of dietary guidelines that was aimed at increasing the daily intake of fruit and vegetables as a part of healthy eating and disease prevention (12). This recommendation was based on several reports that people in the US do not have sufficient dietary intake of fruits and vegetables. In response to the challenge to increase fruit and vegetable consumption, the food industry is posed with the development of new, exciting, healthy, convenient and safe fruit and vegetable products for consumers. Juices and beverages have met previous criteria, not only ease of delivery and high concentration of antioxidant and functional ingredients, but also a natural human requirement of fluids (14). Consequently, juices and beverages have increased in popularity by all-age groups of consumers due to their potential health benefits and convenience. Juice production and consumption have increased in the last twenty years; however, they can impact world economy not only positively such as market profits and overseas trades but also negatively as well when foodborne disease associated with juices and beverages and spoilage issues have been reported. According to the Economic Research Service (5), orange and apple juice are the first and second largest juice consumption in the US, respectively. However, 21 juice-associated outbreaks caused by pathogenic microorganisms associated with health problems in the U.S. including apple juice, apple cider, orange juice, and other juices, with 1,366 illnesses from 1995-2005 were reported according to Center of Disease Control and Prevention (CDC). Apple juice, apple cider, and orange juice were responsible

for 85% of these outbreaks. *Salmonella* and *Escherichia coli* O157:H7 were the causative agents for these outbreaks (13). Besides these significant health-related bacteria, *Cryptosporidium parvum* is also an important agent to juice outbreaks. Outbreaks associated with juices not only lead to economic loss, but also health consequences which can include mortalities. Therefore, food safety of juices and beverages is a critical concern.

Food safety has become the most important concern for food industries and consumers since it might cause severe health problems for consumers and economic losses (1). Based on the study of food safety, there are three categories of hazards causing food safety issues which include physical, chemical, and microbiological food hazards. Microbiological food hazards are different from physical and chemical food hazards because the hazards can increase in magnitude and severity with time and improper handling conditions. Physical hazards that include glass and wood, and chemical hazards such as chemical residues, and additives, usually enter the food products at predictable steps, while as microorganisms can potentially enter at any step of food processing (7). Moreover, microorganisms commonly cause large scale or multi-state outbreaks resulting in health problems and economic losses compared to physical and chemical hazards (13). Therefore, microbiological food safety is viewed as the most important issue for the food industry and consumers alike. As a consequence, the food industry has been challenged with improving the safety of food products, while retaining the physicochemical properties and sensory aspects that are expected by consumers.

Fruit juices such as apple juice and orange juice are considered as high acid beverages with their pH values in the range of 3.0-4.0 in apple juice and orange juice (4, 10). Therefore, yeast and mold are considered major sources of spoilage

in high acid juices such as apple juice and orange juice, since they are tolerant to high acidic conditions. These spoilage microorganisms are the targets for the juice and beverage industry to establish their thermal processing regimes (9). Due to the acidic pH of fruit juices and beverages, and high temperature treatment during production processes, it is sufficient to inactivate all non-spore forming microorganisms and bacterial spores such as *Bacillus coagulans* and *Clostridium pasteurianum* are unable to germinate under these acidic conditions (9). However, the large scale spoilage incident of processed apple juice associated with *Alicyclobacillus* contamination in Germany in 1982 changed the perception of the juice and beverage industry (2). As a consequence, the juice and beverage industries now consider *Alicyclobacillus* as a potential cause of spoilage in acidic juices or beverages with significant economic losses.

In addition to *Alicyclobacillus*, patulin is an important chemical hazard for the juice industry, particularly for apple juice and apple cider. Patulin is a heat stable mycotocin product as a secondary metabolite produced by certain species of *Penicillium*, *Aspergillus*, and *Byssochlamys* (6). A variety of acute and chronic effects in human have been associated wtih patulin. As a cause of adverse health effects, patulin has a maximum limit of 50 µg/l in fruit juices and their products, based on recommendations from *Codex Alimentarius* (3). Due the heat resistance of patulin and limited processing methods to reduce patulin levels in juice, the removal of patulin from finished juices is limited. Processing methods to prevent and/or eliminate patulin from finished juice are needed by the juice industry.

Intervention methods such as antimicrobial substances and non-thermal techniques to enhance the safety and quality of juices have been become an area of research interest in the last decade. These alternative approaches are directed

towards the prevention of bacterial and fungal spoilage, and to reduce the risk of foodborne illness without a loss of product quality (8). Therefore, antimicrobial substances or protective cultures may be a means to control *A. acidoterrestris* spoilage in juices, and non-thermal processing methods such as ultraviolet radiation may allow for the reduction of patulin in juices.

Additional research is necessary to investigate the inhibitory effects of antimicrobial substances that include dimethyl dicarbonate, papain, and bromelain as a means to control the growth of *A. acidoterrestris* in apple juice and orange juice over the shelf life of these products. In addition, UV radiation for patulin reduction in apple cider has been shown to be effective, but the vast majority of worldwide consumption of apple juice is from concentrate. Additional research is needed to validate UV processing as a patulin reduction method for apple juice from concentrate. Most importantly, chemical and physical properties and sensory evaluation are also critical criteria to ensure the quality of juice samples after new treatments or techniques are being used.

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CHAPTER 2
LITERATURE REVIRW

2.1 *Alicyclobacillus*

2.1.1 History

Uchino and Doi (78) first isolated this spore-forming bacterium from hot spring water in Japan and they also reported that this bacterium was able to grow under acidic conditions and its characteristics were similar to *Bacillus coagulans*. A few years later, Darland and Brock (22) reported the isolation of bacteria from a variety of thermal and acidic conditions at the Hawaiian Volcano National Park and Yellow Stone National Park with characteristics that were similar to the bacterium that Uchino and Doi reported. They named this new bacterium as a new species of *Bacillus*; *Bacillus acidocaldarius* based on taxonomical properties and also reported that it contains unusual ω-cyclohexyl fatty acid as a main component of the cellular membrane.

In the early 1980's, an acidothermophilic bacillus was isolated from spoiled apple juice and a relationship was proposed between this bacillus and *B. acidocaldarius*. This organism can be grown in acidic pH (2.5-5.5) and survive pasteurization (160°F for 6 seconds). Cell membrane analysis has shown the presence of ω-cyclohexane fatty acids and hapanoid characters in the cell membrane of *Alicyclobacillus* spp.. A report from Hippchen et al. (34) showed that acidothermophilic bacteria could be found in soils and the spoiled apple juice incident in Germany (15). The G + C content and composition of this newly discovered bacillus were further investigated and it was found that the G + C composition of this bacillus was similar to other bacilli and the G + C content of

this bacillus was 7% lower than *B. acidocaldarius* at 51.0-53.3 % mol (22, 23).

Additional differences between these two bacteria were thirteen of the acids

produced from these unknown bacterial strains were from different carbon sources;

the temperature range for growth was 35-53°C with the optimum temperature of

42-53°C, while the temperature range for growth of *B. acidocaldarius* was

approximately 45-70°C. Cluster analysis revealed that there were two different

clusters between the isolates and *B. acidocaldarius*. After taxonomic analysis, the

isolates from Hippchen et al. (34) and Cerny et al. (15) were found to significantly

differ from *B. acidocaldarius*; consequently, a new species of *Bacillus*

acidoterrestris was proposed (23). Further taxonomic analysis disclosed closely-

related three species of *B. acidocaldarius*, *B. acidoterrestris*, and *B.*

cyclohepatanicus. Comparative 16S rRNA sequence analyses of these three

species showed their sequences to be very similar (>92%). Comparison of the

similarity in 16S rRNA sequences between these three species and *B. subtilis*, *B.*

coagulans, and *B. stearothermophilus*, indicated that *B. acidocaldarius*, *B.*

acidoterrestris, and *B. cycloheptanicus* were closely related and remarkably

different from other *Bacillus* species. On the basis of these findings, these three

bacilli were proposed to be reclassified in a new genus of *Alicyclobacillus* gen.

nov. (88).

2.1.2 *Alicyclobacillus* species

Alicyclobacillus species isolated from soils and geometric landscape

include *Alicyclobacillus hesperidium*, *Alicyclobacillus sendaiensis*, and

Alicyclobacillus vulcanalis (2, 71, 77); whereas *Alicyclobacillus acidophilus*

isolated from spoiled orange juice and *Alicyclobacillus acidoterrestris* isolated

from spoiled apple juice and orange juice are important species in the juice industry (54).

Table 2.1. *Alicyclobacillus* species.

Alicyclobacillus species	Source	References
A. *acidocaldarius*	Acid soil	Wisotzkey et al. (88)
A. *acidophilus*	Orange juice	Matsubara et al. (53)
A. *acidoterrestris*	Acid soil, juices	Wisotzkey et al. (88)
A. *cycloheptanicus*	Acid soil	Wisotzkey et al. (88)
A. *disulfidooxidans*	Waste water sludge	Karavaiko et al. (44)
A. *hesperidium*	Sulfur-containing soil	Albuquerque et al. (2)
A. *herbarius*	Dried hibiscus flowers	Goto et al. (28)
A. *pomorum*	Mixed fruit juice	Goto et al. (29)
A. *sendaiensis*	Acid soil	Tsuruoka et al. (77)
A. *tolerans*	Oxidizable lead-zinc ores	Karavaiko et al. (44)
A. *vulcanalis*	Acid hot springs	Simbahan et al. (71)

Note: This list is compiled from a report from Walker and Phillips (80).

2.1.3 Morphology, Physiology, and Biochemical properties

Alicyclobacillus is acidothermophilic, aerobic, spore forming Gram-positive bacterium with a tendency to be Gram-variable. The cells show Gram-positive characters at the early stage of cultivation and become Gram-negative or Gram-variable at the end of cultivation. Cell size varies depending on growth medium and can reach to 2-5 mm on yeast-starch-glucose (YSG) agar at optimum temperature (89). Colony color ranges from white to beige and becomes darker with age. Spores are formed under unfavorable environmental and nutritional conditions. The rod-shaped cells are 2.9-4.3 µm in length and 0.9-1.0 µm in width, while oval-shaped spores are 1.5-1.8 µm in length and 0.9-1.0 µm in width (23).

All *Alicyclobacillus* species are able to grow at temperatures in the range of 20-70°C, with the optimum temperature between 40-60°C, except for *A. disulfidooxidans*, *A. tolerans*, and *A. ferrooxidans,* which are able to grow at less

than 20°C (30, 41, 44, 88), while optimum pH range is between 2.0-6.0, with an optimum pH between 3.5 and 4.5 for all species with the exception for *A. disulfidooxidans*, and *A. tolerans*. These two species are able to grow at a pH < 1.50 (44).

There is a large variation within species of the permissive growth pH and temperature range of *Alicyclobacillus* spp. as presented in Table 2.2.

Factors affecting *Alicyclobacillus* growth are nutritional state of the cells, salt concentration, organic acids, oxygen concentration, polyphenols, and alcohol concentration. The inhibition effect can be detected if over a limit concentration of these factors (93). Since *Alicyclobacillus* is a strict aerobe bacterium, the oxygen level in growth medium considerably influences the growth of these bacteria; however, some reports have revealed that low oxygen concentration (0.1% dissolved oxygen) can allow for cell growth, resulting in their ability to survive under minimal oxygen environments, but it is inhibited by absence of oxygen (16).

Moreover, vegetative cells of *Alicyclobacillus* can be completely inhibited by 6% ethyl alcohol, while there was no effect on spores of these strains at the same alcohol concentration (93).

Table 2.2. Range of growth pH and temperature of *Alicyclobacillus* species.

Alicyclobacillus species	pH range	Optimum pH	Temperature range (°C)	Optimum temperature (°C)
A. *acidocaldarius*	2.5-6.0	4.0-5.0	35-70	55-60
A. *acidophilus*	2.5-5.5	3.0	20-55	50
A. *acidoterrestris*	3.0-6.0	3.5-4.0	20-55	40-50
A. *cycloheptanicus*	3.0-5.5	4.0	30-55	50
A. *disulfidooxidans*	0.5-6.0	1.5-2.5	4-40	35
A. *hesperidium*	2.5-5.5	3.5-4.0	35-60	50-53
A. *herbarius*	3.5-6.0	4.5-5.0	35-65	55-60
A. *pomorum*	2.5-6.5	4.5-5.0	30-60	45-50
A. *sendaiensis*	2.5-6.5	5.5	40-65	55
A. *tolerans*	1.5-5.0	2.5-2.7	20-55	37-42
A. *vulcanalis*	2.0-6.0	4.0	35-65	55

Note: This list is compiled from reports from governmental and institutional sources (29, 30, 44, 71, 77).

2.1.4 Function of ω-alicyclic fatty acid on heat resistance of *Alicyclobacillus*

ω-alicyclic fatty acid, especially ω-cyclohexane fatty acid, found as a major membrane component, is the most unique characteristic of *Alicyclobacillus* spp., which might be associated with outstanding acid and heat resistance properties of *Alicyclobacillus* spp. (88). There have been several reports suggesting that ω-cyclohexane fatty acid may control a high acyl chain density in the core of the membrane which stabilizes the membrane structure and maintains the membrane permeability that is an important mechanism for membrane stability under acidic and high temperature condition for thermoacidophilic bacteria (43).

In addition to ω-alicyclic fatty acid, dehydration, dipicolinic acid (DPA) content, and mineralization have also been involved in heat resistance of spores. Many studies have suggested that heat resistance has been decreased by the demineralization of spores, and heat resistance can be increased by divalent cations such as calcium and manganese in re-mineralized spores (6). These reports suggested that the heat stability of *Alicyclobacillus* spores is significantly affected by spore mineralization. Yamazaki et al. (92) reported that *A. acidoterrestris* showed stronger binding properties to cations (calcium and manganese) than to *Bacillus* spp. spores at low pH conditions, and concluded that calcium and manganese play an important role in the heat resistance of *A. acidoterrestris* by chelating with DPA.

2.1.5 Pathogenicity and spoilage

Alicyclobacillus, one of the most heat-resistant bacteria, surviving heat treatments under acidic conditions of most fruit juices, has become an important microorganism in the past decade due to its characteristics causing spoilage in

juices (17). Endospores of *A. acidoterrestris* can survive commercial pasteurization and under acidic conditions commonly used for fruit and vegetable juices which might provide the heat-shock environment that stimulates spore germination (90). *Alicyclobacillus* has been identified from a wide range of fruit juices and probably originates from soil during postharvest procedures and production (55, 89). Although, *A. acidoterrestris* is considered non-pathogenic because there are no reports that *A. acidoterrestris* and its metabolites may pose human health problems (10). As a potential spoilage bacterium, pathogenicity of *Alicyclobacillus* spp. was naturally a concern, leading to its pathogenicity studied by Wall and Chuyate (83). Spores were directly injected into mice and spoiled juices with 5 x 10^6 CFU/ml of *A. acidoterrestris* were fed to guinea pigs. No disease symptoms were reported in mice, and no illness symptoms or death was reported in guinea pigs, indicating the non-pathogenicity of these strains of *A. acidoterrestris*. These researchers concluded that *A. acidoterrestris* is non-pathogenic bacterium at the level tested and no reports on illnesses of human have been associated to the consumption of spoiled juices. It periodically causes spoilage in fruit products, especially acidic fruit juices such as apple juice and orange juice, associated with an off-flavor due to guaiacol, which was identified as a significant cause of spoilage in acidic beverages (85). The visual detection of spoilage by *A. acidoterrestris* is difficult to examine because this organism does not produce gas, so no swelling of containers is detected; however, slight sediment might be seen in clear juices (83). Precursors of formation of guaiacol are vanillin and tyrosine; for example, apple juice contains approximately 4.1 µg tyrosine/ml which is a major factor of taint in apple juice (90). Heat-shock treatment, oxygen concentration, and storage temperature are considered important factors of guaiacol production (17). A study by Orr et al.

(64) suggested that guaiacol production in apple juice is not always associated with the number of cells and the sensitivity limit of guaiacol in best estimate threshold (BET) is lower than chromatographic method. Temperature was also identified as a significant factor for guaiacol production. Detection of guaiacol was found more quickly at 37°C compared to 21°C in apple juice, suggesting that guaiacol production is limited at room temperature as *Alicyclobacillus* does not grow well at room temperature (5, 64).

2.1.6 Controls of *Alicyclobacillus*

2.1.6.1 Antimicrobial substances

2.1.6.1.1 Organic acids

Sorbic and benzoic acids are used individually or in combination in juices and beverages to prevent the spoilage by yeast, mold, and *Alicyclobacillus* (81). Sodium benzoate is widely used in juices and beverages as a preservative due to its antimicrobial activity and is compatible for use in juice products due of its water solubility and non-volatility (81). Its antimicrobial activity relies on the undissociated acid form and the concentration declines as pH increases (25). Potassium sorbate also shows good antimicrobial activity against spoilage microorganisms in juices and beverages, and is less affected by pH compared to benzoic acid; consequently, it can be used in products with pH values higher than 3.0 (39). Sodium benzoate and potassium sorbate have been shown to be effective antimicrobials against *Alicyclobacillus* (83); however, sodium benzoate and potassium sorbate might cause sensitivity reactions in humans, especially asthmatics, and symptoms which include chest tightness, or a scratchy feeling at the back of the throats, particularly for sodium benzoate (84), and diarrhea is occasionally reported in people who consume excessive amounts of potassium

sorbate in foods (82). Therefore, the use of sodium benzoate and potassium sorbate

has been avoided and alternative treatments to effectively control against

Alicyclobacillus spoilage have been a topic of interest.

2.1.6.1.2 Oxidizing agent: chlorine dioxide

The concentration of 50-200 ppm of chlorine dioxide is recommended by

the FDA to wash fruits and vegetables to achieve a 2-log reduction of

contaminating microorganisms; subsequently, this solution was reported to reduce

the initial spore concentration *A. acidoterrestris* on apple surfaces and in an

aqueous suspension (48). Its effectiveness is related to contact time and

concentration, for example, spores were reduced more than 4.8-log after a

treatment of 120 ppm chlorine dioxide for 1 min (48). No synergistic effect

between chlorine and heat on spore reduction was investigated in aqueous

suspensions.

2.1.6.1.3 Nisin and other bacteriocins

Nisin is an antimicrobial peptide produced by *Lactococcus lactis* subsp.

lactis, exhibiting a wide range of antimicrobial activity against most Gram-positive

bacteria, including *A. acidoterrestris* vegetative cells and spores. Little or no

inhibitory effect was observed with Gram-negative bacteria, yeast, and fungi (13,

47, 91). Nisin can be used in juices directly or incorporated in a swellable polymer

and then released during storage (13, 47, 91). The concentration of nisin was

reported to range from 1.25 to 100 IU/ml and its effectiveness was related to the

pH of the media, juice types, and the solid content (47, 66, 91). Nisin's

effectiveness could be enhanced by acidic pH and low water activity, and it targets

spores more effectively than vegetative cells, with a sporostatic effect rather than

sporicidal effect (91).

2.1.6.1.4 Lysozyme

Lysozyme, also known as N-acetylmuramide glycanhydrolase, showed strong inhibitory effect on both vegetative cells and spores of *A. acidoterrestris*. Vegetative cells were more resistant than spores, as spores decreased to undetectable levels immediately after lysozyme was added (7). The mode of action for lysozyme against vegetative cells of *A. acidoterrestris* is potentially due to lysozyme catalyzing the hydrolysis of β 1,4 linkages between N-acetylmuramic acid and N-acetyl-D-glucosamine residues in peptidoglycan which is a main component of bacterial cell walls (7).

2.1.6.1.5 Essential oils

Takahashi et al. (75) proposed the use of essential oils to control *A. acidoterrestris* and reported the antimicrobial activity effectiveness of eucalyptus leaf extracts and flavonoids from *Eucalyptus maculata*. The use of combinations of cinnamaldehyde and eugenol to control the germination of *A. acidoterrestris* (strain c8 and c24) was reported by Bevilacqua et al. (8). Combinations of those two compounds could be a promising treatment to control the germination of *A. acidoterrestris* spores. Eugenol strengthened the inhibitory activity of cinnamaldehyde and poses a reduction of cinnamaldehyde level in the system, since cinnamaldehyde at the concentration of 100-120 ppm showed an impact on organoleptic properties of apple juice by untrained panelists.

2.1.6.2 Non-thermal methods to control *Alicyclobacillus*

2.1.6.2.1 High hydrostatic pressure (HHP)

HHP is an application that allows for the microbiological preservation of common foods without any changes in their organoleptic quality compared to thermal treatment with the equivalent preservation effect. Therefore, HHP is

becoming a promising processing method to decontaminate microorganisms in foods while maintaining fresh quality characteristics. Alpas et al. (3) reported the inactivation effect of HHP against vegetative cells of *A. acidoterrestris* in orange, apple, and tomato juices. They showed that, generally, log reduction was significantly enhanced with increasing pressures and temperatures. All juices were inoculated with 10^6 CFU/ml *A. acidoterrestris* cells and treated at 350 MPa at 50°C for 20 min. A greater than 4-log reduction in *A. acidoterrestris* was achieved immediately after pressurization for all juices. Pressurized juices were also stored for 3 weeks and viable cells of *A. acidoterrestris* in orange, apple, and tomato juice were 3.79, 2.59, and 2.27 CFU/ml, respectively. This study concluded that HHP is capable of inactivating *A. acidoterrestris* even at the optimum growth temperature for this spoilage bacterium.

2.1.6.2.2 High homogenization pressure

Bevilacqua et al. (7) reported the inhibitory effect of high homogenization pressure on vegetative cells and spores of *A. acidoterrestris* strains DSMZ 2498, γ4, and c8 in a laboratory medium (malt extract broth) with a pressure range of 500-1700 bars. The effect of high homogenization pressure was strain-dependent and 1-2 log reductions were achieved with the highest pressure. DSMZ 2498 appeared to be the most sensitive strain, while c8 was the most resistant one, and vegetative cells were shown to be more susceptible than spores for all strains tested.

2.1.6.2.3 Radiation

The use of radiation (electron beam and gamma rays) combined with conventional heat treatment against *A. acidoterrestris* in citrus juice was reported by Nakauma et al. (60). The survival rate of spores decreased exponentially with

increasing radiation dose of both electron beam and gamma rays. Authors

concluded that radiation is effective against spores of *A. acidoterrestris* in citrus

juice. The mode of action of irradiation on spore susceptibility was documented by

many authors; some authors suggested that the inactivation mode of action of

radiation was probably due to the formation of hydroxyl radicals and damage to

DNA (62). It has been proposed that the decrease in water content is one important

factor affecting spore resistance/susceptibility due to the reduction in hydroxyl

radicals generated by irradiation (62).

2.2. Patulin

Besides *Alicyclobacillus*, patulin, a mycotoxin associated with juice,

represents an economic loss threat for the juice industry.   Patulin is a chemical

hazard that is found in fruit products, particularly in apple juice and apple products.

Patulin is a secondary toxic metabolite produced by various species of *Penicillium*,

Aspergillus, and *Byssochlamys* (31). *Aspergillus* and *Penicillium* are potential

mycotoxin producing microorganisms in the pre-process stages of fruits, including

pre- and post-harvest, while *Byssochlamys* species are responsible for the

production of mycotoxin at the post-process stages due to their ability to survive

the thermal treatment typically used by the juice industry.

2.2.1 Characteristics

Patulin is one of the most reported mycotoxins found in apple juice and

apple products. It is an unsaturated heterocyclic lactone [4-hydroxy-4-H-furo(3,2-

c)pyran-2(6H)-one] that is water soluble with a molecular weight of 154 (71). A

variety of acute and chronic effects including cellular level effects of patulin have

been reported. The acute symptoms include lung congestion, convulsions, nervousness, hyperemia, gastro-intestinal tract distension while neurotoxic, genotoxic, immunotoxic, and teratogenic effects have been reported as chronic symptoms (35). Some examples of cellular level effects are protein synthesis inhibition, and DNA and RNA synthesis inhibition (21, 58). Based on the reports of adverse effects of patulin, the Codex Alimentarius (20) and the FDA (79) have set a maximum limit of 50 µg/l of patulin for fruit juices and fruit products, while a level of 25 µg/l for apple products and 10 µg/l for juice and infant foods have been recommended according to the regulation of 1425/2003 of the European Common Market (26).
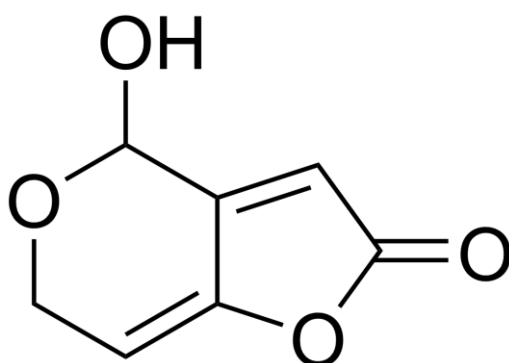
Figure 2.1. Structure of patulin.

Patulin is mainly found in apple and its products and in other fruits occasionally such as peaches apricots, pears, and grapes that have been processed from decayed parts of these fruits (19). The incidence of patulin in commercial apple juice and its products throughout the world indicates that patulin is stable after thermal treatment steps in processing, and it is also tolerant to acidic conditions; thus it is difficult to eliminate if a patulin contaminated apple or its products are already in the production lines (11). Codex (20) suggests that the careful actions of good agricultural practices (GAPs) and good manufacturing

practices (GMPs) during juice processing can decrease the contamination of patulin in juice and its finished product.

2.2.2 Effect of apple juice processing stage on patulin

Even though patulin is primarily found in fungus-damaged fruits, the absence of external fungal growth by visual inspection does not guarantee the absence of patulin because patulin-producing fungal growth may occur on the interior of the fruit. In some scenarios, internal patulin-producing fungal growth can take place due to insect invasion or other type of damage resulting in the presence of patulin in externally undamaged fruits. Although spores of most patulin-producing fungi appear on the fruit, they do not usually grow until the fruits are harvested. The growth of these fungi and patulin production can be seen post-harvest if they are damaged by insects or diseases, or if the ground harvested fruit are used for fruit processing (20). Codex (20) recommends special practices for fruit for juice processing to guarantee the safety and quality of juice, with regards to patulin. Those considerations are i) removal of rotten fruit; ii) space between trees needs to be adequate for good air and light access; iii) control of insects and diseases that might enable entrance for patulin-producing fungi; iv) use of fungicide to inhibit the growth of patulin-producing molds during and post harvest; v) application of fertilization composed of calcium and phosphorus to strengthen cell structure and reduce susceptibility to fruit decay; vi) store fruits with good mineral composition because minerals help fruit be less susceptible to physical disorders and decay; vii) an update of rot indices in each orchard, since the orchard records are the best indicator of fungicide application and fruit storage potential.

2.2.2.1 Pre-harvest and harvest

Good care needs to be taken during fruit harvest to avoid fruit damage regardless of whether they are going to be used for processing, storage prior to processing, or fresh fruits for consumer markets. Marín et al. (53) showed the importance of avoiding damaged-fruits during the handling stage prior to processing, and found that diameter of damaged-area of apple in both groups (rotten and sound) was associated with patulin concentration. Therefore, the better practices of harvesting prior to processing, the higher possibility of producing finished products within the maximum established limits for patulin. Juice produced from apples that were directly harvested from trees showed no patulin, while 40.2-374 µg/l of patulin was reported in juice from apples harvested from the ground (37).

2.2.2.2 Fruit storage before apple juice processing

Orchards around the world exclusively select fruit with rigid quality selection criteria for fresh fruit markets and others not approved by these criteria are typically used for juice or other processing. Thus, apples after harvest may be used immediately for juice processing or fresh apple market, while other fruits are stored for subsequent different types of commercial processing. Apples rejected from fresh market quality criteria are used for juice processing. *Penicillium expansum* is a phychotrophic fungus that grows and produces patulin under refrigerated conditions. With regards to refrigerated storage, *P. expansum* has been shown to be capable of producing patulin in apples stored at 4°C and 25°C for 90 and 20 days, respectively (76). Patulin concentration of 300 µg/l was reported in apples stored at 4°C for 120 days compared to 310 µg/l in apples after 30 days of storage at 25°C (56), whereas no patulin was detected in apples stored after 6

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weeks at 1°C, even though, decay from fungal growth was observed (57). *P. expansum* was reported to grow and produce patulin when apples were stored at 0.5°C containing 1% CO_2, 3% O_2 and 96% N_2 with relative humidity over 90%. Patulin production was shown to be lower when stored in controlled atmosphere compared to stored in only refrigerated temperature (52), showing the diversity and ability of different patulin producing *P. expansum* strains to grow on apples stored at controlled atmosphere. In a recent study, it has been shown that CO_2/O_2 ratio in the storage atmosphere has an impact on patulin production. The CO_2/O_2 ratio must to be kept constant during storage because O_2 can activate the growth of some species of mold, resulting in patulin production. It was reported that some *P. expansum* strains are capable of growing and producing patulin at 0°C, 3°C, 6°C, 17°C, and 25°C, but not when stored in controlled atmosphere containing 3% CO_2 and 2% O_2 at 25°C (65). Because of a storage period that might be up to 12 months prior to processing, patulin production in fruits depends on the storage conditions and fruit varieties. It could be concluded that the higher the amount of damaged fruits in stored lots, the greater the patulin found in the final products.

Deck storage for long periods of time is sometimes performed due to the financial limitation of refrigerated and controlled atmosphere conditions resulting in high levels of patulin production (73). Sydenham et al. (74) reported that patulin concentration in apples stored at ambient temperature increased with the storage time indicating that longer time the apples are stored on the deck, the higher possibility of patulin levels exceeding the maximum limit in derived products.

2.2.2.3 Fungicides for patulin reduction

Storage at refrigerated temperatures and controlled atmosphere are not sufficient to prevent the growth of patulin producing fungi. Additional treatments

which include fungicide applications, are required to limit the growth of patulin producing fungi. Benzylmidazol-based fungicides and organo-phosphorated insecticides have been abandoned because of safety issues and fungal mutations (24). Trans-2-hexanal vapors (an aroma of some fruits and vegetables) has been shown as a potential treatment to control *P. expansum* (blue rot) growth, patulin levels, and apple quality of Golden Delicious variety (61). Trans-2-hexanal vapors are also used to reduce infections of *P. expansum* with a significant difference compared to the control; however, the effectiveness of trans-2-hexanal is variety-variable dependent on phytotoxic symptoms, off-flavor development, and fungicidal activity (61).

2.2.2.4 Fruit washing

Limitations of fungicides due to toxicity and fungal resistance have lead to a search for alternative treatments such as "natural" sanitizers or biological control agents. Sodium hypochlorite (NaOCl) solution showed complete growth inhibition of *Aspergillus flavus*, *Aspergillus niger*, *Aspergillus alternata*, *Cladosporium cladosporioides*, *Fusarium* spp., *P. expansum*, and *Rhizopus stolonifer* in apples at 25°C by immersion of 5% of NaOCl solution for 5 min (32). Acetic acid solution with 2-5% (vol/vol) was reported to be effective against the growth of *P. expansum* and patulin production, and it can be applied to apples by immersion or spraying methods (18). "Natural" compounds such as lemon and orange essential oils have been shown to be effective for the inhibition of *P. expansum* and patulin production (32). Careful selection of fruit, avoiding fruits picked from a ground, and storage conditions, were shown to be significant and important practices from harvest which influence the final quality of fruit juice. Jackson et al. (37) reported no patulin was found in apple juice processed from apples when careful selection

was performed on in-coming apples and then stored at 0-2°C for 46 weeks, while 0.97 to 64.0 µg/l patulin was detected in apple juice pressed from unculled apples that were stored under the same conditions. Patulin levels of 0 to 15.1µg/l and 59.9 to 120.5 µg/l were detected in juice processed from careful and non-careful selection stored in controlled atmosphere conditions, respectively.

Washing treatment plays an important role in reduction of patulin in apples and finished products. Sydenham et al. (73) showed a significant patulin reduction by washing treatment (tap water) of deck-stored apple with 80% patulin reduction when compared to the control (no washing treatment). High-pressured water with 100 ppm and 200 ppm active chlorine was also shown to be effective for patulin reduction in apples with 10-100% depending on the initial concentration of patulin (1). Due to the limitations of using chlorinated compounds that include high corrosiveness on equipment, organic matter susceptibility, unsafe byproduct formation (chorophenols), and effectiveness with narrow pH range (18, 68) to control pathogens in post harvest stage, alternative treatments have been studied as an application for patulin reduction in the washing of fruit. Electrolyzed oxidizing water (EO) is an alternative to chlorinated treatments as a means to decontaminate the infection of *P. expansum* on apples during handling and processing operations; however high capital cost and electrical utilization are possible limitations for large-scale use of this technique (63).

2.2.2.5 Juice processing

Patulin can also be removed during juice processing steps such as juice pressing, clarification, filtration, pasteurization, and concentration. Conventional clarification techniques have been reported to be efficient methods for patulin reduction. Kadakal and Nas (42) reported the use of activated carbon at the

clarification step to reduce patulin in apple juice. Activated carbon ranging from 0-3.0 g/l of apple juice with stirring for 0-30 min showed that 3 g/l of activated carbon was the most effective treatment with approximately 50% patulin reduction in the apple juice. The activated carbon treatment resulted in a decrease in color, increase in clarity, and slight decrease in fumaric acid content, pH, and °Brix. Variations in patulin reduction by activated carbon were affected by type of carbon, type of activation (physical or chemical), and solid content of juices (49). The recycled adsorption system of patulin by activated carbon has been reported as a successful technique with 99% patulin reduction in apple juice; but juice qualities were remarkably altered, indicating that further study is necessary for a better design to maximize the patulin reduction with minimal changes in the juice quality (59). Ultrafine activated carbon (a composite of adsorbent carbon) has been developed and was found to be effective for patulin reduction in aqueous solution and apple juice but this technique lead to alterations in the product appearance and changes in flavors (36). In addition to the quality alterations, environmental impacts should be taken in account since excess residues of activated carbon were generated (27). Activated carbon treatment represents a considerable increase in cost for the food industry and its effectiveness depends on operation time. Therefore, it is important to study the efficacy of different types of activated carbon (49). Gökman et al. (27) showed that activated carbon and resin-based on polystyrene divinyl benzene (synthetic polymer) were capable of patulin reduction by 40.9% and 11%, respectively, with changes in color and phenolic content, whereas other synthetic polymers were able to reduce patulin levels by more than 45% in apple juice (14). Rotary vacuum pre-coat filter, a conventional clarification method for juice, was able to reduce patulin levels by 39% in apple juice

concentrate and has been reported to be more efficient for reducing patulin levels than ultrafiltration methods (29% patulin reduction) (1). Moreover, Bissessur et al. (9) showed the effect of conventional techniques during clarification steps on patulin reduction in apple juice and found that centrifugation, refining with bentonite, enzyme treatment (pectinase), and filtration with diatomaceous earth, were effective treatments in patulin reduction by 20.5%, 8.5%, 4.5% and 3%, respectively.

Controversial results have been reported on the effect of pasteurization on patulin levels in apple juice. The first report of patulin heat stability in aqueous solution was conducted by Wiesner (86) and subsequently reported by Heatley and Philpot (33) that patulin was stable under the heat condition of 100°C /15 min at pH 2.0 in a model system, whereas, Scott and Somers (70) reported the effect of pasteurization on patulin in apple juice containing added ascorbic acid (vitamin c) (35 mg/100 ml juice) resulted in 45% and 55% patulin reduction after heat treatment at 80°C for 10 min and 20 min, respectively. In addition, Taniwaki et al. (76) conducted experiments on apple juice contained patulin of 1,500 µg/l using a heat treatment at 90°C for 2 min, followed by a 5-min heat treatment in boiling water, and a cooling step at room temperature, resulted in a 60% reduction of patulin.

The impact of juice concentration on patulin content is variable depending on the unit operations used. Concentration using vacuum distillation has been reported for patulin reduction and the mechanism for this reduction was due to exposure time and temperature with the transformation of patulin to substances that were identifiable as non-mycotoxin. Kadakal and Nas (42) studied the effect of concentration (evaporation) at 70°C and 80°C for 5, 10, 15, 20 min on patulin

levels in apple juice and reported that patulin levels reduced as time and temperature increased. However, evaporation caused a reduction in juice clarity compared to the heat treatment stage. Leggott et al. (50) reported that no patulin reduction was observed in apple juice samples collected during the concentration step from a juice plant. This suggests that discrepancies in patulin reduction are variable due to processing conditions and equipment used for concentration, when compared to laboratory data where conditions are well controlled.

2.2.2.6 Juice storage

Apple juice concentrate is usually stored under refrigeration or frozen conditions to maintain their quality prior to dilution and commercial processing for single strength juice. Apple juice quality will not change for at least 6 months if 70 °Brix is maintained, and for 40 °Brix apple juice that is held under frozen temperatures (equal or below -20°C) to prevent enzyme reactions and prevent yeast growth. Koca and Eksi (46) studied the effects of storage temperature and time on patulin in apple juice concentrate. Storage temperature (22°C and 30°C) and storage time for a period of 6 months with apple juice concentrate (70-74 °Brix) were used in this experiment. Results showed that time and temperature were important factors on patulin reduction during storage of apple juice concentrate. Patulin reduction of between 45-64% and between 66-86% were observed after 1 month of storage at 22°C and 30°C, respectively, and patulin levels were below detection levels (10 ppm) after 4 months of storage at both temperatures. Ascorbic acid likely contributed to patulin reduction in apple juice concentrate and some studies have reported that added ascorbic acid in apple juice or buffer solutions increased in patulin reduction rates (12). The mechanism of patulin reduction by ascorbic acid is still unknown, but it is postulated that singlet oxygen or free

radicals generated from metal-catalyzed oxidation of ascorbic acid might interact with conjugated double bonds of patulin which would cause an alteration in patulin reduction, resulting in a decrease in patulin detection (12).

Conventional thermal processing techniques to inactivate microorganisms to improve the microbiological safety of foods are considered effective methods but can result in deleterious effects on flavor, color and nutrient changes in foods (4). Moreover, consumer's expectations associated with health benefits are in need of more fresh and natural qualities (40), resulting in alternative non-thermal treatments recently becoming a major interest for the food industry. Non-thermal treatments, referred to non-thermal technologies, cover all food preservation treatments that are effective in microbial inactivation at ambient or room temperature, including pH modification, pressure alteration, irradiation techniques, and antimicrobial additives (69). Non-thermal treatments such as high hydrostatic pressure (HHP) and pulsed electric field (PEF) have recently received particular attention and are the most extensively studied in terms of microorganism inactivation in various food products that include dairy, meat, and juice products. However, HHP and PEF can cause alteration in the structure of proteins and polysaccharides, which are associated with changes in texture and color of foods (45, 87). These emerging technologies are considered expensive or costly processing methods that result in an increase in the cost of the finished product (67). In addition, irradiation treatments can cause free radical production in high fat-containing and high antioxidant-containing foods, affecting flavor and color properties in foods (38). Therefore, antimicrobial compounds and non-thermal technologies may be effective alternative treatments for food preservation to

achieve the desired microbial destruction and safety enhancement, but with minimal changes in the organoleptic qualities of the finished food product.

Therefore, the objectives of this thesis are to investigate alternative non-thermal methods or treatments to improve the microbiological safety and quality of juices focusing on patulin reduction in apple juice from concentrate and inhibition of *A. acidoterrestris* in apple and orange juices.

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CHAPTER 3

PATULIN REDUCTION IN APPLE JUICE FROM CONCENTRATE
BY UV IRRADATION AND COMPARISON OF KINETIC
DEGRADATION MODELS BETWEEN APPLE JUICE AND
APPLE CIDER[1]

ABSTRACT

Patulin, a mycotoxin produced by several genera of fungi, including *Byssochlamys*, *Aspergillus*, and *Penicillium*, has been an important concern in apple cider and apple juice due to its toxicity and health consequences. In this study, the effects of UV on the patulin level, physical and chemical properties, and sensory attributes in apple juice from concentrate were investigated. Kinetic modeling of patulin reduction by UV radiation in apple juice from concentrate was calculated and compared with the degradation rate observed previously in apple cider. From an initial patulin contamination of approximately 1,000 ppb (μg/liter), the UV exposure, ranging from 14.2 mJ/cm^2 (one pass) to 99.4 mJ/cm^2 (seven passes), was successful in reducing patulin levels by 72.57% ± 2.76% to 5.14% ± 0.70%, respectively. Patulin reduction by UV radiation followed first-order kinetic modeling in a fashion similar to first-order microbial inactivation.

An exponential correlation between UV exposure and the percentage of patulin remaining was observed, giving an r^2 value of 0.9950. Apple juice was repeatedly exposed to 14.2 mJ/cm^2 for each treatment, and patulin levels were significantly decreased when compared with the level obtained with the previous UV exposure treatment. While there were no significant differences in the percentages of titratable acidity and ascorbic acid ($P > 0.05$), there were minor yet random sampling differences in pH and degrees Brix (1 °Brix is 1 g of sucrose in 100 g of solution; the °Brix represents the soluble solids content of the solution as percentage by weight [%, wt/wt]) ($P \leq 0.05$). A significant difference ($P \leq 0.05$) in sensory perception for the finished apple juice was detected between the control and the full seven-pass UV radiation treatment using an experienced consumer panel and a triangle test. Patulin reduction by UV radiation from both the current study and a previous study involving apple cider was compared, which showed that both matrices strongly fit a first-order kinetic degradation model. However, the kinetic constant for degradation in apple juice was approximately 5.5 times greater than that observed in an apple cider matrix.

INTRODUCTION

The ß-unsaturated lactone patulin (4-hydroxy-4H-furo[3,2c]pyran-2(6H)-one) is a water-soluble and heat stable mycotoxin produced by several species of *Penicillium*, *Aspergillus*, and *Byssochlamys* (33, 39). *Penicillium expansum* is considered the most important species responsible for "blue mold rot" and patulin production (10). Patulin can be found in a variety of food types, including fruits and vegetables, juices, jam, cheese, and bread (5, 6, 18, 32). Apples are considered as an important source of patulin since they are easily infected by *P. expansum* and

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also display optimum factors for patulin production (39). Patulin production can be variable depending on the apple variety and stage of ripeness (36). Intrinsic factors such as water activity and pH are also associated with patulin production (8, 26). Furthermore, the severity and area of apple spoilage infected by *P. expansum* are related to patulin production. Therefore, removing damaged apples before further juice processing is necessary for patulin reduction (16). Postharvest handling methods, including the removal of contaminated apple sections and high-pressure water spraying, have been reported to reduce the risk of patulin contamination in apple juice and apple cider (16, 24). It also has been reported that there was a significant reduction in patulin levels in juice processed from sound apples compared to the levels in juice processed from contaminated apples (24). Based on health studies, there is evidence that patulin is able to cause acute and chronic health problems in laboratory animals (13, 15).

Patulin levels in apple products, including apple cider and apple juice concentrate, is regulated in many countries, including the United States, where the U.S. Food and Drug Administration has set a maximum allowable level of 50 ppb µg/liter) in single-strength juice equivalent (41). Since this regulation has been established, juice industries are required to reduce the patulin levels of their finished product to 50 µg/liter or below before it is allowed to be sold to the public.

Food irradiation is an alternative process that can be used to inactivate selected pathogenic and spoilage microorganisms, especially bacteria, by exposing target food to ionizing radiation. It is also used to destroy insect larvae, inhibit sprouting in some root vegetables, postpone the ripening process, and increase the juice yield while retaining the chemical and nutritional properties (14). However, there is no previous research aimed at determining the effect of UV radiation on

patulin in apple juice made from concentrate. Thus, the objectives of this study were to investigate the effects of UV radiation on patulin stability, assess the finished apple product's physical and chemical properties, perform a sensory evaluation of the treated apple juice produced from concentrate, and compare the kinetic modeling of patulin degradation by UV radiation in apple juice from concentrate and apple cider.

MATERIALS AND METHODS

Chemicals. Patulin (4-hydroxy-4H-furo[3,2c]pyran-2(6H)one), 2,6-dichlorophenolindophenol, and pure L-ascorbic acid were obtained from Sigma Chemical company (St. Louis, MO). Ethyl acetate (high-performance liquid chromatography [HPLC] grade), sodium hydroxide (NaOH), and sodium sulfate anhydrous (Na_2SO_4) were obtained from Fisher Scientific (Pittsburgh, PA), whereas acetonitrile (99.5% HPLC grade) was obtained through Acros Organics (Morris Plains, NJ).

Apple juice concentrate. Commercial apple concentrate was supplied by Dr. Olga Padilla-Zakour (Department of Food Science, New York State Agricultural Experiment Station, Cornell University, Geneva, NY). It was manufactured on 10 December 2004 and had a pH of 2.2 and 70.6 °Brix (1 °Brix is 1 g of sucrose in 100 g of solution; the °Brix represents the soluble solids content of the solution as percentage by weight [%, wt/wt]). It was kept at -2.2°C prior to being used in the experiments described below. Apple concentrates were diluted to 12 °Brix with distilled water. Patulin was introduced by adding the appropriate amount of patulin-contaminated apple cider (initial concentration, approximately 17,600 ppb) to achieve an approximate final patulin concentration of 1,000 ppb.

UV radiation processing unit. The CiderSure 3500 commercial UV machine (FPE, Inc., Macedon, NY) was used both to study the patulin reduction ability of UV light and to conduct a sensory evaluation study of the treated juice. Technical information concerning the design and operation of the machine has been previously discussed by Quintero-Ramos et al. (31). In brief, the device is composed of a stainless steel outer unit containing three inner chambers of quartz tubes connected in sequence. Apple juice was pumped through a thin layer between the outer steel unit and inner quartz tubes. Eight germicidal low-pressure mercury lamps were used as the source of UV light exposure. Every 50 ms, two UVX-25 UV light sensors monitored the desirable amount of UV energy required for consistent radiation. Time exposure was dependent on UV dose. The juice's UV exposure was calculated by the following equation:

$$\text{UV dose } (\text{mJ/cm}^2) = [\text{irradiance}] \times [\text{exposure time}]$$

where mJ/cm^2 is the unit for measuring the UV dose and irradiance is the product of multiplying the system's internal UV sensor reading by radial factors described as a reflection and absorption factor. According to this equation, the UV dose for one pass can be calculated as 14.2 mJ/cm^2 and a cumulative dose of seven passes as 99.4 mJ/cm^2.

Thermal processing. We evaluated the organoleptic changes in the juice due to UV treatments against conventionally processed, shelf-stable apple juice. Patulin-free apple juice was thermally processed using a UHT/HTST Lab-25HV tubular heat exchanger (MicroThermics, Inc., Raleigh, NC) at a flow rate of 1 ml/min and a hot pack temperature of 88°C, followed by a hot hold time of 3 min prior to forced cooling to room temperature. Heat-treated apple juice was stored in

32-oz (907-ml) polyethylene terephthalate bottles and kept refrigerated at 2°C until needed.

Sample preparation for patulin determination. Sample preparation for patulin analysis was adapted from the method described by the AOAC International official method 2000.02 (25). Briefly, 10 ml of apple juice was transferred to a separatory funnel and extracted three times with 20 ml of ethyl acetate. The ethyl acetate extractions were pooled in a separatory funnel, and the aqueous phase discarded. This ethyl acetate collection was extracted with 4 ml of a 1.5% (wt/vol) solution of Na_2CO_3. After layer separation, the ethyl acetate was quantitatively transferred to a round-bottom flask, first passing through a funnel containing filter paper loaded with anhydrous Na_2SO_4. The ethyl acetate was evaporated under a stream of nitrogen gas until the residue was dry. The residue was dissolved in 1 ml of pH 4 water and filtered through a 0.45-µm-pore-size polytetrafluoroethylene filter (Fisher Scientific) prior to analysis via HPLC. Standard patulin solutions of 50, 100, 250, 400, and 500 mg/liter were prepared every week in the same fashion as apple juice samples in order to construct the patulin standard curve.

Patulin analysis. Patulin content was analyzed via HPLC using the procedure described previously, with a detection limit of 1 ppb (9). The system consisted of a Varían model 2510 HPLC pump connected to an in-line Spectroflow 757 UV/visible light absorbance detector with the wavelength set at 276 nm. Patulin was identified and quantitated by an isocratic elution of 10% acetonitrile (vol/vol) in deionized water at a flow rate of 0.5 ml/min. Fifty microliters of resuspended extract or standard was injected onto a 250-mm Thermo Scientific

Aquasil Q8 column with 4-mm internal diameter, 100-nm pore size, and 5-μm particle size (Thermo Scientific, Waltham, MA).

Determination of physical and chemical properties. In order to determine the effect of UV radiation on the physical and chemical properties of apple juice, the pH, °Brix, percent titratable acidity (%TA), and ascorbic acid were investigated. pH was measured by using a pH meter (Orion PerpHect pH LogR meter model 310, Thermo Scientific, Waltham, MA). °Brix was assessed with a hand refractometer (AOAC method 932.12) with a workable range from 0 to 30 °Brix (model 10430, Reichert, Depew, NY). Titratable acidity was described as grams of malic acid per 100 ml juice and was determined by titration using 0.1 N NaOH (AOAC method 925.10). Ascorbic acid concentration was determined by titrimetric analysis using 2,6-dichlorophenolindophenol (AOAC method 967.21).

Sensory evaluation. Using a triangle test, 20 experienced panelists collected from faculty and students from the Department of Food Science at Cornell University were used to investigate the effect of UV radiation on sensory perception. Apple juice preparation was the same as described above except that no patulin-laden cider was added to the apple juice samples. All samples were pasteurized and kept at 2°C overnight prior to the sensory tests. Panelists were served with three 3-digit-coded samples. Two of them were a control (no UV radiation), while the third sample was a UV-treated sample exposed to seven passes through the UV apparatus. Panelists were asked to taste all samples from left to right and choose the odd one. Statistically significant differences were determined by comparing the number of correct answers to the table of minimum number of correct judgments established for statistical comparison (23).

Kinetic modeling and comparison of patulin degradation via UV radiation in apple juice from concentrate versus apple cider. Data from the current study involving an apple juice matrix prepared from concentrate was compared against this laboratory's previously published (9) patulin degradation data assessed in an apple cider matrix produced on-site. The experimental setup, UV exposure rates, analytical measurement, and statistical analysis were identical for each data set. The patulin degradation kinetics from each data set were modeled according to zero-, first-, and second-order kinetic equations, below, to determine the best linear fit and, thus, the best rate model. An extrapolation of relevant models was also performed in an attempt to model and compare the complete degradation of patulin for each study's matrix.

Statistical analysis. This experiment was conducted in triplicate with completely randomized design. The experimental data were evaluated by the one-way analysis of variance method using Statistical Package for the Social Sciences (SPSS) version 11.5 (SPSS, Inc., Chicago, IL). The statistical significance of differences between mean values was established at $P < 0.05$ with the Tukey honestly significant difference test.

RESULTS

Patulin reduction by UV radiation. The concentration of patulin in apple juice concentrate was reduced by UV radiation in a UV exposure-dependent manner. The percentages of patulin remaining after one pass (14.2 mJ/cm^2) and seven passes (99.4 mJ/cm^2) were 72.57% ± 2.76% and 5.14% ± 0.70%, respectively (Fig. 3.1). The rate of patulin reduction followed first-order kinetics modeling similar to the UV-mediated microbial inactivation kinetics with a correlation coefficient (r^2) of 0.9950. Each subsequent exposure to UV radiation

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resulted in a significantly lower (P < 0.05) amount of patulin remaining compared

with each preceding pass. For example, the percentage of patulin remaining in the

control (0 mJ/cm^2) was significantly higher than the amount remaining after the

single pass (14.2 mJ/cm^2), whereas the percentage of patulin remaining after the

single pass was significantly higher than the amount remaining after two passes

(28.4 mJ/cm^2), and so on.
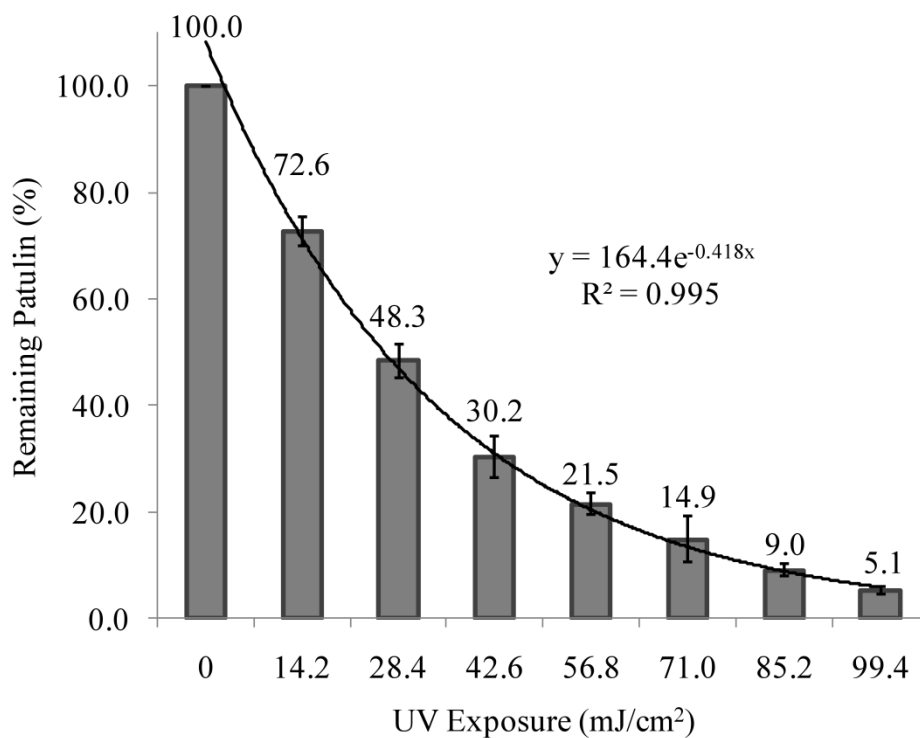
Figure 3.1. The percentage of patulin remaining as a function of UV exposure.

Physical and chemical properties (pH, °Brix, %TA, and ascorbic acid).
While there were no significant differences between the mean values of %TA and

ascorbic acid throughout the seven cumulative UV treatments, there were

significant differences between the mean values of pH and °Brix. The pH values

ranged from 3.590 ± 0.010 to 3.563 ± 0.006, whereas the °Brix ranged from 11.98

± 0.13 to 12.48 ± 0.24 (Table 3.1).

Table 3.1. Measurement of pH, °Brix, % titratable acidity, and ascorbic acid in the apple juice samples of control (no UV treatment),

one pass, and seven–pass treatments.

UV Exposure (mJ/cm^2)	pH[a]	°Brix[a]	Ascorbic Acid (mg/100 ml)[b]	Total Acids (% Malic Acid)[b]
0	3.590 ± 0.010^B	12.08 ± 0.23^{AB}	0.7853 ± 0.0878	0.4331 ± 0.0151
14.2	3.563 ± 0.006^A	12.13 ± 0.18^{AB}	0.7475 ± 0.0575	0.4376 ± 0.0127
28.4	3.568 ± 0.003^A	12.40 ± 0.13^{AB}	0.7858 ± 0.0664	0.4342 ± 0.0102
42.6	3.577 ± 0.003^{AB}	12.48 ± 0.08^B	0.7475 ± 0.0575	0.4320 ± 0.0067
56.8	3.573 ± 0.003^A	12.48 ± 0.24^B	0.6900 ± 0.0000	0.4365 ± 0.0051
71.0	3.573 ± 0.003^A	12.30 ± 0.17^{AB}	0.6900 ± 0.0000	0.4331 ± 0.0108
85.2	3.575 ± 0.005^A	12.22 ± 0.15^{AB}	0.7092 ± 0.0878	0.4286 ± 0.0101
99.4	3.573 ± 0.006^A	11.98 ± 0.13^A	0.7475 ± 0.0000	0.4119 ± 0.0089

[a] Within a column, a different letter (A, B) indicates significant differences in mean values were observed at $\alpha = 0.05$ ($P \leq 0.05$).
[b] No significant differences of mean values were detected between treatments.

Kinetic modeling and comparison of patulin degradation. The current

data set was modeled and compared alongside similar data produced when

examining patulin degradation rates in an apple cider matrix. Zero-, first-, and

second-order rate models were examined. Each model was assessed using the

relevant integrated rate equation for each, as follows:

$$\text{Zero-order: } [A] = [A]_0 - kt \tag{1}$$

$$\text{First-order: } [A] = [A]_0\, e^{-kt} \tag{2}$$

$$\text{Second-order: } [A] = [A]_0/1 + kt[A]_0 \tag{3}$$

where A is the patulin concentration, k is the rate constant, and t is the UV

exposure. The determined best linear fits for the zero- and first-order models are

detailed in Figure 3.2. Constructed as such, the units for each rate constant become

$ppb/mJ\cdot cm^{-2}$, $1/\ mJ\cdot cm^{-2}$, and $l/[ppb\cdot mJ\cdot cm^{-2}]$, for the zero-, first-, and second-order

models, respectively. Table 3.2 describes the correlation coefficients and kinetic

rate constants for all three models for each matrix.

When comparing kinetic modeling of apple juice from concentrate and

apple cider using first-order kinetic modeling, as shown in Table 3.2, the kinetic

constant for the apple juice matrix is approximately 5.5 times greater than the rate

constant for the apple cider matrix. Using the kinetic equations from the zero-order

and first-order models, we constructed the theoretical degradation of patulin

throughout a total of 50 passes through the CiderSure apparatus, or an accumulated

UV dose of 710 kJ/cm^2.
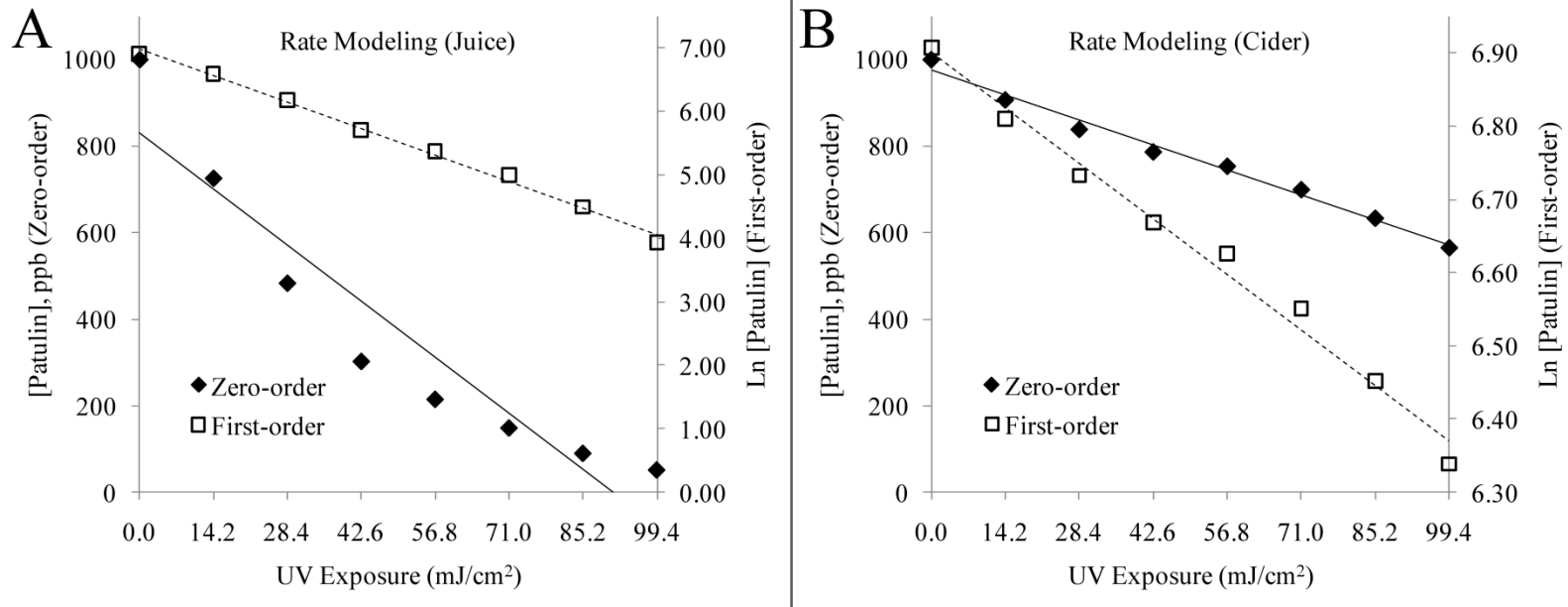
Figure 3.2. Kinetic modeling plot describing zero- and first-order rate models for both apple juice reconstituted from concentrate (current study) (A) and apple cider (previous study) (B). The left-side y axis for each describes the zero-order modeling attempt (closed diamonds), while each right-side y axis describes the first-order modeling attempt (open squares).

Referring to Figure 3.3, an extrapolated plot describes the accepted first-order degradation model for the apple juice matrix compared against both the zero-order and first-order potential models for the apple cider matrix. The shaded area of the figure refers to the actual data points recorded between the current and previous studies. The extrapolated region following these points comprises the theoretically derived data points ascertained by using either the zero-order or first-order kinetic equation calculated from the existing data.

Sensory evaluation. A triangle test was used to investigate the effect of UV radiation on sensory perception. For the number of participants in this trial, if the number of correct answers is greater than 11, it means panelists were able to recognize the difference between a control and a sample treated with the full seven-pass dosage of UV radiation (giving $\alpha = 0.05$). Fourteen panelists were able to discern the odd sample correctly from the other two control samples, indicating that there was a significant difference in organoleptic quality between the control and the seven-pass UV-treated sample.

Table 3.2. Kinetic rate constants and linear correlation coefficients for zero-, first-, and second-order modeling assessments[a].

Degradation Model	Juice		Cider	
	Correlation Coefficient (R^2)	Rate Constant (k)	Correlation Coefficient (R^2)	Rate Constant (k)
Zero-order	0.8929	9.1475	0.9880	4.0686
First-order	0.9950	0.0294	0.9867	0.0053
Second-order	0.7989	0.0002	0.9684	7.0E-06

[a] Modeled as seen in Figure 3.2, the units for each rate constant become ppb/mJ·cm^{-2}, 1/ mJ·cm^{-2}, and 1/[ppb·mJ·cm^{-2}], for the zero-,

first-, and second-order models, respectively.
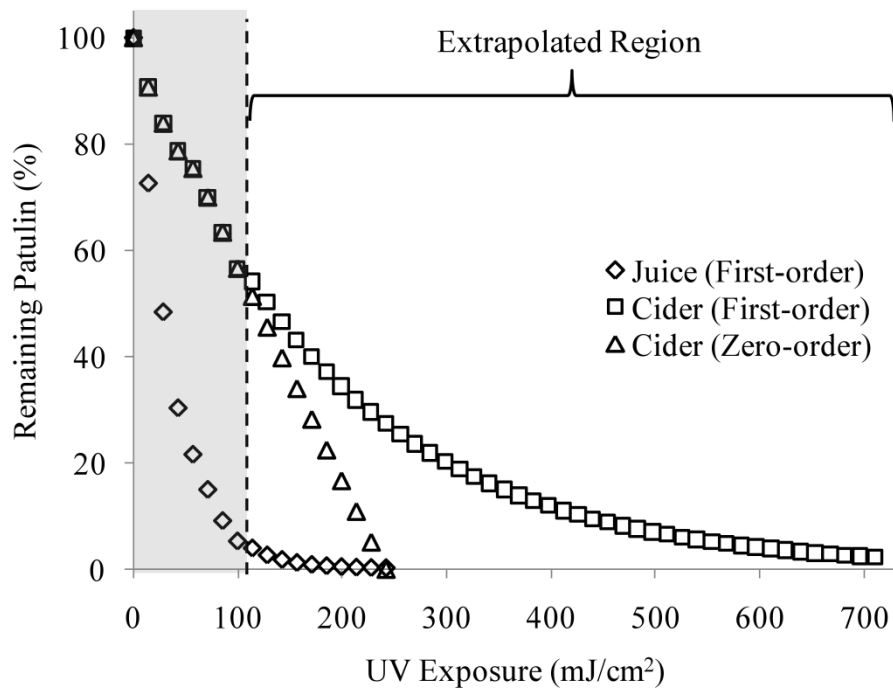
Figure 3.3. An extrapolation of both the zero-order and first-order kinetic models for the apple cider matrix from the previous study compared against the accepted first-order kinetic model of the current study. The shaded region on the left represents actual data points recorded, while the region to the right represents the mathematically calculated patulin levels following each kinetic model.

DISCUSSION

P. expansum is a common patulin-producing fungus typically associated with apples that results in the loss of fresh and processed apple juice products (36). The heat resistant mycotoxin patulin has been an interesting concern in fresh apple, apple cider, and apple juice products due to serious long-term health concerns seen in laboratory animals. Observed toxic endpoints have been carcinogenic, teratogenic, and/or mutagenic in nature (11). Previous studies have reported several potential methods used to decontaminate *P. expansum* for patulin removal,

including various wash and clarification treatments, vaporizing with tran-2-hexanal, and activated charcoal (12, 16, 28, 37).

Through selective removal of contaminated apples, patulin can also be eliminated or reduced during apple harvesting, processing, and storage (27). Based on good agricultural practices (7), the Joint FAO/WHO Food Standards Program recommends that careful apple selection before processing is one of most effective means for patulin control. Indeed, it has been shown that a selective trimming step can reduce patulin burdens up to 99% (24). Standard postharvest processing, particularly a washing step, is also commonly used for both microorganism and patulin control. Depending on the apple variety, it has been reported that apples washed using high-pressure water can reduce patulin up to 54% in the finished apple juice (1). Furthermore, a conventional clarification process using a rotary vacuum precoat filter has been shown to be more effective than ultrafiltration in reducing patulin burdens (1).

Storage practices, in particular storage temperature, also affect patulin production in apples. Apples contaminated with *P. expansum* stored at 20.5°C for 21 to 93 days were found to have a significantly high patulin level compared with the patulin level in apples incubated at 11°C (35). Ripened apples are usually stored at low temperatures under a modified atmosphere environment in order to delay senescence development normally required for apple juice processing. However, patulin production by select species of *Penicillium* can still be observed under these conditions and at storage temperatures below 5°C (30).

Fresh apples are essential for the production of apple juice concentrate to ensure the quality of the final product. Therefore, uninfected apples are preferable for patulin-free apple juice production. However, patulin contamination can occur

during processing through the inadvertent or negligent inclusion of so much as a single contaminated apple (36). As such, effective technologies and/or methodologies are necessary for effective patulin reduction during processing. Due to the effectiveness of non-thermal processing and its low operational cost, UV radiation has been used in the juice industry to eliminate *Escherichia coli* contamination in apple juice (4). This technique generally results in a product with very minimal if any observable differences in quality and organoleptic characteristics (2). Recently, studies have shown that UV radiation can be an alternative method used to reduce patulin in apple cider with no alterations of pH, °Brix, and %TA (9). In contrast, the current study using apple juice from concentrate as a matrix resulted in significant differences in juice treated with a seven-pass UV treatment strategy ($P \leq 0.05$) when compared against controls. This might be due to the fact that apple cider has a more complex "fresh" flavor than apple juice, which might conceal potential deleterious flavor alterations upon UV radiation.

Thermal processing and low-heat UV radiation are two commonly used processing methods designed to produce a microbiologically stable end product that is safe and organoleptically pleasing for the consumer. Depending on the matrix, each of these methods can adversely affect the flavor, color, or nutrient content of the final product. The challenge comes when optimizing the processing protocols to best deliver a safe product in the most unaltered state possible while maintaining the product's natural or added nutrient composition. For a matrix as complex and robust as apple cider, losses of natural compounds, including vitamins, color pigments, and antioxidant compounds, are minimal, leading to a less altered UV-treated product (42). However, in apple juice, UV radiation can

alter the properties of select antioxidants, such as phenolic compounds, which might be responsible for the significant flavor profile changes in apple juice detected by panelists (29).

Based on this study and work by Dong et al. (9), vitamin C appears to be a compound of concern when modeling an effective UV radiation protocol in various apple-based matrices. Ascorbic acid (vitamin C) is an essential multifunctional dietary antioxidant required by humans in a number of diverse biochemical pathways, including the production of collagen in connective tissue. Several diseases, most notably scurvy, are seen in people whose diets contain insufficient ascorbic acid. Fruits and vegetables are important sources of ascorbic acid. However, processing of juice products using heat treatments or high oxygen atmospheric storage conditions can result in ascorbic acid degradation (3, 22, 34). Apple juice concentrate usually contains a small amount of ascorbic acid, depending on the apple variety and which process is applied for concentrate production. Therefore, apple juice producers may add ascorbic acid in their final juice products to compensate for the loss of ascorbic acid during juice processing and storage. Ascorbic acid also has the added benefit of being an antibrowning agent and antioxidant agent (38). However, ascorbic acid strongly absorbs UV light, resulting in an inhibition of UV germicidal function (40). As such, ascorbic acid additions would have to be made after UV radiation in order to achieve maximum germicidal effect.

In liquid food matrices, such as fruit juices, microbial inactivation due to UV radiation relies on the specific product composition, including the physical and chemical properties of each juice system (20). Besides ascorbic acid, several other factors affect the efficacy of UV radiation treatment, including many natural

compounds found in juices, such as polyphenols and insoluble solids including pectin, cellulose and hemicellulose, all of which have some capacity to absorb UV radiation (19). These types of soluble solids, suspended particles, and phenolic compounds, as well as variable amounts of ascorbic acid commonly added to apple juice, are unique matrix-dependent variables that affect the UV absorption coefficient of each matrix differently (19), thereby altering the individual effectiveness of any UV radiation treatment used.

The best-fit model of patulin degradation for the current study of an apple juice matrix reconstituted from concentrate indicates that patulin reduction by UV radiation follows a first-order kinetic degradation model, similar to the pattern observed in microbial burden reduction. Based on this notion, this first-order kinetic model, defined in "Results," can be further described using the same log-linear model seen in UV-mediated microbial inactivation given by the following equation:

$$\text{Log } (N/N_0) = -kD \tag{4}$$

where in this case, N_0 is the initial concentration of patulin, N is the concentration of remaining patulin, N/N_0 is the percentage of patulin remaining, D is the UV fluence, and k is a constant based on the wavelength of the detector, molecular species, and absorption coefficient (17). The behavior of the percentage of patulin remaining and, thus, its first-order degradation rate is affected by many important factors, such as UV dose, exposure time, and the physical properties of the samples (31).

A comparison of the kinetic degradation models between the current study and our previous study involving an in-house-produced apple cider matrix (9) details how many of these matrix-specific components affect the efficacy of UV-

mediated patulin reduction. In regard to the previous study, our initial observations prior to the kinetic modeling undertaken here point to a simple zero-order model of patulin reduction, and as such, a simple linear extrapolation was performed that best described the relevant conditions needed to theoretically achieve a complete reduction in patulin. However, a thorough understanding of the potential degradation kinetics modeled in either matrix can only be appreciated when allowing for the possibility of a zero-, first-, or second-order rate model. As shown, both sets of data show a strong linear fit describing first-order degradation kinetics. While the kinetic fit for the current study is decidedly unambiguous, a complication arises when attempting to assign a definitive kinetic model to the apple cider matrix from our previous study. Both the zero- and first-order plot show a similar fit to each kinetic model. In cases like this, the confusion arises when attempting to model data that have only begun to show their trend. In order to state a completely unambiguous assignment for patulin degradation rates in the apple cider matrix, we would need to understand how the UV treatment affected the patulin concentration at doses beyond what were initially recorded. Without this data, we can simply offer both rate models as potential indicators of patulin degradation.

Based on the two most probable kinetic models, we can extrapolate a curve for the theoretical patulin degradation in apple cider for both the zero-order and first-order predictions (Fig. 3.3). In either kinetic model, it is clear that patulin reduction rates are greatly hindered in the apple cider matrix when compared directly against the apple juice matrix. If we continue to assume a zero-order kinetic, the extrapolated degradation of patulin eventually matches that of the first-order kinetics found in an apple cider matrix, albeit at a severely reduced rate,

point for point. The most obvious problem in this scenario would be the need for a UV exposure of over 225 kJ/cm^2, or at least 16 passes through the CiderSure apparatus.

In the most simplistic (and likely) case, as well as for general comparative purposes, it will become useful to describe the patulin degradation in the two matrices using the first-order model. The "cleaner" apple juice matrix, at least as understood from a UV radiation standpoint, clearly shows that patulin reduction is more effective when its UV-mediated degradation is attempted in a matrix lacking oxidized polyphenolics or other UV-absorbing components. It is further possible that insoluble solids, including pectin, cellulose, and hemicellulose, found in significantly higher quantities in apple cider than in apple concentrate, might be responsible for the diminished UV-mediated reduction in patulin burdens (21). Therefore, the required UV dose in apple cider is markedly higher than that required for apple juice in order to obtain the same patulin reduction. Irrespective of the mechanism, when comparing first-order patulin degradation models, the kinetic rate constant was estimated to be approximately 5.5 times greater in the apple juice matrix. The greater efficacy of UV radiation has the added consequence of altered chemical and physical changes to the final product, as shown by °Brix, total acidity, and pH measurements. However, these changes appear to be minor at best, still falling in the range of typical or average apple juice products.

A seven-pass UV radiation treatment showed a very successful patulin reduction in reconstituted apple concentrate, lowering the levels from an unrealistically high initial patulin concentration of approximately 1,000 ppb to below 50 ppb, the maximum limit in apple concentrate regulated in the United States (41). Based on the first-order modeling, more realistic initial patulin burdens

would not require such an extended treatment of UV radiation. For instance, from a starting patulin burden of 200 ppb, only four passes, or approximately 56.8 kJ/cm^2 of germicidal UV radiation would be required to lower patulin levels below federally mandated action levels. An initial burden of 150 ppb would only require three passes (approximately 42.6 kJ/cm^2).

Since we report a very efficient reduction in patulin, UV radiation promises to be an effective alternative processing method for use in the apple juice and cider industry. We show that at moderate treatment levels within seven times that of the normal germicidal dose, UV radiation is effective at reducing patulin levels with only minor changes to the physical and chemical properties. Juice matrices in particular are looking to be an attractive target matrix based on the high susceptibility to UV-mediated patulin degradation. However, there remains a concern that the finished product may have altered organoleptic qualities. Therefore, a better understanding of how UV radiation influences the sensory attributes in finished apple juice products and how these alterations can be minimized will be essential if this method can be broadly applied to improve juice safety while maintaining quality.

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CHAPTER 4

EFFECT OF DIMETHYL DICARBONATE ON INACTIVATION AND HEAT RESISTANCE OF *Alicyclobacillus acidoterrestris* POPULATIONS IN JUICE SYSTEMS

ABSTRACT

Alicyclobacillus acidoterrestris, a chronic spoilage and non-pathogenic bacterium, causes spoilage in fruit, vegetable, and beverage products due to the production of off-flavors. It is capable of growing under acidic conditions and its spores survive typical thermal processing regimes used for pasteurized and shelf stable products. Thus, it is considered the primary spoilage microorganism when determining processing conditions to guarantee shelf stability for acidic fruit juices. In this study, the effects of dimethyl dicarbonate (DMDC) ranging from 0 – 250 ppm on vegetative cells and spores of *A. acidoterrestris* strains VF, WAC, and SAC, were investigated in potato dextrose broth (PDB), apple juice, and orange juice. DMDC was added directly to all treatment samples at room temperature and then samples were taken at 0, 2, 4, 6, 12, and 24 hours for microbial analysis and enumeration. Chemical and physical properties, including sensory evaluation, of apple juice treated with DMDC were performed. Heat resistance parameters including D- and z-values were determined at 90°C, 95°C, and 98°C in apple juice and orange juice. Results showed DMDC had a bactericidal effect on vegetative cells and spores for all strains of *A. acidoterrestris* in PDB, apple juice, and orange juice control and DMDC treated. It was observed that higher DMDC concentrations resulted in greater log reductions for both vegetative cells and

spores, but vegetative cells were more sensitive to DMDC than spores in all samples tested. A greater than 3, 4, and 5 log reduction was achieved with vegetative cells of SAC strain with 250 ppm DMDC treatment in orange juice, PDB, and apple juice samples, respectively. Approximately 1 and 0.20 log reductions were achieved after 250 ppm treatment for all strains tested in apple and orange juice samples, respectively. No significant differences (α = 0.05) between mean values of pH, total soluble solids (°Brix), titratable acidity, ascorbic acid, total phenolic compounds, and color values (L^*, a^*, and b^*) were detected in apple juice and orange juice samples. Sensory evaluation by 30 experienced panelists showed no significant differences between the control (no DMDC) and 250 ppm DMDC treated apple juice or orange juice samples using the triangle test. No significant differences (α = 0.05) were detected between the D-value of control and 250 ppm DMDC in all treatments tested except 90°C for VF in both juices and 95°C for SAC in orange juice.

INTRODUCTION

The juice and beverage industries have grown rapidly and their products have gained popularity in recent years due to health benefit claims and consumer's convenience. According to consumer's trends, orange and apple juice are the first and second most consumed juices in the US, respectively (11). Due to the acidic pH of fruit juices and beverages, it was believed that thermal processes for pasteurization and commercial sterility were sufficient to inactivate all non-spore forming microorganisms and the acidic conditions would prevent the outgrowth of bacterial spores such as *Bacillus coagulans* and *Clostridium pasteurianum* (39). However, in 1982 a large-scale spoilage incident in Germany involving shelf-stable apple juice identified *Alicyclobacillus* as the causative spoilage

microorganism (6). Since that incident, the juice industry has considered *Alicyclobacillus* as a primary spoilage microorganism in acidic juices or beverages. For 25 years *Alicyclobacillus* spp. was considered a contaminant causing fruit and vegetable juice spoilage in raw fruits and vegetables. Surveys of beverages not containing juice identified the presence and potential of *Alicyclobacillus* to cause spoilage (47). The source of *Alicyclobacillus* spp. in these beverages was identified to be a variety of sugar sources that included high fructose corn syrup, liquid sugar, and granulated sugar (47).

Due to the identification of *Alicyclobacillus acidoterrestris* as a spoilage microorganism for juice and beverages, this microorganism has been actively investigated by researchers and the food industry over the past two decades (7). Spores of *Alicyclobacillus* spp. can survive the hot fill processes (185°F for 3 min) normally employed for fruit and vegetable juices (48). *Alicyclobacillus* has been identified from a wide range of fruit juices where contamination originates from soil during postharvest procedures and production (46). Fortunately, *Alicyclobacillus* is a non-pathogenic bacterium and does not represent any food safety hazards for consumers. It causes spoilage in juice and beverages due to the production of off flavors. Spoilage due to *Alicyclobacillus* is difficult to detect visually because it causes only light sediment. The only characteristic that can be used for detection of *Alicyclobacillus* spoilage in juice and beverage products is the presence of off odors which are described as medicinal or antiseptic (48). Guaiacol has been identified to be the primary compound produced by *Alicyclobacillus* that is responsible for off odor. Due to the heat resistant characteristics of *Alicyclobacillus*, it has been used as the target microorganism for designing thermal processing regimes of shelf-stable high acid fruit juice (34).

Dimethyl dicarbonate (DMDC) is a colorless liquid at room temperature with fruity-smell characteristics. It is soluble in organic solvents and slightly soluble in water. In 1980, it was reported that DMDC was used as a fungicide in alcohol-free beverages. The first approval for using DMDC was in wine as a yeast inhibitor in 1988 (41). In 2001, FDA changed the term of "inhibitor of yeast" to "microbial control agent" for safe use in the beverage industry. Recently, DMDC has been approved as direct food additive as a microbial control agent in many type of beverages including wines, ready-to-drink teas, and juices, with different maximum limits of DMDC for different applications, such as 200 ppm for wine and 250 ppm for carbonated or diluted juice and tea (40). However, there are no published reports on the effect of DMDC on *A. acidoterrestris* in apple and orange juices. Therefore, the objectives of this study were to study the antimicrobial activity of DMDC against vegetative cells and spores of *A. acidoterrestris* strains VF, WAC, and SAC, in aqueous suspension (potato dextrose broth, PDB), apple juice, and orange juice, to determine the effect on physical and chemical properties including pH, percent total soluble solid, percent titratable acidity, ascorbic acid content, total phenolic compound, color values, to determine the sensory evaluation in apple juice and orange juice, and to determine heat characteristics of *A. acidoterrestris* spores in apple and orange juices.

MATERIALS AND METHODS

Materials, chemicals, and media. Shelf-stable apple juice and refrigerated pulpy orange juice were purchased from a local market in Geneva, NY (Wegmans Inc., Rochester, NY). Potato dextrose broth (PDB) and Bacto™ peptone were obtained from Becton, Dickinson and Company (Spark, MD). Potato dextrose agar (PDA) was purchased from Weber Scientific (Hamilton, NJ). Tartaric acid was

obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). 2,6 dichlorophenolindophenol and pure L-ascorbic acid was obtained from Sigma Chemical company (USA). Sodium hydroxide was obtained from Fisher Scientific (Pittsburgh, PA).

Bacterial strains. Three strains of *Alicyclobacillus acidoterrestris*; VF, WAC, and SAC were used in this study. These cultures were originally isolated from spoiled fruit juices and are part of the culture collection in Dr. Worobo's laboratory.

Bacterial cell and spore preparation:

-   Vegetative cells: Strains were grown at 50°C for 24 hours in PDB, pH 3.5, acidified by sterile 10% tartaric acid solution (wt/vol). Each stationary phase culture was transferred into fresh PDB, apple juice, or orange juice, in sterile flasks to achieve final cell numbers of 10^5-10^6 organisms per ml sample (CFU/ml).

-   Spore: Strains were grown at 50°C in PDB pH 3.5 acidified with sterile 10% tartaric acid solution (wt/vol) for 24 hours. Then, this culture was transferred into a sterile flask containing fresh PDB. After inoculation, flasks were put in the incubator at 50°C for 7 days. To achieve only spores of *A. acidoterrestris*, heat treatment of 80°C for 10 min was applied to inactivate vegetative cells. For sample preparation, this suspension was transferred to a sterile flask containing PDB, apple juice, or orange juice. The final spore number was approximately 10^5-10^6 spores per ml sample (CFU/ml).

Dimethyl dicarbonate treatment. Four different DMDC concentrations were used to investigate the inhibitory effects of DMDC on three strains of *A. acidoterrestris*; VF, WAC, and SAC, in PDB. DMDC was added directly to the sample (PDB) to achieve the final concentration of 250, 125, 50 ppm with 0 ppm

as a control, whereas 250, 125, and 0 ppm (control) DMDC were added to apple and orange juice samples.

Microbiological analysis. Samples were taken at 0, 2, 4, 6, 12, and 24 hours of the incubation time at ambient temperature for all samples (PDB, apple juice, and orange juice). Serial dilutions of the samples were immediately performed in 0.1% (wt/vol) peptone water. Serial dilutions and pour plate techniques were performed in duplicate onto PDA, pH 3.5, and then incubated at 50°C for 72 hours, followed by enumeration. Counts were averaged and expressed as the log number and then log reduction calculations were performed. This experiment was conducted in triplicate.

Heat resistance parameters. Apple juice and orange juice inoculated with spores of *A. acidoterrestris* strains VF, WAC, and SAC treated with DMDC (250 ppm) and 0 ppm DMDC (control) were transferred into sterile microcapillary tubes (1.5 x 90 mm). Microcapillary tubes were sealed by flaming with a gas burner and then submerged in water-filled test tubes and placed in water baths at different temperatures: 90°C, 95°C, and 98°C. Each test tube was filled with 5 microcapillary tubes and each test tube was sampled at different time points for the different temperatures and immediately immersed into the 70% ethanol-filled test tube stored in an ice bath to quickly cool the microcapillary tubes. Each microcapillary tube was cleaned by gently placing on filter paper to absorb the excess 70% ethanol and immediately transferred to 20 ml of sterile deionized water. The microcapillary tubes were crushed using a sterile pestle. The suspension was serial diluted in 0.1% (wt/vol) peptone water and cell growth and enumeration were the same as previously described in microbiological analysis. Heat resistance parameters were expressed as D- and z-values which are defined as the time

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required to reduce the microbial population by 90%, or 1 log reduction at a given temperature and as a temperature, in Fahrenheit or Celsius, required for 1 log reduction in the D-value, respectively.

- **Spore preparation** To investigate the heat resistance parameters, strains were grown at 50°C in PDB pH 3.5 acidified by sterile 10% tartaric acid solution (wt/vol) for 24 hours. To stimulate sporulation, this culture in PDB was spread plated onto PDA (pH 3.5) and incubated at 50°C for 7 days, until at least 80% cell sporulation was achieved, based on microscopic evaluation. Spores were harvested by adding approximately 3 ml sterilized deionized water onto the surface of the PDA plates and the spores were collected by scraping with a sterile swab. The suspension was centrifuged at 5,000 x g for 5 min and the supernatant was discarded. This procedure was repeated 3 times and the resulting pellet was resuspended in sterile-deionized water with an approximate concentration of $10^7 - 10^8$ spores/ml. The spore suspension was stored at -80°C until needed.

Physical and chemical properties analysis. pH, percent total soluble solids (°Brix), percent titratable acidity (%TA), ascorbic acid content, total phenolic compounds, and color values, were investigated to determine the effect of DMDC on the physical and chemical properties of apple and orange juices. DMDC (250, 125, 50, and 0 ppm) was directly added to apple juice and orange juice without any bacterial inoculation and samples were kept at ambient temperature for 2 hours before analyses. pH was measured by using a pH meter (Orion PerpHect pH LogR meter Model 310, Thermo Scientific, Baverly, MA). Total soluble solids was assessed with digital refractometer ranged from 0-30 °Brix (Reichert Model 10430, Depew, NY). Titratable acidity was expressed as gram of malic acid/100 ml apple juice and as gram of citric acid/100ml orange juice by titrating with 0.1 N

NaOH (1). Ascorbic acid was determined by titrimetric analysis using 2,6 dichlorophenolindophenol (1). Total phenolic compounds was measured by Folin Ciocalteau method using gallic acid as a standard, described by Singleton and Rossi (35). Color values (L^*, a^*, and b^*) were measured by Hunter Lab model UltraScan XE spectrophotometer (Reston, VA).

Sensory evaluation. In order to investigate the effect of DMDC on sensory characteristics of apple juice and orange juice, 30 semi-trained panelists were recruited from the Department of Food Science (New York State Agricultural Experiment Station, Cornell University, Geneva, NY) to perform a triangle test (18). Panelists were served three samples coded with 3-digit random numbers. Two served as controls (no DMDC added) and the odd one was apple juice or orange juice treated with 250 ppm DMDC. Apple juice treated with 250 ppm DMDC was prepared by adding DMDC in apple juice and was kept at room temperature for 24 hours whereas DMDC-treated orange juice was kept at refrigerator temperature (4°C) prior to conducting the sensory test.

RESULTS

Population reduction (log CFU/ml) of vegetative cells and spores of *A. acidoterrestris* strain VF, WAC, and SAC in PDB treated with 250, 125, 50 and 0 ppm DMDC stored for 24 hours at ambient temperature are shown in Tables 4.1 and 4.2, respectively. In general, results showed that higher concentrations of DMDC in PDB resulting in higher population reductions being achieved with both vegetative cells and spores. The maximum population reduction for vegetative cells as obtained with 250 ppm DMDC in VF, WAC, and SAC; 2.42 ± 0.11 CFU/ml at 4 h, 2.75 ± 0.04 CFU/ml at 2 h, and 4.46 ± 0.11 CFU/ml at 24 h, respectively. Whereas, 0.91 ± 0.06 CFU/ml at 2 h, 1.09 ± 0.13 CFU/ml at 4 h, and

0.71 ± 0.09 CFU/ml, at 2 h were achieved after 250 ppm DMDC with the spores of VF, WAC, and SAC, respectively. Similarly, DMDC had an inhibitory effect on both vegetative cells and spores in apple juice and orange juice. A 2.68-2.86 log reduction (log CFU/ml) for vegetative cells was achieved with 250 ppm DMDC in VF and WAC samples, respectively, whereas >5 log reduction for SAC in apple juice samples was achieved (Table 4.3). A 2.5-2.7 log reduction (log CFU/ml) in vegetative cells of VF and WAC, and 3.7 log reduction in SAC was achieved with 250 ppm DMDC-treated orange juice samples (Table 4.5). Approximately 1 log reduction was achieved with the spores for all strains tested in apple juice, whereas only a 0.20 log reduction in orange juice samples was achieved with 250 ppm DMDC treatment (Table 4.4 and Table 4.6). The negative log reduction in some samples, primarily in control samples, indicates that the population increased during the incubation period. The effectiveness of DMDC was rapidly achieved within 2 hours after DMDC treatments for all the samples tested.

D-values of *A. acidoterrestris* were found to be between 49.14 min and 59.30 min at 90°C, 13.86 min and 15.48 min at 95°C and 5.22 min and 6.03 min at 98°C in apple juice. For orange juice, the D values ranged between 41.44 min and 59.31 min at 90°C, 8.66 min and 13.69 min at 95°C, and 3.79 min and 5.73 min at 98°C (Table 4.7). Some significant differences ($\alpha = 0.05$) were observed for the D-values of control (0 ppm DMDC) and 250 ppm DMDC with strain VF at 90°C in apple juice and orange juice, and SAC at 95°C in orange juice. The z-values of *A. acidoterrestris* were between 6.6°C and 8.96°C (Table 4.8).

There were no significant differences between control and treated mean values of pH, °Brix, ascorbic acid, % titratable acidity, total phenolic compounds,

and color values (L^*, a^*, and b^*) in all DMDC treatments in apple juice and orange juice (Table 4.9 and Table 4.10).

Nine experienced panelists and seven experienced panelists out of thirty in apple juice and orange juice, respectively, chose the odd sample correctly, indicating that there were no significant differences ($\alpha = 0.05$) between control (0 ppm DMDC) and DMDC-treated apple juice and orange juice samples (250 ppm DMDC).

Table 4.1. Population reduction of *Alicyclobacillus acidoterrestris* vegetative cells strain VF, WAC, and SAC in PDB treated with

DMDC stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	2.37 ± 0.17	2.42 ± 0.11	2.36 ± 0.17	2.37 ± 0.19	2.35 ± 0.13
125 ppm	0	2.08 ± 0.10	2.07 ± 0.08	2.10 ± 0.08	2.01 ± 0.11	1.77 ± 0.10
50 ppm	0	1.82 ± 0.28	1.73 ± 0.29	1.82 ± 0.18	1.70 ± 0.27	1.42 ± 0.25
0 ppm	0	-1.27 ± 0.25	-1.62 ± 0.25	-1.57 ± 0.20	-1.14 ± 0.19	-0.50 ± 0.26
WAC						
250 ppm	0	2.75 ± 0.04	2.49 ± 0.02	2.56 ± 0.04	2.41 ± 0.03	2.10 ± 0.03
125 ppm	0	2.16 ± 0.08	2.36 ± 0.10	2.24 ± 0.16	2.24 ± 0.14	2.36 ± 0.15
50 ppm	0	2.31 ± 0.20	2.30 ± 0.21	2.29 ± 0.21	2.22 ± 0.19	1.85 ± 0.15
0 ppm	0	-0.56 ± 0.14	-0.61 ± 0.19	-0.56 ± 0.15	-0.21 ± 0.14	-0.16 ± 0.36
SAC						
250 ppm	0	4.38 ± 0.13	4.31 ± 0.15	4.36 ± 0.03	4.38 ± 0.05	4.46 ± 0.11
125 ppm	0	4.27 ± 0.08	4.31 ± 0.03	4.30 ± 0.10	4.41 ± 0.13	4.41 ± 0.08
50 ppm	0	4.24 ± 0.08	4.28 ± 0.12	4.28 ± 0.15	4.27 ± 0.10	4.44 ± 0.14
0 ppm	0	-1.17 ± 0.22	-1.09 ± 0.17	-1.06 ± 0.11	-0.99 ± 0.17	-1.18 ± 0.17

Table 4.2. Population reduction of *Alicyclobacillus acidoterrestris* spores strain VF, WAC, and SAC in PDB treated with DMDC

stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	0.91 ± 0.06	0.73 ± 0.06	0.73 ± 0.04	0.56 ± 0.05	0.49 ± 0.03
125 ppm	0	0.63 ± 0.09	0.57 ± 0.05	0.62 ± 0.06	0.25 ± 0.03	0.24 ± 0.02
50 ppm	0	0.27 ± 0.05	0.37 ± 0.03	0.29 ± 0.02	0.25 ± 0.04	0.31 ± 0.06
0 ppm	0	0.04 ± 0.01	0.02 ± 0.01	0.05 ± 0.05	0.04 ± 0.02	0.04 ± 0.03
WAC						
250 ppm	0	1.08 ± 0.08	1.09 ± 0.13	1.00 ± 0.10	0.99 ± 0.07	1.08 ± 0.10
125 ppm	0	0.35 ± 0.01	0.38 ± 0.10	0.39 ± 0.07	0.40 ± 0.07	0.35 ± 0.09
50 ppm	0	0.34 ± 0.03	0.37 ± 0.06	0.31 ± 0.07	0.34 ± 0.08	0.34 ± 0.06
0 ppm	0	0.03 ± 0.01	0.03 ± 0.01	0.10 ± 0.01	0.08 ± 0.01	-0.11 ± 0.04
SAC						
250 ppm	0	0.71 ± 0.09	0.56 ± 0.06	0.61 ± 0.08	0.60 ± 0.06	0.60 ± 0.07
125 ppm	0	0.31 ± 0.01	0.34 ± 0.03	0.24 ± 0.03	0.25 ± 0.04	0.28 ± 0.04
50 ppm	0	0.11 ± 0.01	0.16 ± 0.02	0.13 ± 0.02	0.11 ± 0.03	0.14 ± 0.00
0 ppm	0	0.02 ± 0.01	0.04 ± 0.02	0.02 ± 0.01	0.02 ± 0.01	0.06 ± 0.02

Table 4.3. Population reduction of *Alicyclobacillus acidoterrestris* vegetative cells strain VF, WAC, and SAC in apple juice treated

with DMDC stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	2.85 ± 0.09	2.86 ± 0.13	2.82 ± 0.17	2.73 ± 0.15	2.78 ± 0.12
125 ppm		2.55 ± 0.13	2.47 ± 0.05	2.45 ± 0.06	2.51 ± 0.04	2.50 ± 0.03
0 ppm	0	-0.37 ± 0.10	-0.38 ± 0.08	-0.36 ± 0.09	-0.32 ± 0.10	-0.36 ± 0.05
WAC						
250 ppm	0	2.76 ± 0.33	2.68 ± 0.28	2.70 ± 0.32	2.69 ± 0.34	2.77 ± 0.18
125 ppm		2.04 ± 0.09	2.03 ± 0.10	1.99 ± 0.01	2.02 ± 0.05	2.04 ± 0.02
0 ppm	0	-0.32 ± 0.05	-0.19 ± 0.04	-0.41 ± 0.07	-0.22 ± 0.09	-0.53 ± 0.04
SAC						
250 ppm	0	5.28 ± 0.04	5.28 ± 0.04	5.28 ± 0.04	5.28 ± 0.04	5.28 ± 0.04
125 ppm		3.87 ± 0.19	3.76 ± 0.21	3.69 ± 0.16	3.72 ± 0.17	3.81 ± 0.19
0 ppm	0	0.04 ± 0.05	-0.09 ± 0.07	-0.56 ± 0.01	-0.63 ± 0.10	-0.34 ± 0.04

Table 4.4. Population reduction of *Alicyclobacillus acidoterrestris* spores strain VF, WAC and SAC in apple juice treated with DMDC

stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	1.01 ± 0.02	1.04 ± 0.06	1.05 ± 0.02	1.08 ± 0.07	0.98 ± 0.02
125 ppm	0	0.28 ± 0.04	0.31 ± 0.06	0.29 ± 0.06	0.26 ± 0.04	0.24 ± 0.01
0 ppm	0	0.03 ± 0.03	0.01 ± 0.04	0.01 ± 0.01	-0.04 ± 0.05	-0.05 ± 0.02
WAC						
250 ppm	0	0.99 ± 0.08	1.01 ± 0.05	1.00 ± 0.05	1.03 ± 0.08	1.03 ± 0.05
125 ppm	0	0.39 ± 0.10	0.32 ± 0.10	0.37 ± 0.12	0.37 ± 0.14	0.36 ± 0.05
0 ppm	0	-0.03 ± 0.02	-0.05 ± 0.03	-0.05 ± 0.01	-0.05 ± 0.04	-0.10 ± 0.02
SAC						
250 ppm	0	1.00 ± 0.09	1.02 ± 0.11	1.03 ± 0.10	1.07 ± 0.09	0.84 ± 0.03
125 ppm	0	0.36 ± 0.08	0.32 ± 0.05	0.34 ± 0.06	0.38 ± 0.05	0.36 ± 0.05
0 ppm	0	-0.04 ± 0.05	-0.09 ± 0.01	-0.08 ± 0.04	-0.09 ± 0.05	-0.16 ± 0.07

Table 4.5. Population reduction of *Alicyclobacillus acidoterrestris* vegetative cells strain VF, WAC and SAC in orange juice treated

with DMDC stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	2.54 ± 0.06	2.61 ± 0.14	2.52 ± 0.18	2.60 ± 0.09	2.64 ± 0.13
125 ppm	0	2.30 ± 0.06	2.32 ± 0.09	2.29 ± 0.19	2.28 ± 0.16	2.38 ± 0.15
0 ppm	0	0.16 ± 0.09	0.24 ± 0.02	0.25 ± 0.04	0.24 ± 0.05	0.37 ± 0.08
WAC						
250 ppm	0	2.69 ± 0.07	2.67 ± 0.06	2.70 ± 0.06	2.73 ± 0.03	2.63 ± 0.10
125 ppm	0	2.43 ± 0.05	2.35 ± 0.03	2.41 ± 0.03	2.41 ± 0.06	2.41 ± 0.06
0 ppm	0	0.20 ± 0.11	0.27 ± 0.13	0.41 ± 0.07	0.44 ± 0.05	0.48 ± 0.04
SAC						
250 ppm	0	3.72 ± 0.14	3.73 ± 0.22	3.72 ± 0.18	3.74 ± 0.25	3.71 ± 0.22
125 ppm	0	3.27 ± 0.12	3.31 ± 0.32	3.19 ± 0.15	3.15 ± 0.16	3.14 ± 0.16
0 ppm	0	0.05 ± 0.01	0.27 ± 0.05	0.37 ± 0.04	0.56 ± 0.05	0.71 ± 0.10

Table 4.6. Population reduction of *Alicyclobacillus acidoterrestris* spores strain VF, WAC and SAC in orange juice treated with

DMDC stored for 24 hours at ambient temperature (20°C).

DMDC concentration	Population reduction (log CFU/ml)					
	0 h	2 h	4 h	6 h	12 h	24 h
VF						
250 ppm	0	0.20 ± 0.08	0.21 ± 0.03	0.24 ± 0.01	0.22 ± 0.03	0.24 ± 0.02
125 ppm	0	0.05 ± 0.02	0.06 ± 0.01	0.06 ± 0.02	0.05 ± 0.02	0.08 ± 0.01
0 ppm	0	-0.01 ± 0.02	0.01 ± 0.05	0.02 ± 0.02	0.03 ± 0.04	0.08 ± 0.05
WAC						
250 ppm	0	0.22 ± 0.02	0.21 ± 0.01	0.22 ± 0.04	0.24 ± 0.04	0.25 ± 0.02
125 ppm	0	0.06 ± 0.04	0.04 ± 0.02	0.07 ± 0.02	0.12 ± 0.05	0.10 ± 0.03
0 ppm	0	-0.01 ± 0.03	-0.01 ± 0.01	0.01 ± 0.01	-0.00 ± 0.04	0.02 ± 0.01
SAC						
250 ppm	0	0.19 ± 0.02	0.22 ± 0.01	0.23 ± 0.04	0.23 ± 0.06	0.24 ± 0.05
125 ppm	0	0.10 ± 0.04	0.08 ± 0.03	0.10 ± 0.03	0.14 ± 0.01	0.15 ± 0.03
0 ppm	0	-0.01 ± 0.03	0.00 ± 0.02	0.03 ± 0.01	0.01 ± 0.01	0.08 ± 0.04

Table 4.7. D-value (min) of *Alicyclobacillus acidoterrestris* spores in apple juice and orange juice at different temperature of 90°C, 95°C, and 98°C.

Temperature	DMDC concentration	Apple juice			Orange juice		
		VF	WAC	SAC	VF	WAC	SAC
90°C	0 ppm	49.93 ± 1.01^{A}	59.30 ± 2.63	49.15 ± 0.98	41.44 ± 0.43^{A}	62.45 ± 2.76	49.61 ± 1.28
	250 ppm	52.26 ± 0.58^{B}	59.30 ± 3.32	49.85 ± 0.93	45.59 ± 0.32^{B}	59.31 ± 4.88	50.18 ± 1.00
95°C	0 ppm	14.93 ± 0.62	13.86 ± 0.58	15.25 ± 0.44	9.40 ± 0.90	9.54 ± 0.70	8.66 ± 0.05^{A}
	250 ppm	15.42 ± 0.81	14.08 ± 1.49	15.49 ± 0.40	10.18 ± 0.76	9.42 ± 0.56	13.69 ± 1.04^{B}
98°C	0 ppm	6.22 ± 0.40	5.16 ± 0.84	4.95 ± 0.19	5.73 ± 0.10	3.99 ± 0.40	5.20 ± 0.48
	250 ppm	5.81 ± 0.47	6.03 ± 0.91	5.22 ± 0.26	5.11 ± 0.48	3.79 ± 0.36	4.91 ± 0.25

A,B: within the column at the same temperature; a difference letter in mean values indicates significant differences were observed at $\alpha = 0.05$ ($P \leq 0.05$).

Table 4.8. z-value (in Celsius) of *Alicyclobacillus acidoterrestris* spores in apple juice and orange juice.

Alicyclobacillus acidoterrestris	Apple juice		Orange juice	
	Control	250 ppm DMDC	Control	250 ppm DMDC
VF	8.89	8.43	8.96	8.30
WAC	7.57	8.05	6.60	6.63
SAC	8.04	8.19	7.76	7.97

Table 4.9. Chemical and physical properties of apple juice treated with DMDC (250, 125, 50, and 0 ppm).

DMDC (ppm)	pH [ns]	Degree Brix (°Brix) [ns]	Ascorbic acid (mg/100ml) [ns]	% Titratable acidity (%malic acid) [ns]	Color			Total phenolic compound [ns 1]
					L^{*} [ns]	b^{*} [ns]	a^{*} [ns]	
250	3.626 ± 0.002	11.70 ± 0.02	51.95 ± 0.59	0.3810 ± 0.0026	86.67 ± 0.03	30.50 ± 0.07	-3.90 ± 0.02	69.33 ± 2.56
125	3.633 ± 0.003	11.73 ± 0.02	51.77 ± 0.41	0.3855 ± 0.0068	86.53 ± 0.10	30.51 ± 0.04	-3.92 ± 0.07	65.85 ± 1.57
50	3.632 ± 0.002	11.68 ± 0.02	52.36 ± 0.18	0.3810 ± 0.0052	86.62 ± 0.13	30.49 ± 0.04	-3.93 ± 0.03	68.74 ± 1.80
0 (control)	3.628 ± 0.002	11.66 ± 0.03	52.07 ± 0.12	0.3765 ± 0.0052	86.57 ± 0.06	30.52 ± 0.07	-3.96 ± 0.00	68.69 ± 1.90

ns: no significant differences ($\alpha = 0.05$) of mean values were detected between treatments.

[1] : total phenolic compound were expressed as a mg gallic acid /100 ml juice sample.

Table 4.10. Chemical and physical properties of orange juice treated with DMDC (250, 125, 50, and 0 ppm).

DMDC (ppm)	pH [ns]	Degree Brix (°Brix) [ns]	Ascorbic acid (mg/100ml) [ns]	% Titratable acidity (%citric acid) [ns]	Color			Total phenolic compound [ns 1]
					L^{*} [ns]	b^{*} [ns]	a^{*} [ns]	
250	3.890 ± 0.005	12.17 ± 0.03	29.09 ± 1.58	0.7467 ± 0.0133	43.49 ± 0.02	-0.73 ± 0.01	13.80 ± 0.13	75.68 ± 1.54
125	3.890 ± 0.005	12.18 ± 0.03	29.86 ± 1.75	0.7403 ± 0.0148	43.57 ± 0.13	-0.74 ± 0.04	13.99 ± 0.14	76.16 ± 0.32
50	3.893 ± 0.003	12.22 ± 0.06	28.86 ± 1.64	0.7424 ± 0.0128	42.83 ± 0.88	-0.77 ± 0.11	14.32 ± 0.14	77.61 ± 1.83
0 (control)	3.892 ± 0.005	12.23 ± 0.05	29.94 ± 2.93	0.7339 ± 0.0133	43.13 ± 0.66	-0.76 ± 0.05	14.24 ± 0.30	76.70 ± 0.83

ns: no significant differences ($\alpha = 0.05$) of mean values were detected between treatments.

[1] : total phenolic compound were expressed as a mg gallic acid /100 ml juice sample.

DISCUSSION

Changes in consumer consumption habits and current food-processing technologies have created conditions that have selected for the spoilage-associated microorganism, *A. acidoterrestris*, a spore forming bacterium. *Alicyclobacillus* spp. are commonly found in soils and their spores are able to germinate and grow under high acid conditions, such as those found in apple, orange, and grapefruit juices (16, 27). *A. acidoterrestris* vegetative cells are heat resistant and more intense heat treatments are required for inactivation compared to other vegetative cells, such as lactic acid bacteria and yeasts, that are common microbial contaminants in fruit and vegetable juices. Moreover, *Alicyclobacillus* spp. also produce spores which are highly heat resistant. For example, D-values of greater than 60 min at 85°C and approximately 8 min at 95-97°C have been reported (28). Hot fill processes during commercial pasteurization for juices usually hold the products at temperature between 88-96°C for approximately 2 min (22); therefore, *A. acidoterrestris* spores have become an important spoilage concern for juice industries. Fruit juices and fruit-containing beverages are highly susceptible to *A. acidoterrestris* in either fresh or pasteurized juice products (27). Consequently, alternative treatments or processing to control this bacterium are necessary if less heat treatment is going to be used to satisfy consumers demands for a fresh-taste finished product.

DMDC has been approved by the FDA for the use in wines, juices, and beverages (40). DMDC was primarily used as a yeast inhibitor in wines, but recently it has been shown that DMDC is not an effective treatment in wines due to the lack of effectiveness against lactic acid bacteria in wines (8). Conversely, the antimicrobial effects of DMDC against *E. coli* O157:H7 in apple cider has been

shown to be capable of achieving > 5 log reductions within 6 hours with 250 ppm DMDC, the maximum limit approved by FDA (4). In addition, combination treatments of ozone, DMDC, and hydrogen peroxide showed the antimicrobial effects on *E. coli* O157:H7 and *Salmonella* in apple juice and orange juice (45). Moreover, Van der Riet et al. (42) investigated the effect of DMDC at 10°C and 30°C in apple juice against vegetative cells and ascospores of *Byssochlamys fulva* strains WG and BFW, with the initial cell concentrations of approximately $10^2 - 10^3$ CFU/ml and they found that DMDC showed inhibitory effect against vegetative cells of both strains but not with theirs ascospores at the concentration of 25 to 75 ppm. A greater log reduction was achieved at 30°C than 10°C and more than 2 log reduction was achieved after 60 min of adding DMDC of treated apple juices (50 ppm and 75 ppm) with both strains.

Our studies showed that vegetative cells for all *Alicyclobacillus* strains tested were more sensitive than their spores. Vegetative cells of SAC were the most sensitive to DMDC of the strains tested. A >4 log reduction (log CFU/ml) was achieved in PDB and >5 log reduction in apple juice, and >3 log reduction in orange juice, were achieved with 250 ppm DMDC treatments. Furthermore, 250 ppm DMDC was the most effective concentration to inhibit *A. acidoterrestris* growth with both vegetative cells and spores, compared to 125 and 50 ppm DMDC. Thus, 250 ppm DMDC was selected to investigate the effects on the organoleptic properties with apple and orange juices.

The inhibitory effect of DMDC on vegetative cells and spores of *A. acidoterrestris* in apple juice and orange juice were slightly greater than in PDB samples for all strains tested except SAC vegetative cells in orange juice, and WAC spores in apple juice. The lower inhibitory effect observed with PDB is

likely due to the fact that PDB is an enriched medium for *A. acidoterrestris* and total phenolic compounds found in juices might have a synergetic inhibitory effect on *A. acidoterrestris* (36). DMDC has a rapid inhibitory effect on both spores and vegetative cells, with as short as 2 hours of treatment for all menstra (PDB, apple juice and orange juice). Moreover, DMDC was shown to be very efficient against both vegetative cells and spores of all strains and samples tested within 10 min after DMDC treatments from preliminary data (data not shown). DMDC rapidly hydrolyzes and non-specifically methylates the proteins of spores and vegetative cells of *A. acidoterrestris*, resulting in a highly efficient inhibitory effect. This attribute can be used by the juice and beverage industries to improve the safety and reduce the spoilage incidence of juice products with limited processing time.

Growth of *Alicyclobacillus* spp. vegetative cells and spores are influenced by juice type. Splittstoesser et al. (36) reported the growth of *Alicyclobacillus* spp. for VF and WAC spores in tomato juice, apple juice, and orange juice, was increased while their growth was decreased in prune juice after 2-day incubation at 43°C. Moreover, no growth of *Alicyclobacillus* was observed in red grape juice, and it was proposed that specific phenolic compounds contained in red grape juice has an inhibitory effect on *Alicyclobacillus* spore germination and growth (36). Different growth behavior by *Alicyclobacillus* in different juices might be due to the natural composition of juices, including phenolic compounds and physical properties of juice, such as pH and soluble solids content (7). *Alicyclobacillus* growth has been shown to be inhibited by the sugar content in juices when the sugar content is above 18 °Brix and it is completely inhibited by 6% ethanol (33).

D-and z-values are valuable parameters used in commercial food processing when attempting to either decrease time to achieve product safety and

stability or decrease the temperature of processing to achieve the same or better product quality (2). Most of antimicrobial substances, such as nisin and lysozyme, used to inhibit *Alicyclobacillus* spores commonly decrease heat resistance or D-value of its spores. Nisin with 50 International Unit/ml (IU/ml) in apple juice was able to reduce heat resistance ($D_{80°C}$ and $D_{90°C}$) of *A. acidoterrestris* spores by about 40% while 0.005% lysozyme (vol/vol) in commercial orange juice decreased $D_{89°C}$ of *A. acidoterrestris* AB-1 by approximately 50% (16, 49). On the contrary, our results showed that the D-values for *A. acidoterrestris* VF treated with 250 ppm DMDC were significantly higher ($\alpha = 0.05$) than of control at 90°C in apple and orange juice, and with strain SAC at 95°C in orange juice, when compared to other treatments. It is obvious that D-values are greatly affected by the combined effects of temperature and DMDC treatment. The mechanism for this significant increase in D-values of DMDC-treated samples compared to controls is not clearly established, but is probably due to DMDC-induced stress on *A. acidoterrestris* spores and hydrophobicity on the spore surface that results in clump formation of the cells, thus resulting in the apparent increase in the heat resistance or D-value (12, 44). In addition, it has been reported that some bacterial species that includes *E. coli*, *Listeria monocytogenes*, and *Lactobacillus plantarum*, develop stress responses when exposed to sub-lethal temperatures and produce heat-shock protein (HSPs), leading to higher thermotolerance in the cells (9, 15, 20). The z-values of *A. acidoterrestris* from literature by Sapers et al. (30) were reported to be relatively similar within the range of 8.3 ± 1.9°C. Our results were also in agreement of these conclusions. Thermal inactivation of *A. acidoterrestris* spores is affected by several environment factors that include temperature, total soluble solids, pH, nutrient composition, water activity, divalent cations, and antimicrobial

compounds (5, 26). According to the study by Silva et al. (33), it was reported that the D-value of *A. acidoterrestris* spores decreased with increasing temperature (85-97°C), decreasing total soluble solid (5-60 °Brix) and decreasing pH (2.5-6.0).

The mechanism of inactivation of DMDC on microorganism is related to the inhibition of essential enzymes via DMDC methylation. It has been reported that lactate dehydrogenase, a critical enzyme for bacteria, was completely inactivated by diethyl dicarbonate, which is similar to DMDC (13). Lactate dehydrogenase is an essential enzyme in the glycolysis pathway to convert pyruvate, the final product of glycolysis, to lactate in low oxygen levels or in the absence of oxygen (23). DMDC activity is dependent on the physical and chemical characteristics of foods, such as temperature, pH, and ethanol content. In general, reaction rates increase with increasing temperature and ethanol concentration, whereas lower pH lowers the reaction rate (25, 29). It has been reported that an increase in temperature from 20°C to 40°C resulted in a 100-fold increase in inhibitory activity against *Lactobacillus plantarum* (37). DMDC is currently used in juice and beverage industries as a microbial inhibitor and it is usually added to finished juices or beverages before bottling (40). DMDC is not required to be declared on the product label because it is a processing aid and is rapidly hydrolyzed to carbon dioxide and small amounts of methanol (17). However, one disadvantage is the capital cost associated with the dosing unit that is needed for the DMDC addition to the juice or beverage.

Humans cannot synthesize gluconolactone oxidase, which is the specific enzyme for vitamin C synthesis. Therefore, fruits and vegetables are necessary parts of the human diet since they are the most abundant source of vitamin C in foods (3). Vitamin C is the most abundant vitamin in apple juice and orange juice,

related to antioxidant activity and health benefits such as risk reduction in coronary heart disease and anticancer treatment (24, 43). In addition, vitamin C has been used as an antibrowning agent in the food industry, including apple products (31). However, vitamin C is rapidly degraded by high temperatures and oxygen during juice processing (19). Therefore, most juice processors supplement vitamin C in the final products to retain the vitamin C content and serve as a good source of vitamin C.

Color is one of the most important characteristics of food products since it is the first feature exposed to consumers. As a result, it is critical for any new processing treatment or technique to have no or limited effect on the color characteristics of the food products. Color is represented by three values; L^*, a^*, and b^* (14). L^* color indicates brightness; the maximum L^* is 100, which represents a perfect reflection, whereas minimum L^* which is 0 represents black color. Positive a^* indicates red and green for negative a^* while positive b^* indicates yellow and blue for negative b^*. Apple juice showed an L^* value of approximately 86, indicating the color was reasonably bright due to the fact it was a clear juice. a^* and b^* values were approximately -3.90 and 30, respectively, indicating green and yellow color for the apple juice. Apple juice color was measured using a transmittance technique, described as the light passing through the samples compared to the light that is not. This is commonly used for clear juice, whereas the reflectance technique, the color measurement of reflected or scattered light of samples, is used for orange juice (10). The different color measurement techniques were applied because of the different nature of juice in terms of cloudiness and clearness.

Polyphenols and phenolic compounds commonly found in fruit and vegetable diets have become of interest in the last 20 years due to their health benefits relating to the prevention of a cardiovascular disease and cancers by the protection of oxidation reactions taking place in cells and tissues (32). The majority of phenolic compounds found in apple juice are flavanols (quercitins) and proanthocyanidins (cathechin and epicathechin) which are responsible for the bitterness and astringency (21), whereas flavonoids are the dominant phenolic compounds in orange juice (38). Our results showed that DMDC did not alter the total phenolic compound content of apple juice and orange juice resulting in antioxidant compounds in apple and orange juices were preserved after DMDC treatment.

Sensory attributes play an important role for all food products and sensory evaluation of the effects of DMDC on the sensory characteristics of apple juice and orange juice were performed. A triangle test was used to determine if panelists were able to detect the differences between the untreated control and DMDC treated juices. DMDC at the highest concentration allowed by FDA (250 ppm), demonstrated that it did not affect the sensory characteristics in apple juice and orange juice based on the triangle test conducted. It may be that the typical flavors in apple juice and orange juice are not affected by DMDC due to the rapid hydrolysis of DMDC to methanol and carbon dioxide (17). Twenty one and twenty three panelists in apple and orange juice, respectively, were unable to select the odd sample correctly, indicating there were not significant differences ($\alpha = 0.05$) between the control and the 250 ppm DMDC treated juice. Thus, the quality of both apple and orange juice were retained after DMDC treatment.

Since DMDC exhibited a bactericidal effect against the *A. acidoterrestris* strains tested, VF, WAC, and SAC, it may be an alternative treatment for the apple juice and orange juice industry to improve the microbiological safety and quality without any deleterious effects on the physical, chemical, and sensory properties. However, spores were shown to be more resistant than vegetative cells, and were only reduced by approximately 1 log and 0.2 log reduction in apple juice and orange juice, respectively. Therefore, combination treatments between DMDC and other treatments are necessary to control *A. acidoterrestris* spoilage in juices and beverages.

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CHAPTER 5

ANTIMICROBIAL ACTIVITY OF PAPAIN AND BROMELAIN TO PROTECT AGAINST *Alicyclobacillus acidoterrestris* SPOILAGE IN FRUIT JUICES

ABSTRACT

Alicyclobacillus acidoterrestris **is thermoacidophilic, spore-forming, non-pathogenic bacterium, that is associated with spoilage of shelf-stable juices and beverages due to the extreme thermal resistance of their spores. The antimicrobial effects of papain and bromelain were investigated against vegetative cells and spores of** *A. acidoterrestris* **strains; VF, WAC, and SAC, in potato dextrose broth, apple juice, and orange juice. Papain and bromelain were added to apple and orange juice to achieve final concentrations of 100 ppm and 1,000 ppm for each enzyme, and the survival of** *A. acidoterrestris* **was monitored over the incubation period. Papain and bromelain exhibited matrix-dependent antimicrobial activity against vegetative cells for all stains of** *A. acidoterrestris* **tested but no sporicidal activity at any of the enzyme concentrations tested. Papain showed greater antimicrobial activity with vegetative cells of all strains tested compared to bromelain at the same concentrations. Approximately 3-log and 1-log reductions were achieved for all strains tested with 1,000 ppm bromelain in apple and orange juice, respectively, whereas approximately 3-4 log reduction was achieved for all strains tested with 1,000 ppm papain in the various menstra. No significant differences ($\alpha = 0.05$) were observed between mean values of control samples and treated samples for any of the physicochemical properties of the juices tested except °Brix and turbidity. Sensory evaluation showed no significant**

differences ($\alpha = 0.05$) between control and 100 ppm papain and 1,000 ppm

papain in apple and orange juices, respectively.

INTRODUCTION

Orange juice and apple juice are the first and second largest juices consumed in the US, respectively, and have recently gained popularity with consumers due to their health benefits (10). High water activity, sugar content, minerals, and vitamins are all factors that influence the microbial populations that are associated with fruit juices. Contamination with spoilage microorganisms in juices may take place at any of the steps during juice processing. Microbial spoilage results in changes in odor, flavor, or appearance of juices, leading to limited shelf life due to an unacceptable product (29). In general, microbial spoilage is limited by natural characteristics of juices such as pH of the juices and high temperature treatment during the commercial juice processing (18, 44). *Clostridium, Bacillus*, and foodborne pathogens are important microorganisms associated with the safety and stability in juices and beverages. However; the spores of *Clostridium* and *Bacillus* are unable to germinate, and vegetative cells of pathogens are unable to grow under the acidic conditions naturally found in fruit juices (42). However, *Alicyclobacillus* spp. have been isolated from a variety of spoiled fruit juice products since its first report of causing spoilage in fruit juices was in 1982 (6).

Alicyclobacillus is a thermoacidophilic, spore-forming, non-pathogenic bacterium, that has been isolated mainly from soil and hot springs (44). Spores are able to survive the typical pasteurization processes used for juice products, and the vegetative cells are capable of growing under acidic conditions as those found in fruit juices. The genus of *Alicyclobacillus* is composed of several species;

however, *A. acidoterrestris* is the primary species associated of concern in the juice industry due to its high heat resistant characteristic compared to other species (43). Spoilage by *A. acidoterrestris* has been primarily identified in apple juice but also in a wide range of juices and beverages including tomato products, tea, and herbal beverages (37, 38, 47). It is difficult to visually detect the spoilage by *A. acidoterrestris* because only slight cloudiness may be found in clear juices and there is no swelling of containers from gas or acid production. *A. acidoterrestris* is responsible for the spoilage of several juices and beverages by the production of off-flavors, described as medicinal or antiseptic, mainly due to guaiacol (2-methoxyphenol) and 2,6 dibromophenol, which are considered as the two most important compounds contributing to juice taint (7, 14, 19, 29, 46).

Papain and bromelain, protein-digesting (proteolytic) enzymes, are commonly known as tenderizing agents for meat products by hydrolysis of muscle protein (16). Papain is primarily derived from unripe papaya fruit and pawpaw tree (9), whereas bromelain is derived from the stem and juice of pineapple. Bromelain has been studied in animals and has been found to have many pharmacological benefits such as prevent blood platelets aggregation, act as an anti-inflammatory agent, enhance the membrane permeability of antibiotics, and reduce the thrombus formation (24). Papain, a sulfhydryl protease with molecular weight of 23 kDa, is widely applied in food, textile, and cosmetic industries. Papain and bromelain have been used for medical applications that include anti-inflammatory, lysing would lesions, inhibit parasites, and promote digestion (32, 39). Some of the therapeutic applications of papain and bromelain are associated with the antibacterial activity and may be potential compounds to improve the microbial safety and quality of juice by inhibiting the growth of spoilage and foodborne pathogens. The objectives

of this study were to investigate the effect of papain and bromelain on preventing

the growth of *A. acidoterrestris* in apple and orange juices.

MATERIALS AND METHODS

Materials, chemicals, and media. Shelf-stable apple juice and refrigerated

orange juice were locally purchased in Geneva, NY (Wegmans, Rochester, NY).

Potato dextrose broth (PDB) and Bacto[TM] peptone were obtained from Becton,

Dickinson and Company (Spark, MD). Potato dextrose agar (PDA) was distributed

from Weber Scientific (Hamilton, NJ). Tartaric acid was obtained from J.T. Baker

Chemical Co. (Phillipsburg, NJ). 2,6 dichlorophenolindophenol and pure L-

ascorbic acid was obtained from Sigma Chemical company (USA). Sodium

hydroxide and sodium benzoate was obtained from Fisher Scientific (Pittsburgh,

PA). Folin & Ciocalteu's phenol reagent was obtained from MP Biomedical, LLC

(Solon, OH). Gallic acid and was obtained from Sigma-Aldrich Co. LLC. (USA).

Papain and bromelain were purchased from Acros Organics (Fair Lawn, NJ)

Bacterial strains. *A. acidoterrestris* strains VF, WAC, and SAC used in

this study were originally isolated from spoiled shelf-stable fruit juices and

maintained in Dr. Worobo's culture collection.

Bacterial cell and spore preparation:

- **Vegetative cells:** *A. acidoterrestris* was cultivated overnight in

PDB pH 3.5, acidified by sterile 10% tartaric acid (wt/vol) at 50°C. This culture

was transferred into fresh PDB, apple juice, or orange juice, in sterile flasks to

achieve final cell concentration of 10^5-10^6 organisms per ml sample (CFU/ml).

- **Spores:** *A. acidoterrestris* was cultivated in PDB pH 3.5, acidified

by sterile 10% tartaric acid (wt/vol) and incubated at 50°C for 7 days. Heat

treatment at 80°C for 10 min was performed to inactivate any vegetative cells of *A.*

acidoterrestris. This suspension was transferred to sterilized flasks containing PDB, apple juice or orange juice to achieve the final spore concentration of approximately 10^5-10^6 spores per ml sample (CFU/ml).

Treatments. Papain and bromelain stock solutions (10,000 ppm) were prepared by dissolving each enzyme in sterile deionized water and filtered through 0.22 μm nitrocellulose membrane with 13 mm diameter (CELLTREAT Scientific Products, LLC, Shirley, MA). Enzyme stock solutions were directly added to PDB, apple juice, or orange juice, to achieve the final concentration of 100 ppm and 1,000 ppm for each enzyme, with 0 ppm as a control. Sodium benzoate was prepared in the same way as the enzyme preparations.

Microbiological analysis. All samples were stored at ambient temperature and were tested at 0, 2, 4, 6, and 24 hours after inoculation. Serial dilution in 0.1% (wt/vol) peptone water was performed immediately after sampling. Pour plate technique was used with PDA pH 3.5, acidified by sterile 10% (wt/vol) tartaric acid. Following incubation at 50°C for 72 hours, populations were enumerated. The duplicate plate count averages for the three replications were converted to log scale and the log reductions for the various treatments were calculated.

Stability of *Alicyclobacillus* during storage. Bacterial cultures were prepared same as above and were inoculated in apple juice and orange juice to achieve the final cell concentration of approximately 10^2-10^3 CFU/ml. This experiment was performed with both vegetative cells and spores. Treatments were 200 ppm sodium benzoate and papain (100 ppm papain for apple juice and 1,000 ppm papain for orange juice) with no-added enzyme treatment as a control. After adding the enzymes and treatment, juice samples were stored at room temperature (ambient temperature). The samples were analyzed in duplicate for microbiological

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properties as previously described at day 0, 1, 2, 5, and every week until 6 weeks of storage.

Analysis of physical and chemical properties of apple and orange juice.
Juice samples were analyzed as described below without any microbial inoculation. Samples were kept at ambient temperature for 6 hours before measurements were performed. pH was measured using a pH meter (Orion PerpHect pH LogR meter Model 310, Thermo Scientific, Baverly, MA). °Brix was determined using a digital refractometer (Reichert Model 10430, Depew, NY). Titratable acidity was described as grams of malic acid/100 ml apple juice and as grams of citric acid/100 ml orange juice by titrating with 0.1 N NaOH (1). Ascorbic acid was determined by titrimetric analysis using 2,6 dichlorophenolindophenol (1). Total phenolic compounds were measured by the Folin Ciocalteau method using gallic acid as a standard, as described by Singleton and Rossi (36). Color values (L^*, a^* and b^*) were measured using a Hunter Lab model UltraScan XE spectrophotometer (Reston, VA). Turbidity was measured using portable turbidimeter (Model 2100AN, Hach Co., Loveland, CO) with formazin as a standard solution. Turbidity was reported as Nephelometric Turbidity Units (NTU).

Turbidity during storage. Juice samples treated with papain or bromelain, and the control (0 ppm enzymes) were prepared the same as in papain and bromelain treatments without bacterial inoculation. Samples were kept at ambient temperature and were taken at 0, 1, 2, 4, 6, 24, 48, and 72 hours. Samples were transferred to the cell holder for turbidity measurement. Haze formation in juices was gently mixed by vortexing (Model Vortex-Genie 2, Scientific Industries, Inc.,

Bohemia, NY) prior to sample turbidity measuring. Turbidity was measured and reported as previously described.

Sensory evaluation. 30 experienced-panelists from the Department of Food Science (New York State Agricultural Experiment Station, Cornell University, Geneva, NY) were used for the sensory evaluations using a triangle test and acceptance test (21). A blind triangle test was described as follows: papain stock solution was added to samples without microbial inoculation to achieve a final concentration of 100 ppm and 1,000 ppm in apple juice and orange juice respectively, with 0 ppm serving as a control. Each opaque sample cup (88 ml) containing approximately 50 ml of juices and was covered with aluminum foil. Three samples coded with 3-digit number were served to panelists. Two of the samples were controls (no papain added) and the odd one was apple juice or orange juice containing 100 ppm or 1,000 ppm papain treatment, respectively. Apple juice treated with papain was kept at room temperature for 24 hours, whereas orange juice was kept at room temperature for 6 hours followed by 4°C for 18 hour prior to performing the sensory test. Panelists were asked to test all samples in the order presented, from left to right, and check the number of sample that was different. Acceptance test was performed in both apple juice and orange juice by asking 30 experienced-panelists to score the 5 treated samples, including 100 ppm and 1,000 ppm bromelain, 100 ppm and 1,000 ppm papain, and the control (no enzyme added). All samples were prepared the same as the triangle test. A 9-point hedonic scale questionnaire was used in the acceptance test and panelists were asked to score each samples in three categories; color liking, appearance liking, and overall liking, with the score range of 1 to 9 (1 = dislike

extremely, 3 = dislike moderately, 5 = neither like nor dislike, 7 = like moderately, 9 = like extremely).

Statistical Analysis. The analyses of physical and chemical properties of juices were repeated three times. An analysis of variance (ANOVA) was carried out to test the differences between the mean values of physical and chemical properties, and sensory evaluation using the Tukey HSD test at $\alpha = 0.05$.

RESULTS

Tables 5.1, 5.2, and 5.3 represent the population reduction (log CFU/ml) of vegetative cells of *A. acidoterrestris* strains VF, WAC, and SAC in PDB, apple juice, and orange juice, respectively. In general, results showed that at same concentration, a greater log reduction was achieved with papain compared to bromelain for all the samples tested. Higher concentrations resulted in higher log reductions with both papain and bromelain treatments for all samples tested.

The highest log reduction against vegetative cells was achieved with 1,000 ppm bromelain treatment in apple juice. The same bromelain treatment with PDB resulted in an approximate 3-4 log reduction, whereas approximately 1-log reduction was observed with orange juice samples for all the strains tested. A 3-4 log reduction was achieved using 100 ppm and 1,000 ppm papain treatments for all samples and all strains tested with apple juice and PDB. Whereas, a 1-log reduction was observed with 100 ppm papain treatments, and 3-4 log reduction resulted with 1,000 ppm papain treatment in orange juice with VF, WAC, and SAC, respectively. Preliminary results showed that papain and bromelain did not have any bacterial inhibitory effect against *A. acidoterrestris* spores for strains VF, WAC, and SAC in all samples tested (data not shown). Papain and bromelain at all concentrations tested showed no inhibitory activity against any of the

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Alicyclobacillus spores over time period of 6 weeks for the strains tested.
Whereas, the vegetative cells for all strains tested showed a decrease in cell
numbers until they were below the detection limit after day 1 of incubation at
ambient temperature for both apple juice and orange juice (data not shown).

Physical and chemical properties of apple juice and orange juice treated
with papain and bromelain are shown in Table 5.4 and 5.5, respectively. It can be
clearly seen in Tables 5.4 and 5.5, apple juice and orange juice quality parameters
including pH, % acidity, ascorbic acid content, total phenolic compounds, and
color values (L^*, a^*, b^*) were not significantly ($\alpha = 0.05$) affected by papain or
bromelain treatments, but significant differences ($\alpha = 0.05$) were observed between
the mean values for °Brix and turbidity between control and treated samples.

Turbidity in all treated samples of apple juice increased rapidly in the first 6
hours and then slightly increased until the end of storage while turbidity for all
treated samples of orange juice decreased over the storage time (Figure 5.1 and
5.2).

Sensory evaluation using triangle test demonstrated that seventeen and
eigthteen experienced panelists out of thirty could not choose the odd sample
correctly in apple juice and orange juice, respectively. Thus, no significant
differences between control (no enzyme) and treated samples (enzyme treatment)
were detected ($\alpha = 0.05$). Color liking, appearance liking, and overall liking scores
of apple juice and orange juice samples are shown in Figure 5.3. There were no
significant differences between samples ($\alpha = 0.05$) for orange juice treatments for
all attributes, whereas, significant differences were observed for all scores between
treated samples in apple juice ($\alpha = 0.05$). All the scores for the 1,000 ppm
bromelain treated samples were the lowest for all the treatments, followed by 100

ppm bromelain, 1,000 ppm papain, and 100 ppm papain, respectively, whereas the

control sample showed the highest score in all categories for apple juice.

Table 5.1. Log reduction of *Alicyclobacillus acidoterrestris* vegetative cells (strains VF, WAC, and SAC) in PDB treated with papain

and bromelain for 24 hours at ambient temperature (20°C).

Papain and bromelain Concentration	Log reduction (log CFU/ml)				
	0 h	2 h	4 h	6 h	24 h
VF					
0 ppm (control)	0	-0.15 ± 0.06	-0.10 ± 0.07	-0.15 ± 0.05	-0.78 ± 0.15
100 ppm bromelain	0	0.41 ± 0.08	0.54 ± 0.14	0.69 ± 0.11	0.68 ± 0.03
1,000 ppm bromelain	0	2.30 ± 0.21	3.14 ± 0.12	3.42 ± 0.13	3.29 ± 0.20
100 ppm papain	0	2.27 ± 0.05	3.27 ± 0.12	3.41 ± 0.06	3.47 ± 0.12
1,000 ppm papain	0	3.15 ± 0.19	3.39 ± 0.09	3.51 ± 0.13	3.51 ± 0.14
WAC					
0 ppm (control)	0	0.07 ± 0.06	0.04 ± 0.03	0.07 ± 0.09	-0.08 ± 0.12
100 ppm bromelain	0	0.27 ± 0.05	0.45 ± 0.07	0.68 ± 0.13	0.69 ± 0.12
1,000 ppm bromelain	0	1.29 ± 0.35	2.13 ± 0.16	3.04 ± 0.20	2.95 ± 0.27
100 ppm papain	0	2.92 ± 0.17	3.13 ± 0.31	3.14 ± 0.22	3.17 ± 0.40
1,000 ppm papain	0	3.03 ± 0.16	3.18 ± 0.34	3.65 ± 0.13	3.67 ± 0.17
SAC					
0 ppm (control)	0	-0.09 ± 0.14	-.0.20 ± 0.17	-0.21 ± 0.21	-0.36 ± 0.21
100 ppm bromelain	0	0.16 ± 0.07	0.12 ± 0.05	0.32 ± 0.04	0.30 ± 0.06
1,000 ppm bromelain	0	1.70 ± 0.10	2.28 ± 0.11	3.58 ± 0.25	3.52 ± 0.26
100 ppm papain	0	3.23 ± 0.12	3.47 ± 0.23	3.64 ± 0.16	3.64 ± 0.12
1,000 ppm papain	0	3.64 ± 0.23	4.33 ± 0.17	4.35 ± 0.25	4.27 ± 0.11

Table 5.2. Log reduction of *Alicyclobacillus acidoterrestris* vegetative cells (strains VF, WAC, and SAC) in apple juice treated with

papain and bromelain for 24 hours at ambient temperature (20°C).

Papain and bromelain Concentration	Log reduction (log CFU/ml)				
	0 h	2 h	4 h	6 h	24 h
VF					
0 ppm (control)	0	-0.32 ± 0.14	-0.32 ± 0.03	-0.31 ± 0.04	-0.19 ± 0.20
100 ppm bromelain	0	0.94 ± 0.12	1.16 ± 0.15	1.44 ± 0.08	1.82 ± 0.10
1,000 ppm bromelain	0	1.67 ± 0.26	2.60 ± 0.17	3.02 ± 0.33	3.45 ± 0.07
100 ppm papain	0	2.78 ± 0.23	3.44 ± 0.13	3.74 ± 0.17	4.00 ± 0.16
1,000 ppm papain	0	4.01 ± 0.24	4.31 ± 0.20	4.26 ± 0.19	4.14 ± 0.32
WAC					
0 ppm (control)	0	-0.16 ± 0.19	-0.29 ± 0.12	-0.46 ± 0.34	-0.25 ± 0.09
100 ppm bromelain	0	0.55 ± 0.05	0.82 ± 0.07	1.17 ± 0.20	1.71 ± 0.08
1,000 ppm bromelain	0	1.57 ± 0.20	2.40 ± 0.11	3.63 ± 0.20	3.80 ± 0.17
100 ppm papain	0	1.87 ± 0.08	2.09 ± 0.09	3.42 ± 0.15	3.51 ± 0.04
1,000 ppm papain	0	2.88 ± 0.16	3.51 ± 0.17	3.56 ± 0.10	3.80 ± 0.05
SAC					
0 ppm (control)	0	-0.12 ± 0.22	-0.15 ± 0.24	-0.15 ± 0.23	-0.20 ± 0.52
100 ppm bromelain	0	1.19 ± 0.30	1.77 ± 0.34	2.11 ± 0.10	2.64 ± 0.08
1,000 ppm bromelain	0	2.46 ± 0.41	3.25 ± 0.08	3.46 ± 0.14	3.81 ± 0.06
100 ppm papain	0	2.98 ± 0.27	3.33 ± 0.10	3.40 ± 0.01	3.59 ± 0.05
1,000 ppm papain	0	3.02 ± 0.08	3.43 ± 0.27	3.64 ± 0.19	3.78 ± 0.09

Table 5.3. Log reduction of *Alicyclobacillus acidoterrestris* vegetative cells (strains VF, WAC, and SAC) in orange juice treated with

papain and bromelain for 24 hours at ambient temperature (20°C).

Papain and bromelain Concentration	Log reduction (log CFU/ml)				
	0 h	2 h	4 h	6 h	24 h
VF					
0 ppm (control)	0	-0.23 ± 0.14	-0.15 ± 0.07	-0.21 ± 0.07	-0.25 ± 0.08
100 ppm bromelain	0	0.41 ± 0.10	0.47 ± 0.11	0.45 ± 0.07	0.51 ± 0.05
1,000 ppm bromelain	0	0.98 ± 0.23	1.17 ± 0.07	1.08 ± 0.06	1.06 ± 0.09
100 ppm papain	0	1.69 ± 0.20	2.53 ± 0.36	2.90 ± 0.13	3.04 ± 0.08
1,000 ppm papain	0	3.17 ± 0.14	3.36 ± 0.27	3.50 ± 0.21	3.38 ± 0.14
WAC					
0 ppm (control)	0	-0.15 ± 0.11	-0.02 ± 0.21	-0.23 ± 0.20	-0.19 ± 0.18
100 ppm bromelain	0	0.43 ± 0.07	0.33 ± 0.11	0.57 ± 0.17	0.53 ± 0.08
1,000 ppm bromelain	0	0.95 ± 0.24	1.05 ± 0.18	1.12 ± 0.22	1.23 ± 0.15
100 ppm papain	0	0.95 ± 0.12	0.95 ± 0.15	1.41 ± 0.15	1.12 ± 0.08
1,000 ppm papain	0	3.31 ± 0.30	3.57 ± 0.38	3.70 ± 0.33	3.64 ± 0.34
SAC					
0 ppm (control)	0	-0.13 ± 0.04	-0.17 ± 0.07	-0.24 ± 0.10	-0.33 ± 0.05
100 ppm bromelain	0	0.48 ± 0.08	0.48 ± 0.03	0.48 ± 0.08	0.40 ± 0.02
1,000 ppm bromelain	0	1.13 ± 0.25	1.22 ± 0.38	1.01 ± 0.08	1.14 ± 0.18
100 ppm papain	0	1.18 ± 0.18	0.18 ± 0.13	1.19 ± 0.25	1.15 ± 0.14
1,000 ppm papain	0	2.94 ± 0.17	3.33 ± 0.30	3.39 ± 0.27	3.25 ± 0.29

Table 5.4. Chemical and physical properties of apple juice treated with papain and bromelain (0, 100, and 1000 ppm).

Treatment (ppm)	pH [ns]	Degree Brix (^{o}Brix)	Total phenolic compounds [ns] (mg gallic acid/ 100 ml juice)	Ascorbic acid (mg/100ml) [ns]	% Titratable acidity (g/100 ml) [ns]	Color		
						L* [ns]	a* [ns]	b* [ns]
0 ppm (control)	3.89 ± 0.008	12.68 ± 0.03^a	67.54 ± 3.56	33.20 ± 0.61	0.3895 ± 0.0089	86.42 ± 0.07	-2.77 ± 0.03	31.23 ± 0.26
Bromelain								
100 ppm	3.89 ± 0.003	12.40 ± 0.17^a	63.39 ± 1.98	33.37 ± 0.92	0.3862 ± 0.0070	86.51 ± 0.03	-2.74 ± 0.01	31.15 ± 0.15
1,000 ppm	3.89 ± 0.005	11.50 ± 0.00^b	60.09 ± 2.67	33.02 ± 0.61	0.3594 ± 0.0051	87.45 ± 0.19	-2.76 ± 0.01	29.42 ± 0.09
Papain								
100 ppm	3.89 ± 0.006	12.47 ± 0.15^a	66.10 ± 3.98	33.20 ± 1.10	0.3907 ± 0.0084	86.29 ± 0.00	-2.64 ± 0.01	31.46 ± 0.06
1,000 ppm	3.90 ± 0.001	11.58 ± 0.08^b	64.63 ± 3.44	32.14 ± 1.10	0.3572 ± 0.0102	86.72 ± 0.03	-2.58 ± 0.03	29.91 ± 0.10

ns: no significant differences of mean values were detected between treatments in same column.

a,b : means with different letters in the same column differ significantly at α = 0.05.

[1] : total phenolic compounds were expressed as a mg gallic acid/100 ml juice sample.

Table 5.5. Chemical and physical properties of orange juice treated with papain and bromelain (0, 100, and 1000 ppm).

Treatment (ppm)	pH [ns]	Degree Brix (°Brix)	Total phenolic compounds [ns] (mg gallic acid/ 100 ml juice)	Ascorbic acid (mg/100ml) [ns]	% Titratable acidity (g/100 ml) [ns]	Color		
						L* [ns]	a* [ns]	b* [ns]
0 ppm (control)	3.74 ± 0.005	12.45 ± 0.05^a	71.18 ± 2.57	40.61 ± 0.81	0.9173 ± 0.0297	43.66 ± 0.16	-1.81 ± 0.04	13.03 ± 0.27
Bromelain								
100 ppm	3.73 ± 0.003	12.32 ± 0.03^a	69.44 ± 3.98	39.20 ± 0.53	0.9163 ± 0.0208	43.06 ± 0.11	-1.78 ± 0.05	12.73 ± 0.02
1,000 ppm	3.73 ± 0.007	11.41 ± 0.03^b	68.35 ± 1.51	40.26 ± 1.06	0.8619 ± 0.0129	41.99 ± 0.08	-1.78 ± 0.04	11.58 ± 0.02
Papain								
100 ppm	3.73 ± 0.003	12.38 ± 0.03^a	73.27 ± 4.94	40.08 ± 0.31	0.9365 ± 0.0133	42.96 ± 0.13	-1.78 ± 0.06	12.63 ± 0.12
1,000 ppm	3.73 ± 0.003	11.53 ± 0.06^b	71.18 ± 4.84	39.73 ± 0.53	0.8629 ± 0.0067	42.48 ± 0.14	-1.81 ± 0.05	11.88 ± 0.05

ns: no significant differences of mean values were detected between treatments in same column.

a,b : means with different letters in the same column differ significantly at $\alpha = 0.05$.

[1] : total phenolic compounds were expressed as a mg gallic acid/100 ml juice sample.

Figure 5.1. Turbidity (NTU) of apple juice treated with 100 ppm bromelain, 1,000 ppm bromelain, 100 ppm papain, and 1,000 ppm papain with control (0 ppm) stored at ambient temperature (20°C) for 72 hours.

Figure 5.2. Turbidity (NTUx100) of orange juice treated with 100 ppm bromelain, 1,000 ppm bromelain, 100 ppm papain, and 1,000

ppm papain with control (0 ppm) stored at ambient temperature (20°C) for 72 hours.

ns: no significant differences of mean values were detected between treatments.

a,b : means with different letters in the same sensory categories differ significantly at $\alpha =$ 0.05.

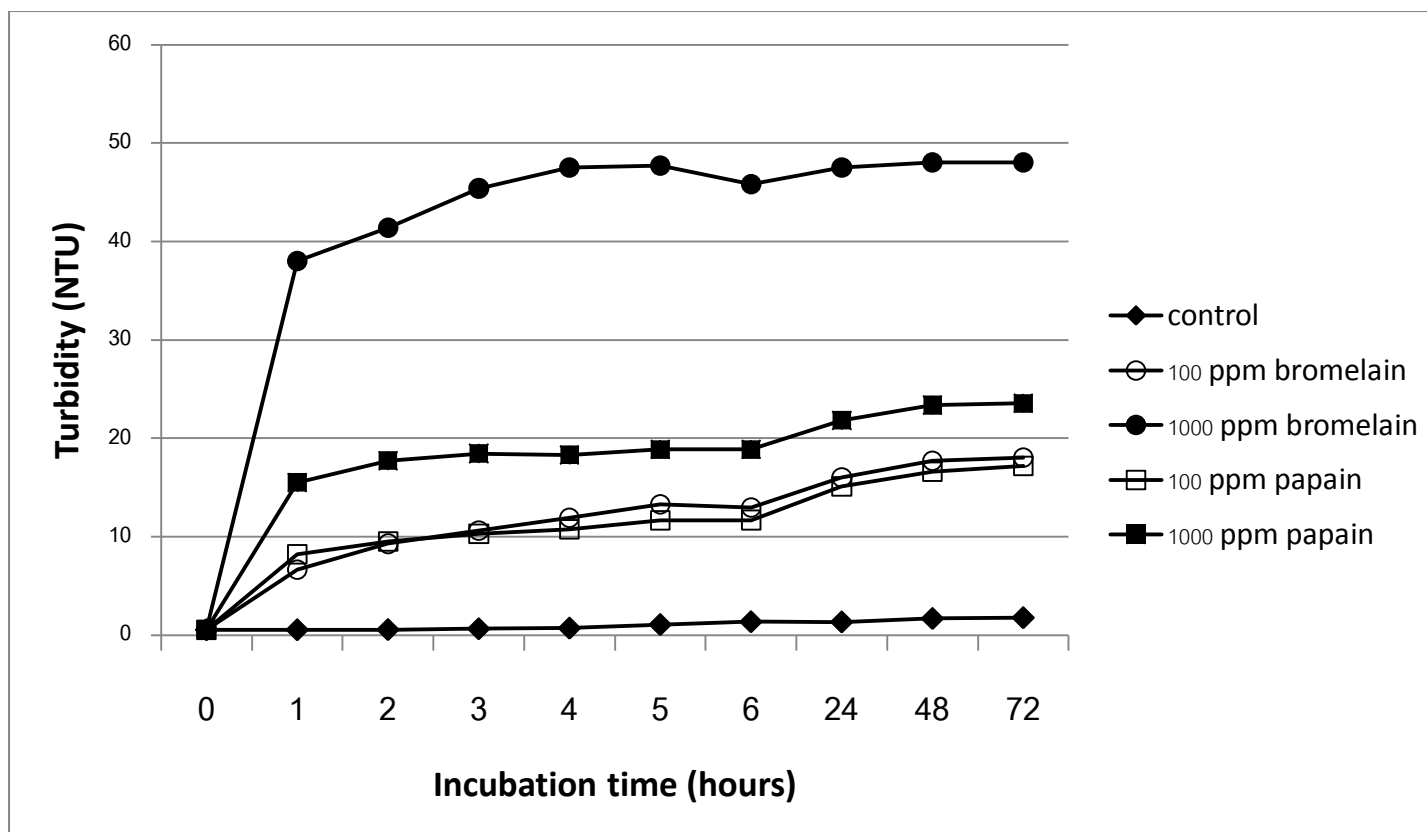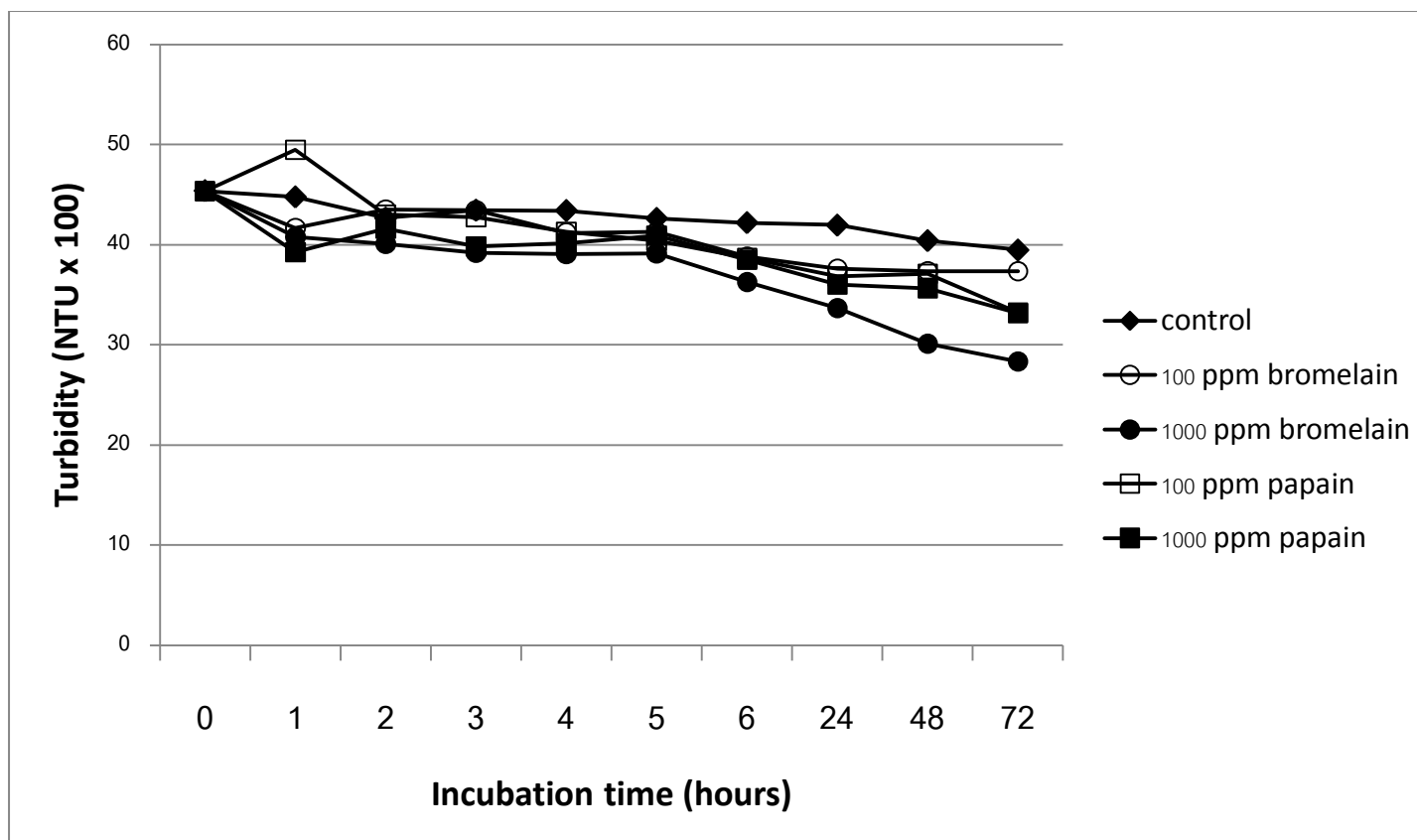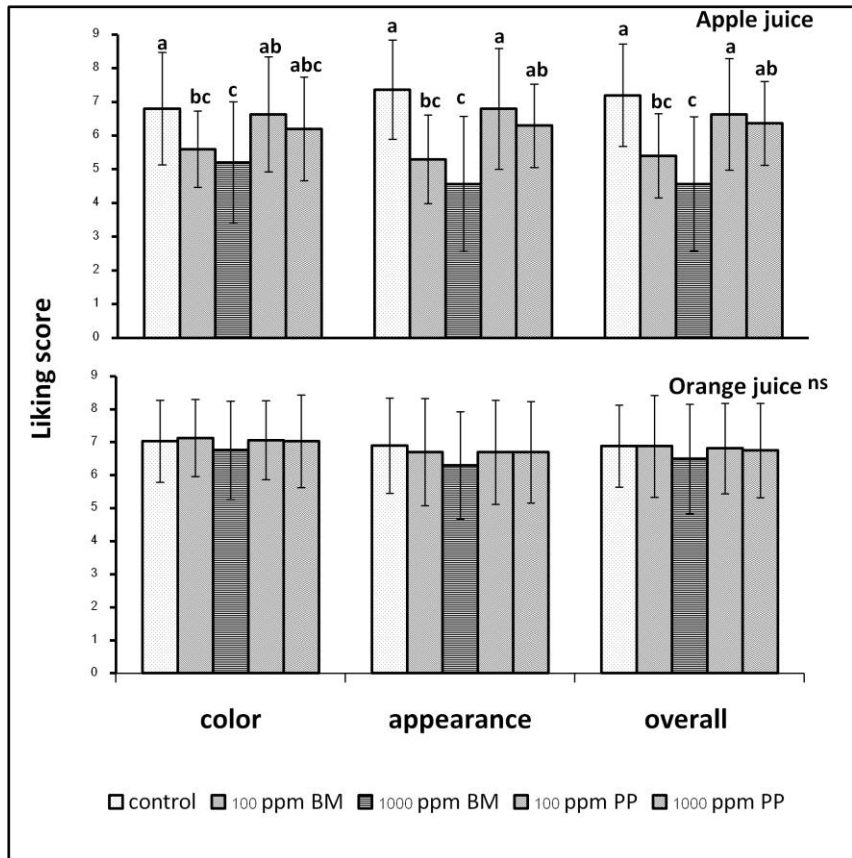Figure 5.3. Sensory scores of apple juice and orange juice treated with 100 ppm bromelain, 1,000 ppm bromelain, 100 ppm papain, and 1,000 ppm papain with control (0 ppm).

DISCUSSION

Due to the adverse effects of chemical preservatives, their use is decreasing in the juice industries. New approaches such as "natural" antimicrobial substances and non-thermal processing have been studied and proposed as promising methods to control the spoilage of juices and beverages caused by *A. acidoterrestris* (22,27). In recent years, several studies have been conducted to investigate methods to control or inactivate the growth of *A, acidoterrestris* and these studies were focused on either apple juice or orange juice because they are the prone to spoilage and are the two largest consumed juices, compared to other juices (3, 22, 27). Shelf-stable apple juice was selected for use in this experiment due to periodic spoilage due to *A. acidoterrestris*, whereas refrigerated orange juice was also as a model juice since *A. acidoterrestris* spores are still able to germinate under low temperatures with slower growth rates compared to ambient temperature or higher (7).

From the results, it is apparent that papain and bromelain had an inhibitory effect on vegetative cells of *A. acidoterrestris* while no inhibitory effect wasobserved with their spores. Samples were kept for the incubation period of 42 days at ambient temperature to determine whether papain and bromelain were capable of preventing the growth of vegetative cells and spores of *A. acidoterrestris* during typical shelf life storage conditions. Results showed papain and bromelain had bacteriocidal effect on *A. acidoterrestris* vegetative cells according to cell numbers of vegetative cells of all strains in apple and orange juice treated with papain and bromelain decreased until undetectable level within 1 day of incubation whereas no bacteriocidal or bacteriostatic effect was found with spores. The inhibitory effect by papain and bromelain against *A. acidoterrestris*

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vegetative cells in apple juice was slightly higher than in orange juice. These might due to the different natural components in juices. Conversely, some studies have shown that ascorbic acid which is normally found higher amount in orange juice than apple juice may have inhibitory effect against *Alicyclobacillus* because ascorbic acid is able to reduce oxygen availability which is a critical factor on *Alicyclobacillus* growth (37). Moreover, an addition of 150 ppm ascorbic acid in apple juice could prevent the growth of *A. acidoterrestris* vegetative cells (5). Therefore, other factors or combinations might be responsible for the differences in inhibiting *A. acidoterrestris* vegetative cells.

The mechanism of microbial inactivation by papain and bromelain has not been clearly established; however, bromelain has been showen to be an inhibition agent of proliferation of tumor cells *in vitro* and later found that bromelain might induce differentiation of leukemic cells while papain has been reported to be a treatment of pinworm (*Enterobiasis vermicularis*) infection (4). Moreover, Weise (45) revealed the digestion action of papain for *in vitro* trials and found that papain reduced pinworm infections primarily by cuticle digestion and cell wall erosion of *E. vermicularis* and secondarily by slow maceration of the viscera of the worm. This evidence may be a possible mechanism of action for papain and bromelain action on bacterial cell wall interruption and subsequent cell disruption (25).

Our results showed that papain and bromelain had no effect on chemical and physical properties of apple juice and orange juice; however, there were significant differences of °Brix and turbidity between control and papain or bromelain treated samples. °Brix was shown to be significantly different between control, 1,000 ppm of papain, or 1,000 ppm bromelain treatments likely because

the enzyme solution addition to the samples diluted the juice concentration causing a resulting decrease in °Brix.

Papain was selected over bromelain for the stability tests with *A. acidoterrestris*. For the storage experiment and sensory evaluation tests, 100 ppm papain was chosen for apple juice, whereas 1,000 ppm papain was chosen for orange juice, as a result of the higher antibacterial activity against *A. acidoterrestris*. Sodium benzoate was used in the stability tests with *A. acidoterrestris* as a preservative that is currently used by the juice and beverages with 0.05% wt/vol for fruit juices and concentrates (8), to allow for comparison with the papain treatments. Our results showed that 200 ppm sodium benzoate had inhibitory effect against *A. acidoterrestris* vegetative cells by approximately 2 log reduction within a day of storage in apple and orange juice, These results were supported by the report by Pettipher et al. (28). They showed that benzoic acid with 150 ppm at ambient temperature in apple juice was shown to decrease *A. acidoterrestris* vegetative cells about 1 log reduction in ten days while about 0.70 log reduction in three months was achieved with spores (28). Moreover, minimum inhibitory concentration (MIC) of benzoic acid was found to be 61 ppm for *A. acidoterrestris* vegetative cells in potato dextrose broth (17). Physical and chemical properties of apple juice and orange juice were investigated after 6 hours of treatment time, due to the inhibitory effect of treatments had reached the maximum inhibitory effect.

Haze formation was observed with clear apple juice treated with enzymes, even the lowest concentration of 100 ppm for both enzymes evaluated. Haze is a cloudiness that results from light scattering of colloidal or large particles such as pigment particles or contaminants suspended in products. Several different causes

of haze formation in juices and beverages have been discussed, including inorganic and organic substances (12, 13, 31, 40). Haze formation after bottling has long been a problem in the beverage industry and is associated with the product perception and quality, particularly in clear juices and wines. Protein-polyphenol interaction is most frequently responsible for the haze formation in beverages, especially clear juices such as apple juice and grape juice (40). Apple juice contains high polyphenol content, and is subject to haze formation causing an increase in turbidity. Protease or proteolytic enzymes commonly cause clarification in juices by proteolysis of haze-active proteins, consequently, preventing the binding with haze-active polyphenols and leading to a decrease in turbidity (20, 30).

The antibacterial activity of papain and bromelain in juices has not been studied; however, some studies showed the antibrowning properties of these enzymes in juices. Tochi et al. (41) reported the effect of bromelain on the browning of apple juice using bromelain concentration ranging from 0.175-0.700 g/l compared with ascorbic acid treatments of 0.3-1.0 mM at 25°C and showed that ascorbic acid at 1.0 mM inhibited browning up to 80%, while up to 60% was achieved in 0.700 g/l bromelain treatement during the 10-hour study period. They concluded that bromelain which has an inhibitory effect on polyphenoloxidase activity, was less effective than ascorbic acid in the inhibition of browning in apple juice.

Appearance of cloudiness or haze in apple juice is perceived as deterioration of quality whereas cloudiness in fresh orange juice is a decisive factor for consumer acceptance which is affected by pectin substances (35). Orange juice displays a "cloudy" appearance due to finely divided particulates that consist of

pectin, protein, and lipid, with the most responsible particle being pectin substances (2, 34). Heat is commercially used to prevent cloud loss in apple juice by inactivation of pectinesterase, a critical enzyme responsible for clarification, in orange juice. Therefore, treatments being investigated for the inhibition of *Alicyclobacillus* need to maintain cloudiness in orange juice and not being a cause of haze formation in apple juice. Papain and bromealain resulted in an increase in turbidity (haze formation) in apple juice, whereas turbidity decreased in orange juice. Results from acceptance test by experienced panelists were in agreement with the turbidity results. Experienced panelists were satisfied of the clear characteristic of apple juice, therefore lower scores of color, appearance, overall of papain and bromelain treatments was obtained when compared to control. Turbidity was shown to be significantly decreased ($\alpha = 0.05$) in enzyme-treated orange juice however, experienced panelists still satisfied the treated samples at the same score as control (no enzyme added) probably due to decrease in cloudiness of treated orange juices (papain and bromelain treatments) cannot be visibly differentiated by experienced panelists. These results were in agreement with the results by Gomez-Lopez et al. (15) who studied the effect of sonication on sensory quality of calcium-added orange juice. Results showed that ultrasonic treatment with an amplitude of 89.25 µm for 8 min affected the sensory qualities of treated orange juice which include changes in color and cloudiness; however, there were no significant differences ($\alpha = 0.05$) on sensory scores, including color, flavor, aroma, and overall quality scores, between control and sonicated orange juice sample. They also suggested that changes in sensory qualities of sonicated orange juice sample could not be visually detected due to the cloudy appearance of orange juice.

Due to the greater log reduction of *A. acidoterrestris* vegetative cells, papain was selected over bromelain for conducting the sensory evaluation, along with the lowest concentration of 100 ppm papain, to minimize the haze formation, which showed almost the same log reduction on vegetative cells compared to 1,000 ppm papain in apple juice, whereas 1,000 ppm papain was used in orange juice to obtain the maximum log reduction on vegetative cells. Higher papain concentrations were used in orange juice compared to apple juice because changes in appearance caused by enzyme treatments would be noticed easier in clear juices such as apple juice compared to cloudy juices such as orange juice.

A blind triangle test was conducted to avoid the visual effect of clearness and cloudiness between the control (0 ppm papain) and 100 ppm papain treated apple juice or 1,000 ppm papain treated orange juice, whereas acceptance sensory tests were carried out to determine whether treated juices were still acceptable by panelists even if light haze formation occurred in apple juice samples or a decrease in cloudiness with orange juice.

Ascorbic acid or vitamin C is an essential vitamin required for human body to maintain normal metabolic functions and plays an important role as a cofactor in several essential enzymes associated with the biosynthesis of collagen, carnitine, and neurotransmitters (26). Fruit variety and thermal processing used for juice production have a significant effect on vitamin C content in juices, therefore, vitamin C is usually added to final juice products to compensate for the loss during processing and storage, and to prevent browning in juices (33). In respect to an antibrowning property, bromelain was reported to be an effective treatment for browning inhibition in refrigerated apple slices (11) while Lozano-de-Gonzalez et al. (23) reported that dipping fresh and dried apple rings in pineapple juice which

contains high amount of bromelain for 2 min was an effective enzymatic browning inhibitor in both samples.

Apple and orange juices are highly sensitive juices. Their physical, chemical, and sensory properties can be affected by various processing treatments. The most significant quality characteristics of juices products are clear or cloudiness appearance, color, and nutritional content, which include antioxidants. Papain and bromelain were shown to possess antibacterial activity against *A. acidoterrestris* vegetative cells; however, no sporicidal effect was observed for any of the strains. Papain and bromelain were shown to have a significant effect on the turbidity of apple and orange juice, which are directly related to consumer acceptance. Therefore, papain and bromelain may be used in juice production as an additional treatment to control spoilage bacteria of juices with minimal changes in the finished product quality. Further research is needed to better understand the mode of action of papain and bromelain on vegetative cells of *A. acidoterrestris* and it is important to ensure the quality of acidic juice products.

ACKNOWLEDGEMENTS

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CHAPTER 6

SCREENING FOR POTENTIAL BACTERIOCINOGENIC STRAINS WITH ANTI-*Alicyclobacillus* ACTIVITY AND THE INFLUENCE OF INHIBITORY MEDIA COMPONENTS

ABSTRACT

Alicyclobacillus, a Gram-positive, spore forming, themoacidophilic, non-pathogenic bacterium, is a spoilage microorganism for the juice and beverage industries. Spoilage due to *Alicyclobacillus* spp. is generally associated with the production of off odors and flavor in juices and beverages. Kimchi and fermented apples were selected as potential sources of bacteriocin-producing lactic acid bacteria (LAB) species against *Alicyclobacillus* growth. Bacteriocin-producing strains isolated from fermented fruit and vegetable products were tested for their inhibitory activity against several spoilage associated *Alicyclobacillus* species. Deferred inhibition assays using various *Alicyclobacillus* species showed media-specific inhibition against the indicator strains. The effect of various media components from potato dextrose agar pH 3.5 (PDA), de Man, Rogosa and Sharpe broth (MRS), all purpose tween (APT), tryptic soy agar (TSA), M9, M17 and APT ingredients were evaluated for their inhibition of *Alicyclobacillus* growth. Results showed that PDA was the only medium that allowed the growth of *Alicyclobacillus* species and sodium citrate and dipotassium phosphate were found to be antimicrobial compounds inhibiting *Alicyclobacillus* in APT. Among 300 isolates screened from kimchi and fermented apples, no potential bacteriocin-producing LAB strains showed activity against *Alicyclobacillus* species. The uniqueness of *Alicyclobacillus*

insensitivity to Gram-positive bacteriocins and the sensitivity to citrate and phosphate may provide insight to the recalcitrant nature of this unusual spoilage bacterial species.

INTRODUCTION

Lactic acid bacteria (LAB) are naturally found in fresh produce and are used as starter cultures in fermentation processes to prevent the growth of undesirable microorganisms (11). LAB produce various types of antimicrobial substances including organic acids, hydrogen peroxide, diacetyl, and bacteriocins (6). These antimicrobial substances are advantageous in term of product shelf life extension, product quality improvement, and human health benefits (4).

Kimchi, an example of a LAB fermented food, is a group of Korean traditional fermented vegetables, primarily cabbage and radish, is gaining popularity in terms of health benefits as a functional food (9). The health benefits of kimchi have been reported to help enhance appetite, relieve constipation, and maintain a healthy intestinal microbiota (8). LAB play an important role in kimchi fermentation and contains high levels of LAB approximately 10^7 - 10^9 CFU/ml (2). Organic acids and vitamins are formed during the LAB fermentation and these organic acids in combination with additional LAB antimicrobial compounds help to inhibit the growth of spoilage microorganisms or pathogens during the fermentation process (12).

Alicyclobacillus is considered as important causative spoilage microorganism in acidic juices and beverages due to the ability of the spores of this genus to survive commercial pasteurization and hot fill conditions for juices and beverages (1). *Alicyclobaacillus* has been identified from a wide range of fruit and vegetable juices with the contamination originating from soil during postharvest

procedures and production (10, 17). Although, *A. acidoterrestris* is non-pathogenic, it causes spoilage in fruit products, especially acidic fruit juices such as apple juice and orange juice. It is also a significant cause of spoilage in acidic beverages, and spoilage is associated with the off odor due to guaiacol production (16).

The objectives of this study were to isolate bacteriocin-producing strains of LAB from fermented foods and determine their antibacterial activity against *Alicyclobacillus* spp., as a possible "natural" biopreservative to prevent the spoilage of juices and beverages due to *Alicyclobacillus* spp.

MATERIALS AND METHODS

Materials, chemicals, and media. Tryptic soy broth (TSB), potato dextrose broth (PDB), and Bacto™ peptone were obtained from Becton, Dickinson and Company (Spark, MD). All purpose tween (APT), M17, and M19 were obtained from Difco laboratories (Detroit, MI). Potato dextrose agar (PDA) and de Man, Rogosa and Sharpe broth (MRS) were obtained from Hardy Diagnostics (Santa Maria, CA). Tartaric acid was obtained from J.T. Baker Chemical Co. (Phillipsburg, NJ). Kimchi was purchased locally from a grocery store (Wegmans, Rochester, NY) while fermented apple were prepared by adding sodium chloride into finely ground apples to achieve final concentration of sodium chloride in the sample of 2.25% (wt/wt).

Bacterial strains. *A. acidoterrestris*, *A. acidocaldarius*, *A. acidophilus*, *A. hesperidium*, *A. fastidious*, *Paenibacillus polyxyma*, *Paenibacillus FI-1*, *Paenibacillus FI-B*, *Enterococcus mundtii* CUGF08, *Lactococcus lastis* 11454, and *Lactococcus lastis* AA4 from culture collections in our laboratory were used in this study.

Cultures preparation. *Alicyclobacillus* species were grown in PDB pH 3.5 acidified by sterile 10% tartaric acid (wt/vol) at 50°C for 24 hours. *Enterococcus mundtii* CUGF08, *Lactococcus lactis* 11454, *Lactococcus lactis* AA4, and *Paenibacillus* species were inoculated in TSB at 30°C for 18, 18, 18, and 24 hours, respectively. *Alicyclobacillus* cultures in PDA pH 3.5, soft agar (0.75% wt/vol) used in the overlay method was prepared by adding 50 µl of 24-hour-old *Alicyclobacillus* species culture to 50°C PDA pH 3.5, soft agar.

Effect of media on the growth of *Alicyclobacillus* species. The pour plate method was used to investigate the effect of media on the growth of *Alicyclobacillus* species. One milliliter of *Alicyclobacillus* spp. cultures were transferred to empty plates and then molten agar of TSA, APT agar, MRS agar, M9 agar, and M17 agar were poured in the Petri plates containing the various *Alicyclobacillus* strains. Following the incubation at 50°C for 72 hours, colonies were enumerated.

Effect of APT ingredients on *Alicyclobacillus* species. Each ingredient of APT broth including yeast extract (0.75% wt/vol), tryptone (1.25% wt/vol), dextrose (1% wt/vol), sodium citrate (0.5% wt/vol), thiamine hydrochloride (0.0001% wt/vol), sodium chloride (0.5% wt/vol), dipotassium phosphate (0.5% wt/vol), manganese chloride (0.014% wt/vol), magnesium sulfate (0.08% wt/vol), and ferrous sulfate (0.004% w/v) were tested for antibacterial activity against *Alicyclobacillus* species. Solutions of APT ingredients were filtered sterilized through 0.22 micron low protein binding membrane (polyethersulfone, PES membrane). Two hundred microliters of these solutions were spotted in well-prepared PDA pH 3.5 agar plates and overlaid with *Alicyclobacillus* species cultures in PDA pH 3.5, soft agar described in culture preparation.

Effect of bacteriocin-producing strains on antimicrobial activity

against *Alicyclobacillus* species. *Enterococcus mundtii* CUGF08 and

Paenibacillus species were inoculated in TSB at 30°C for 18 and 24 hours,

respectively. Supernatants were obtained by centrifuging bacterial cultures at 5,000

rpm for 5 min at 4°C and then filtered through 0.22 micron low protein binding

membrane (polyethersulfone, PES membrane). Two hundred microliters of these

supernatants were spotted in well-prepared PDA agar plates and overlaid with *A.

acidoterrestris* inoculated PDA pH 3.5 soft agar (0.75 % wt/vol agar). Incubation

at 50°C for 72 hours was performed prior to examining for the presence of

inhibition zones due to bacteriocin activity.

Bacteriocin screening. LAB were isolated from kimchi (Korean fermented

cabbage) and fermented apple by plating serial dilutions onto APT agar plates and

incubation at 30°C for 48 hours. All the isolates from the APT agar plates were

screened for their antimicrobial activity against *Alicyclobacillus* species by using

well diffusion and spot method described by Schillinger and Lucke (18). Briefly,

each colony from APT agar plates was inoculated in modified formula APT broth

(without sodium citrate and dipotassium phosphate) and incubated at 30°C for 24

hours. The overnight culture was then streaked onto APT agar plates and each

colony on APT agar plates and then re-streaked for colony purification. Re-

inoculation of each colony into modified formula APT broth at 30°C for 24 hours.

Supernatants were obtained by centrifugation at 5,000 rpm for 5 min at 4°C and

then filtered through 0.22 micron low protein binding membrane

(polyethersulfone, PES membrane). Two hundred microliters of the supernatants

were spotted in well-prepared PDA pH 3.5 agar plates and overlaid with *A.

acidoterrestris* inoculated PDA soft agar (0.75 % wt/vol agar) with a positive

control of *Lactococcus lactis* 11454 and *Lactococcus lactis* AA4. Incubation at 50°C for 72 hours was performed to identify cleared zones caused by bacteriocin activity. The isolates producing the highest level of antimicrobial activity were selected for further characterization.

RESULTS

Table 6.1. shows the effect of various media on the growth of *Alicyclobacillus* species. Of all the media tested, PDA is the only medium that permitted the growth of *Alicyclobacillus* spp. Sodium citrate and dipotassium phosphate were found to be the inhibitory ingredients in APT agar (Table 6.2). *Lactococcus lactis* 11454 and *Lactococcus lactis* AA4 showed antimicrobial activity against all *Alicyclobacillus* species tested when compared to other bacteriocin-producing strains tested (Table 6.3). From the screening experiments, 150 isolates from each kimchi and fermented apple samples were tested and none of the isolates from either fermented foods showed any antimicrobial activity against *Alicyclobacillus* species (data not shown).

Table 6.1. Growth of *Alicyclobacillus* species on various media.

Alicyclobacillus species	PDA (pH 3.5)	MRS	APT	TSA	M9	M17
A. acidoterrestris VF	+	-	-	-	-	-
A. acidoterrestris WAC	+	-	-	-	-	-
A. acidoterrestris SAC	+	-	-	-	-	-
A. acidophilus	+	-	-	-	-	-
A. acidocaldarius	+	-	-	-	-	-
A. fastidious	+	-	-	-	-	-
A. hesperidium	+	-	-	-	-	-

+ presence of *Alicyclobacillus* growth

- absence of *Alicyclobacillus* growth

Table 6.2. Antimicrobial activity of All Purpose Tween (APT) ingredients against *Alicyclobacillus* species.

APT ingredients	*A. acidoterrestris* VF	*A. acidoterrestris* WAC	*A. acidoterrestris* SAC	*A. acidophilus*	*A. acidocaldarius*	*A. fastidious*	*A. hesperidium*
bacto yeast extract	+	+	+	+	+	+	+
bacto tryptone	+	+	+	+	+	+	+
bacto dextrose	+	+	+	+	+	+	+
sodium citrate	-	-	-	-	-	-	-
thiamine hydrochloride	+	+	+	+	+	+	+
sodium chloride	+	+	+	+	+	+	+
dipotassium phosphate	-	-	-	-	-	-	-
maganess chloride	+	+	+	+	+	+	+
magnesium sulfate	+	+	+	+	+	+	+
ferrous sulfate	+	+	+	+	+	+	+

+ presence of *Alicyclobacillus* growth

- absence of *Alicyclobacillus* growth

Table 6.3. Antimicrobial activity of bacteriocin-producing species against *Alicyclobacillus* species.

Bacterial strains	*Paenibacillus polymyma*	*Paenibacillus FI-B*	*Paenibacillus FI-1*	*Lactococcus lactis 11454*	*Lactococcus lactis AA4*	*Enterococcus mundtii* CUGF08
A. acidoterrestris VF	-	-	-	+	+	-
A. acidoterrestris WAC	-	-	-	+	+	-
A. acidoterrestris SAC	-	-	-	+	+	-
A. acidophilus	-	-	-	+	+	-
A. acidocaldarius	-	-	-	+	+	-
A. fastidious	-	-	-	+	+	-
A. hesperidium	-	-	-	+	+	-

+ presence of clear zone on *Alicyclobacillus* growth

- absence of clear zone on *Alicyclobacillus* growth

DISCUSSION

Thermal treatments used in juice production (86-96°C for approximately 2 minutes), along with physicochemical characteristics of juice, such as low pH and presence of organic acids, effectively inhibit most of the growth of undesirable and pathogenic microorganisms except *Alicyclobaillus* spp. (7). Therefore, the juice industry has identified *Alicyclobacillus* as an important spoilage microorganism for acidic juices and beverages. Although *Alicyclobacillus* is non-pathogenic, it causes spoilage in acidic fruit juices and beverages by producing off-flavors and off odors such as guaiacol, which is typically described as having medicinal or antiseptical odors (5).

The use of chemical preservatives has decreased due to consumer health concerns and negative consumer perceptions. Since some unprocessed foods can pose a health risk and food spoilage problems, an alternative solution for this dilemma is the use of antimicrobial substances produced by fermentative microorganism (13). LAB were selected in this experiment as bacteriocin-producing species because they naturally produce a wide range of antimicrobial metabolites such as organic acids, hydrogen peroxide, diacetyl, and bacteriocins which are ribosomally synthesized antimicrobial substances against the growth of other bacteria including pathogenic bacteria (6). Bacteriocins have been increasingly studied and used to improve the safety of food products. Nisin, an example of a bacteriocin, is an antimicrobial peptide produced by certain bacteria *Lactococcus lactis* subsp. *lactis*. It has been used as an antimicrobial substance against a wide range of pathogenic Gram-positive bacteria including *Clostridium*, *Bacillus*, *Listeria monocytogenes* and *Alicyclobacillus* spp. but has limited antimicrobial activity against Gram-negative bacteria and fungi (3).

Alicyclobacillus acidoterrestris are significant *Alicyclobacillus* species associated to the spoilage of acidic fruit juices and beverages while strains VF, WAC, and SAC have been recognized as the most heat resistant strains in the *A. acidoterrestris* genus (14).

.   Various media were tested against *Alicyclobacillus* species to identify the appropriate media used to identify bacteriocin-producing LAB strains from kimchi and fermented apple. Kimchi was selected as a source of LAB which produce bacteriocin due to the high level of LAB in finished products (2) whereas fermented apple was selected as a potential source of bacteriocin-producing LAB strains since *Alicyclobacillus* are natural contaminants on apples (15). Sodium chloride was added to ground apples during the fermentation to select for the growth of LAB and inhibit the growth of undesirable microorganisms (12).

APT medium was selected to grow bacteriocin-producing LAB strains since LAB produce less acid in APT than MRS, therefore, inhibition effect from acid could be avoided. APT broth formula was slightly modified from original formula by excluding sodium citrate and dipotassium phosphate which are the inhibitory ingredients in APT media.

Bacteriocin-producing species were tested against *Alicyclobacillus* species to identify potential antagonistic cultures that exhibit activity against *Alicyclobacillus*. *Lactococcus lactis* 11454 (nisin A producer) and *Lactococcus lactis* AA4 (nisin Z producer) were used as positive controls and confirm the method being used was appropriate to demonstrate antimicrobial effect against *Alicyclobacillus* spp.. Large volumes of supernatant (200 µl) used in the well-diffusion and spot method to test for inhibitory activity against *Alicyclobacillus* species produced visually detectable clear zones on testing plates.

Methods used in this bacteriocin screening experiment identified potential bacteriocin-producing LAB strains from kimchi and fermented apple, but none exhibited activity against *Alicyclobacillus* spp. strains that were tested. Additional research involving a more diverse collection of bacteriocin-producing strains to identify potential bacteriocin-producing LAB strains against *Alicyclobacillus* species is needed.

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CHAPTER 7
DISCUSSION, CONCLUSIONS, AND PROSPECTUS

DISCUSSION AND CONCLUSIONS

The consumption of fresh, pasteurized, or shelf-stable juices has been continuously increasing in the last 20 years. Additionally, there has been an increase in the use of fruit juice as an ingredient in other foods or beverages. The main reasons for the dramatic increase in juice consumption and uses in foods and beverages are largely due to the reported human benefits associated with consumer health concerns (9).

Changes in dietary habits, processing methods, and an increase in fresh fruits being processed, along with mutations of foodborne pathogenic microorganisms with enhanced acid resistance, have lead to an increase in foodborne outbreaks associated with the consumption of fresh fruit juices. On the contrary, shelf-stable fruit juices that have undergone rigorous thermal processing regimes have been confronted with an extreme spoilage issue due to the contamination of *Alicyclobacillus* (1). Although several processing methods have been established to control foodborne illness outbreaks, they are insufficient to control fruit juice spoilage due to *Alicyclobacillus* spp.. Additional research is needed to fully understand the basic physiological characteristics of this unusual bacterium in an attempt to provide better control methods to reduce the incidence of *Alicyclobacillus* spoilage. The understanding of this microorganism's behavior will allow the development of effective strategies to improve the quality and stability of fruit juices. In addition to regulations and preventive programs such as HACCP and GMPs specific to enhance the safety of juices, new preservation

methods aimed at controling microorganisms while retaining the fresh

characteristics of fruit juices, including nutritional and sensory aspects, will be a

challenge for the juice industry (2).

Therefore, microbiological studies of fruit juices should be closely focused

on public health concerns and economic loss reduction of spoilage for juice

products through the implementation of GAPs, HACCP, predictive microbiology,

risk assessment, and new technological processing methods.

UV irradiation has been shown to be an effective method to reduce patulin

in apple juice from concentrate, following first-order kinetics for patulin reduction,

while zero-order kinetics were observed with apple cider (3). However, increasing

UV exposure influenced the sensory characteristics but did not result in an

alteration of the physicochemical properties for apple juice from concentrate.

Optimization of UV exposure and patulin reduction needs to be taken into

consideration to decontaminate patulin below the regulatory action level of 50 ppb

patulin (10), while sensory characteristics and juice quality still need to be

preserved.

DMDC or Velcorin® with the maximum concentration of 250 ppm could be

used as an intervention method in acidic juices and beverages to reduce spoilage

since it was shown to be effective for inhibiting the growth of *A. acidoterrestris*

strains VF, WAC, and SAC in both vegetative cells and spores in apple juice and

orange juice, without affecting the physicochemical and sensory properties.

Papain and bromelain were shown to possess antimicrobial activity against

vegetative cells of *A. acidoterrestris* strains VF, WAC, and SAC but not against

their spores. Minor changes in physicochemical properties and sensory

characteristics in apple juice and orange juice were observed with these treatments.

Turbidity was shown to be the only critical quality property affected when papain and bromelain were applied in juices. This was due to the protein-polyphenol interaction and clarification that resulted and caused changes in the turbidity in apple juice and orange juice (6). The concentration of papain or bromelain was a significant factor affecting the inhibitory activity against *A. acidoterrestris* and changes in turbidity of the juice. Therefore, optimized papain or bromelain concentration is needed for juice application to achieve the highest level of antimicrobial activity, while minimizing changes in turbidity.

Alicyclobacillus were found to be sensitive to common media components except for PDA which was the only medium found to be capable of supporting their growth. Moreover, sodium citrate and dipostassium phosphate, ingredients in APT used for screening bacteriocin-producing strains, exhibited antimicrobial activity against *Alicyclobacillus* species. Kimchi and fermented apple were used to screen for LAB with potential bacteriocin activity against *Alicyclobacillus* species. However, no bacteriocins were found to be active against the *Alicyclobacillus* species tested. The possible explanations for the lack of anti-*Alicyclobacillus* bacteriocins might be i) insufficient isolates were screened ii) potential sources of LAB were not effective sources and iii) methods of bacteriocin screening used were not appropriate.

PROSPECTUS

UV irradiation evidently reduced patulin in apple juice from concentrate and apple cider (3) but negatively affected the sensory characteristics of apple juice from concentrate. The mechanism of patulin reduction by UV irradiation is not clearly understood, but it is proposed to be due to the alteration of patulin structure to an alternative structure which cannot be detected by HPLC and/or patulin

structure has been destroyed resulting in loss of toxicology leading to further investigation of patulin reduction mechanism by UV irradiation. Moreover, further study of sensory attributes of treated apple juice from concentrate by UV irradiation is still important to better understanding of effect of UV on sensory characteristics of apple juice from concentrate.

DMDC is effective intervention treatment to inhibit the growth of *A. acidoterrestris* in apple juice and orange juice but no others juices were tested. Therefore, additional acidic juices and beverages need to be studied for its ability to control *Alicyclobacillus* in juices.

The study of the mechanism of action for papain and bromelain inhibition of *Alicyclobacillus* spp. is an essential key to enhance the effectiveness of these proteolytic enzymes by studying of their effectiveness combined with other factors including pH, total soluble solid, and vitamin C, on the inhibition effect and for other fruit juices are possible future directions.

It is obvious that the use of multiple preservation treatments can successfully control microbial spoilage (hurdle technology) (4). Different hurdles, indeed, could result in different preservation methods due to the effectiveness on different targets such as cell membrane, enzyme, and molecular level target (DNA) leading to microbial homeostasis interruption (4). The use of various hurdles to prevent or control *Alicyclobacillus* contamination has been reported by different authors (5, 7, 8). For example, many studies have shown that pH, water activity (A_w), and environmental temperature could synergistically work to inhibit the germination of *Alicyclobacillus* spores in laboratory media and juices (8). Additionally, pH and total soluble solid content could facilitate to strengthen the inhibitory effect of some antimicrobial compounds such as nisin as an additive

factor (5). Unfortunately, few data exist on the combination approach using different non-thermal methods and/or natural antimicrobial substances. The use of non-thermal approaches to control *Alicyclobacillus* could be employed as a possible way to improve the quality of juices; however, existing data were collected using various media at the laboratory scale. A potential trend could be a scale up to industry scale; in order to verify and validate the proposed non-thermal methods and/or natural antimicrobial compounds to determine whether they could be successfully used at the industrial scale. Interaction between different treatments and food components is another field of great concern since there is little known; therefore, a possible topic of interest for future trend could be the study of combination of non-thermal treatments and natural antimicrobial compounds to improve microbiological safety and quality and their effect on juice quality with the shelf life study.

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