SCREENING OF BIOCONTROL ORGANISMS FOR THE MANAGEMENT

OF PHYTOPATHOGENIC FUNGI AND FOODBORNE PATHOGENS

ON PRODUCE

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ABSTRACT

Screening Of Biocontrol Organisms For The Management Of Phytopathogenic Fungi

And Foodborne Pathogens On Produce

Antoinette de Senna

The multibillion dollar agricultural industry is an important part of the United

States economy, and the management of factors that affect crop and human health is

imperative to maintaining this economic sector. The fungi *Botrytis cinerea*, *Fusarium

pallidoroseum*, and *Fusarium moniliforme* are the causative agents of several plant

diseases and can cause significant crop loss both before and after harvest in commodities

such as strawberries, lettuce, citrus, and grains. Fungicides are employed to control these

phytopathogens, but the use of these chemicals has led to an increase in fungicide

resistance and may negatively affect the environment and human health. In addition to

plant pathogens, foodborne pathogens also have a substantial impact on the agricultural

industry. Foodborne disease outbreaks involving *Listeria monocytogenes*, *Salmonella*,

and *Escherichia coli* O157:H7 not only cause considerable economic losses, but can also

result in devastating health problems for consumers. The increase in fungicide resistance

and number of produce-related foodborne disease outbreaks warrants investigation into

additional methods of microbial control for use in the agricultural industry. Many

bacterial species, including Lactic Acid Bacteria (LAB) and *Bacillus* species, produce

antifungal and antimicrobial compounds, thus the use of biological control agents pre-

and postharvest could augment current methods of pathogen management. The purpose

of this study was to screen 22 bacterial isolates for inhibitory activity against the fungal

phytopathogens *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme* and the foodborne pathogens *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 *in vitro*, then evaluate antimicrobial efficacy of select isolates against the foodborne pathogens on fresh produce.

To evaluate antifungal activity, the bacterial isolates were individually spot-inoculated onto Tryptic Soy Agar, Potato Dextrose Agar, or MRS agar, depending on isolate growth requirements and then a plug of fungal-colonized agar was placed onto the center of the isolate-inoculated plate. Plates were incubated at 24°C for 10 days; fungal growth was evaluated daily, beginning on Day 3. Nine of the 22 isolates screened inhibited all three fungi; inhibition by these isolates ranged from 51-62% for *B. cinerea*, 60-68% for *F. pallidoroseum*, and 40-61% for *F. moniliforme*. Isolates were also screened for biosurfactant activity using the drop-collapse test. Biosurfactant production was detected in seven of the nine isolates. *Bacillus megaterium*, *Bacillus coagulans*, *Bacillus thuringiensis* BT2 and three *Bacillus amyloliquefaciens* isolates demonstrated strong biosurfactant activity and suppression of all three fungi, and therefore are recommended for further study.

Antimicrobial activity of the isolates was assessed using two methods: LAB isolates were screened using a seeded-overlay method and all other isolates were evaluated by spot inoculating the isolate on pathogen-seeded TSA. Three LAB isolates and six *Bacillus* isolates suppressed *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 *in vitro*. Based on the results of the screening, three LAB isolates—*Lactobacillus plantarum*, *Pediococcus acidilactici*, and *Pediococcus pentosaceus*—were selected for further evaluation and use in challenge studies on fresh produce.

The role of organic acids in pathogen inhibition was evaluated by incubating *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 cultures in the cell-free supernatant (CFS; pH 3.81-4.27) or the neutralized cell-free supernatant (pH adjusted to 6.5 -7.0) of each isolate. When neutralized, the antimicrobial activity of the CFS of the three LAB isolates was greatly diminished, illustrating the role of lactic acid in the inhibition of pathogen growth.

To assess antimicrobial efficacy on Iceberg lettuce, a cocktail of the three LAB isolates (7-8 log CFU/g) was sprayed onto lettuce spot-inoculated with *L. monocytogenes* (2-3 log CFU/g); lettuce was incubated at 10°C for 14 d. *L. monocytogenes* levels were 1.84 log lower on LAB-treated lettuce than on untreated lettuce at the end of incubation. Because the LAB cocktail suppressed the growth of *L. monocytogenes* on lettuce, testing on fresh produce continued using DF1, which was a powdered product comprised of the three LAB isolates and media components. Because DF1 caused substantial browning of Iceberg lettuce after 2 d, Gala apples were chosen to evaluate the antimicrobial activity of DF1 against *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7.

The effect of DF1 on *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on Gala apples was determined by spraying a Gala apple spot-inoculated with pathogen (6-7 log CFU/plug) with approximately 3 mL of a 20% DF1 solution, then incubating at 20°C for 5 d. After 5 d incubation, *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 levels on DF1-treated apples were approximately 4, 2, and 2 log higher than the control, respectively. Based on the results of these experiments, DF1 is not the optimal formulation for the biocontrol of foodborne pathogens on fresh produce.

This study identified several bacterial isolates with potential for use in the biocontrol of plant and foodborne pathogens. Further investigation is required to assess possible use in the agricultural industry, including characterization of bioactive compounds, optimization of biocontrol product formulation, and evaluation of the commercial viability of the biocontrol product.

ACKNOWLEDGMENTS

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

1. INTRODUCTION

The United States agricultural industry is a multibillion dollar business. In 2012, vegetable, fruit, and nut production in the U.S. was valued at approximately US$223 billion (USDA-National Agricultural Statistics Service, 2014). Products manufactured by the produce industry include fresh produce, fresh-cut fruits and vegetables, and packaged salads. Fresh produce refers to fruits and vegetables sold as whole pieces. Fresh-cut fruits and vegetables include overwrapped fruits, fresh-cut fruits (chunks, cubes, cored, etc.), fruit in jars and cups packed in juice, and vegetables washed, cut, and packaged for side dishes, trays, snacking, or meal preparation. The term packaged salad refers to pre-cut lettuce, vegetables, and fruits packaged together as a salad. (United Fresh Produce Association, 2013).

Because of the economic importance of the agricultural industry, maintaining the integrity of agricultural commodities both before and after harvest is key to ensuring the vitality of this sector of the U.S. economy. Factors rendering agricultural commodities unfit for consumption, be it commodity loss in the field due to phytopathogenic microorganisms or postharvest due to food safety concerns, have a dramatic economic impact on the agricultural industry. Globally, crop loss caused by pathogens, animals, and weeds is between 20 and 40%; losses in major crops—wheat, maize, and soybeans—due to plant diseases were 10.2, 8.5, and 8.9%, respectively, from 2001-2003 (Oerke, 2005; Savary et al., 2012). In the United States, crop disease losses amount to an estimated US$220 billion annually (Chakraborty and Newton, 2011). Foodborne disease outbreaks

1

are also costly to the agricultural industry. Food safety incidences cost the United States

food industry approximately US$7 billion a year (Hussain and Dawson, 2013); the cost of

a single outbreak can exceed US$100 million (Table 1.1). The mitigation of

phytopathogenic and pathogenic microorganisms on produce is an important part of

ensuring the vitality of the agricultural industry.

This study aimed to determine the potential use of non-pathogenic bacteria as

biocontrol agents for the suppression of plant and foodborne pathogens on fresh produce.

The objectives of this study were to screen several bacterial isolates for antifungal and

antimicrobial activity in vitro and to evaluate the antimicrobial efficacy of select isolates

on fresh produce.

Table 1.1. Estimated economic loss due to major foodborne disease outbreaks in the
United States (adapted from Hussain and Dawson, 2013)

Year	Food product	Pathogen	Estimated economic loss (US dollars)
2009	Peanut products	*Salmonella*	$70 million
2008	Tomatoes	*Salmonella*	$250 million
2007	Peanut butter	*Salmonella*	$133 million
2006	Spinach	*E. coli*	$350 million

2. LITERATURE REVIEW

2.1 Fungal Phytopathogens

2.1.1 Economic Impact of Fungal Phytopathogens

Fungal phytopathogens cause disease in plants and are a major concern to the

agricultural industry; fungi such as *Botrytis cinerea* and *Fusarium* species are the

causative agents of several plant diseases. *Bot. cinerea*, the fungus responsible for the

diseases Gray Mold and Botrytis Rot, is a major issue for strawberry producers, as well

as grape, almond, pistachio, tomato and orange growers. In the United States,

approximately 15% of strawberry crops are lost to disease caused by *Bot. cinerea* (Haydu

and Legard, 2003). *Bot. cinerea* colonizes dead or dying plant tissues, then continues on

to kill and macerate adjacent healthy tissues; it is the ability to destroy healthy tissue that

makes *Bot. cinerea* especially problematic for growers (Moorman, 2015). Fusarium Wilt

and Fusarium Rot, caused by various *Fusarium* species, also affect a wide range of crops

including lettuce, soybeans, strawberries, tomatoes, peppers, potatoes, and oranges.

Fusarium Root Rot caused an average annual loss of 7.72 million bushels of soybeans

between 2006 and 2009 in the United States (Koenning and Wrather, 2010), and

Fusarium Head Blight has cost wheat growers in the Northern Great Plains and central

United States an estimated US$2.7 billion from 1998 to 2000 (Chakraborty and Newton,

2011). The fungus enters the plant through the roots and grows in the vascular system,

clogging the conduits through which water flows, causing wilting and eventual death

3

(Miller, et al., 1996; UC IPM, 2014c). The control of these plant pathogens is essential to maintaining and growing the U.S. agricultural industry.

2.1.2 Pre-harvest Control of Phytopathogenic Fungi

Careful crop management is used to limit the incidence of diseases caused by *Bot. cinerea* and *Fusarium* species in crops. Practices such as clearing fields of plant detritus and creating an open canopy to allow for the quick evaporation of water are used to prevent diseases caused by *Bot. cinerea* (Williamson et al., 2007). The management of *Fusarium* species can be challenging because the fungus persists in soil by forming chlamydospores (large, thickly-walled fungal spores), which are difficult to destroy. Selecting a location to plant crops without a history of *Fusarium* disease is the most effective way of preventing infection (UC IPM, 2014a).

Chemical control methods are also used to limit the impact of *Bot. cinerea* and *Fusarium* species on crops. Fungicides are used to inhibit *Bot. cinerea* with varying efficacy, but the phytopathogen has developed fungicide resistance (Williamson et al., 2007a; UC IPM, 2014b; Moorman, 2015). Fumigation before planting is used to treat fields where *Fusarium* chlamydospores are present (UC IPM, 2014a). The use of some broad-spectrum chemical fungicides, however, is being phased out because of their environmental impact. This, in addition to an increase in consumer demand for more "natural" products, has forced the agricultural industry to investigate other means of fungal phytopathogen suppression.

4

2.2 Foodborne Pathogens

2.2.1 Foodborne Illness Outbreaks in the United States

Foodborne illness affects an estimated 48 million Americans each year. Between 1998 and 2008, the Centers for Disease Control and Prevention (CDC) recorded 13,405 foodborne disease outbreaks, with an average of 968 to 1,403 outbreaks per year. During this ten year period, there was an annual average of 19,951 to 28,895 cases of illness, 593 to 1261 hospitalizations and 9 to 48 deaths (Gould et al., 2013b). The CDC received reports of 1,527 foodborne disease outbreaks in 2009 and 2010—675 and 852 outbreaks, respectively—which resulted in 29,444 cases of illness, 1,184 hospitalizations and 23 deaths (Gould et al., 2013a). The foodborne pathogens *Listeria monocytogenes*, *Salmonella*, and Shiga toxin-producing *Escherichia coli* O157:H7 have all been associated with foodborne disease outbreaks in the United States.

2.2.2 *Listeria monocytogenes*

Listeria monocytogenes is a Gram positive foodborne pathogen. It can grow in temperatures ranging from less than 0°C to 45°C, with optimal growth at 30 to 37°C, and can also proliferate in growth media with up to 20% sodium chloride. *L. monocytogenes* can be isolated from many different environments, including soil, vegetation, sewage, water, and the feces of healthy animals (De Vos et al., 2009).

This pathogen is usually transmitted to humans via contaminated food; its infectious dose is unknown, but is thought to be below 1,000 organisms. In healthy adults, *L. monocytogenes* infection causes gastroenteritis after 1-2 d incubation and

symptoms typically last 1-3 d. However, *L. monocytogenes* infection can also result in the development of listeriosis.

Listeriosis is a much more serious concern among immunocompromised populations (pregnant women, young children, the elderly and those with suppressed immune systems) than for healthy adults. Within these immunocompromised groups, listerial infection can cause meningitis, meningoencephalitis, septicemia and spontaneous abortions in pregnant women (The Center for Food Security & Public Health, 2005). Listeriosis is the third most common cause of death among foodborne diseases in the United States; in immunocompromised populations, the overall fatality rate is 20-30%, and can be as high as 70% (The Center for Food Security & Public Health, 2005; Silk et al., 2013). From 2009 to 2011, *L. monocytogenes* caused 1,651 cases of invasive listeriosis and 292 deaths or fetal loses—21% of foodborne illness-associated deaths (Silk et al., 2013). *L. monocytogenes* is of particular interest to the food industry because of the pathogen's ubiquitous distribution, its ability to proliferate at refrigeration temperatures and high sodium chloride concentrations, and its high mortality rate in immunocompromised populations.

Foodborne *L. monocytogenes* outbreaks are most commonly associated with cheese and dairy products. Twelve *L. monocytogenes* outbreaks were reported to the CDC from 2009 to 2011—the contaminated food in six of the twelve outbreaks was identified as cheese (Silk et al., 2013). However, *Listeria* outbreaks in fresh produce, commodities not traditionally associated with *L. monocytogenes*, are occurring with more frequency (Tables 2.1 and 2.2). Of the 25 foodborne disease outbreaks reported to the CDC from 1998 to 2008, only one was associated with *L. monocytogenes* in fresh

produce (sprouts) (Gould et al., 2013b). From 2009 to 2011 two *L. monocytogenes* outbreaks involving produce occurred—one outbreak associated with pre-cut celery and the other with whole cantaloupes (Silk et al., 2013).

The largest *L. monocytogenes* foodborne disease outbreak in United States history occurred in 2011. The outbreak involved contaminated whole cantaloupes from Jensen Farms' production fields in Granada, CO. During the outbreak, 147 cases of listeriosis across 28 states were reported; 33 deaths and one miscarriage were attributed to the consumption of the contaminated melons (CDC, 2012a). An investigation was conducted by the U.S. Food and Drug Administration (FDA) and Colorado state officials of the Jensen Farms cantaloupe fields and packing facility in September, 2011. Samples collected in the growing field were negative for *L. monocytogenes*, but the four outbreak strains were isolated from various sample sites in the packing facility (FDA, 2011a).

The investigation identified several factors that potentially contributed to *L. monocytogenes* contamination. Even though the outbreak strains of *L. monocytogenes* were not isolated from the growing fields, the fields were identified as a potential source of contamination. Jensen Farms did not precool the melons before refrigerated storage and the cantaloupes, still warm from the field, could have provided a warm, moist environment in which to promote *L. monocytogenes* growth. Poor facility design of the packing house allowed for the pooling of water around employee walkways and packaging equipment, and drains were not easily accessible for proper cleaning; both these conditions could allow for the establishment of harborage sites. Packaging equipment was not designed for easy and complete cleaning and sanitation; three of the four outbreak strains were isolated from the packaging equipment. Finally, Jensen Farms

7

used a truck to haul culled cantaloupe to a cattle operation—this truck could have introduced *L. monocytogenes* into the packing facility (FDA, 2011a). The FDA investigation highlights the need for effective preventative control measures and the importance of sanitary facility and equipment design in produce postharvest processing.

Table 2.1. Number of foodborne disease outbreaks caused by *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* in fresh produce, 1998 to 2008 (adapted from Gould, Walsh, et al., 2013)

Pathogen	Fruits and nuts	Leafy vegetables	Root	Sprouts	Vine-stalk vegetables
L. monocytogenes	--	--	--	1	--
Salmonella	36	11	6	21	21
Shiga-toxin producing *E. coli*	10	23	--	6	1

(--): no outbreaks reported.

2.2.3 *Salmonella* Species

Salmonella is a Gram negative, non-spore forming bacteria commonly associated with animals, especially poultry and swine, but can also be found in water and soil. *Salmonella* infection can result in two kinds of illness depending on serotype: typhoid fever and non-typhoidal salmonellosis. Typhoid fever is caused by *Salmonella enterica* ser. Typhi and *Salmonella enterica* ser. Paratyphi A, with an infectious dose of fewer than 1,000 cells. Symptoms develop 1-3 weeks after exposure and include high fever, lethargy, abdominal pain, diarrhea or constipation, headache, achiness and sometimes a flat, rose colored rash. Typhoid fever generally lasts 2-4 weeks. Non-typhoidal salmonellosis is caused by all other *Salmonella* serotypes and is the disease most frequently associated with foodborne illness. The infectious dose can be as low as one cell, depending on the organism's serotype and the age and health of the host.

Salmonellosis symptoms include nausea, vomiting, abdominal cramps, diarrhea, fever and headache; they develop 6-72 h after exposure and generally last for 4-7 d. Complications with typhoid fever and non-typhoidal salmonellosis can result in septicemia, with the colonization of other tissues and organs, and also cause chronic health problems, such as reactive arthritis (FDA, 2012).

In the United States, approximately 42,000 cases of salmonellosis are reported each year (CDC, 2012b). Between 1998 and 2008 and 2009 to 2010, *Salmonella* was the cause of 26% and 30% of foodborne disease outbreaks with a single confirmed or suspected causative agent, respectively; it was the second most common etiology of foodborne disease outbreak in both time periods. *Salmonella* was the second most frequent cause of foodborne illness and hospitalization from 1998 to 2010 and the cause of 29% of the deaths from foodborne disease outbreaks in this period (Gould et al., 2013a; b). *Salmonella* outbreaks have traditionally been associated with animal products, such as meats, poultry and eggs (FDA, 2012). However, fresh produce has become an increasing source of foodborne *Salmonella* outbreaks.

Salmonella has caused numerous multistate outbreaks in a variety of types of produce (Tables 2.1 and 2.2). From 1998 to 2008, *Salmonella* outbreaks in fruits and nuts, leafy vegetables, root vegetables, sprouts, and vine-stalk vegetables were the cause of 6,764 illnesses (Gould et al., 2013b). In 2008, an outbreak of *Salmonella enterica* ser. Saintpaul—spanning 43 states, the District of Colombia, and Canada—caused 1,442 cases of salmonellosis, 286 hospitalizations and two deaths. An FDA investigation, with the cooperation of state and local agencies, determined the sources of the infection to be jalapeño peppers, serrano peppers, and Roma tomatoes. The FDA traced the

contaminated produce back to a farm in Tamaulipas, Mexico which grew jalapeño

peppers, serrano peppers and Roma tomatoes. Although the outbreak strain was not

isolated from the farm, it was isolated from a nearby farm with whom it shared a packing

facility; the packing facility was likely the source of contamination (Jungk et al., 2008).

Table 2.2. Major *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 outbreaks in fresh produce investigated by the CDC, 2006 to 2013

Pathogen	Year	Commodity	Company	Cases/ deaths
L. monocytogenes[1]	2011	Cantaloupe	Jensen Farms	147/34
S. enterica ser. Saintpaul[2]	2013	Cucumbers	Daniel Cardenas Izabal and Miracle Greenhouse	84/0
S. enterica ser. Braenderup[3]	2012	Mangos	Agricola Daniella	127/0
S. enterica ser. Typhimurium and *S. enterica* ser. Newport[4]	2012	Cantaloupe	Chamberlain Farms Produce, Inc.	261/3
S. enterica ser. Agona[5]	2011	Papayas	Agromod Produce Inc.	106/--
S. enterica ser. Saintpaul[6]	2008	Peppers and tomatoes	Unnamed packing facility in Mexico	1,442/2
E. coli O157:H7[7]	2013	Ready-to-eat salads	Glass Onion Catering	33/0
E. coli O157:H7[8]	2011	Romaine lettuce	No source identified	58/0
E. coli O157:H7[9]	2006	Fresh spinach	Natural Selection Foods	199/3

(--): no deaths reported
[1]CDC, 2012a; [2]CDC, 2013a; [3]CDC, 2012c; [4]CDC, 2012d; [5]CDC, 2011a; [6]Jungk et al., 2008; [7]CDC, 2013b; [8]CDC, 2012e; [9]CDC, 2006.

2.2.4 *Escherichia coli* O157:H7

Escherichia coli is a Gram negative bacteria that is part of the normal intestinal flora of the human gut. Most strains of *E. coli* are non-pathogenic, but a subgroup of enterohemorrhagic *E. coli* (EHEC) strains have been linked to numerous foodborne disease outbreaks. EHEC strains are characterized by the ability to produce Shiga toxin, and the *E. coli* strain O157:H7 accounts for approximately 75% of EHEC infections worldwide. The infective dose of *E. coli* O157:H7 is between 10 and 100 cells; this toxin-mediated infection causes hemorrhagic colitis. Symptoms appear 3-4 d after exposure, last for 2-9 d, and include severe cramping and abdominal pain, nausea or vomiting, no or low-grade fever, and bloody diarrhea. In 3-7% of hemorrhagic colitis cases, the more serious diseases of hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenia pupura (TTP) can develop, which can result in kidney failure (FDA, 2012).

Pathogenic *E. coli* has been associated with foodborne disease outbreaks in a variety of different commodities. Between 1998 and 2010, *E. coli* was responsible for 4% of outbreaks with known etiology, causing 3% of the illnesses, 14% of the hospitalizations, and 12% of the deaths in this period (Gould et al., 2013a; b). Shiga toxin-producing *E. coli* has been associated with many fresh produce commodities (Tables 2.1 and 2.2). From 1998 to 2008, *E. coli* O157:H7 was the cause of approximately 16% of the outbreaks that occurred in leafy vegetables, 7% of the outbreaks in fruits and nuts, and 5% of outbreaks in sprouts (Gould et al., 2013a). Produce-associated *E. coli* outbreaks have caused devastating foodborne disease outbreaks not only in the United States, but also internationally.

A multistate outbreak of *E. coli* O157:H7 involving fresh spinach caused 199 confirmed illnesses, 31 cases of HUS, and 3 deaths in 26 states (CDC, 2006). The source of the contamination was traced to spinach fields in San Juan Bautista, CA operated by Natural Selection Foods, LLC. FDA investigators isolated the outbreak strain in environmental samples from a field, river water, and cattle and wild pig feces from a nearby ranch. The likely source of the contamination was wild pigs in and around the spinach field (California Food Emergency Response Team, 2007).

Although a majority of outbreaks have been linked to *E. coli* O157:H7, non-O157 strains of EHEC have also been identified as the cause of foodborne disease outbreaks. In 2011, an outbreak of *E. coli* O104:H4 sickened 4,075 people across 16 European countries and North America. The outbreak caused 908 cases of HUS and 50 deaths (WHO, 2011). The source of the outbreak was traced back to contaminated sprouts from a farm in Lower Saxony, Germany, and eventually to contaminated fenugreek seeds from Egypt (CDC, 2011a).

2.3 Postharvest Control of Foodborne Pathogens

2.3.1 Temperature Control

Temperature control is the most common means of microbial control on fresh produce; storage at low temperatures is used to slow or inhibit microbial growth. Minimum growth temperatures are a function of growth media. However, the minimum growth temperatures for *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 are

considered to be -0.4, 5.2, and 6.5°C, respectively (FDA, 2011b). While maintaining produce at refrigeration temperatures (approximately 4°C) will not inhibit the growth of *L. monocytogenes*, storage at lower temperatures slows the proliferation of most of the microflora present on produce, both native and pathogenic.

Maintaining produce at low temperatures throughout the entire distribution chain is critical to suppressing microbial growth. The FDA requires that ready-to-eat fruits and vegetables be stored at or below 5°C to suppress the proliferation of pathogens (FDA, 2013a). Temperature abuse during transport and retail storage could allow for the growth of pathogens. A study by Zeng et al. (2014) constructed temperature profiles of refrigerated truck transport and retail storage during fresh produce distribution, and determined the fate of *E. coli* O157:H7 and *L. monocytogenes* on pre-cut romaine lettuce under those temperature conditions. Average temperatures during transport in refrigerated trucks and in retail display cases ranged from ranged from -0.3 to 7.7°C and -1.1 to 9.7°C, respectively. There was no significant increase in *E. coli* O157:H7 levels during transport or retail display storage; *L. monocytogenes* populations increased ≤ 0.6 log and 1.1 log CFU/g, respectively.

The largest temperature fluctuation and the greatest increase in pathogen populations were observed during retail storage. The average temperature ranged from 0.6 to 15.4°C, with a maximum of 18.2°C; both *E. coli* O157:H7 and *L. monocytogenes* populations increased approximately 3 log CFU/g under this temperature profile (Zeng et al., 2014). This demonstrates the importance of good, consistent temperature management throughout the entire distribution chain in minimizing microbial hazards on fresh produce.

2.3.2 Mechanical Removal

Often the first step in postharvest processing is the washing of produce, sometimes with the use of brushes and detergents, to facilitate the mechanical removal of soil and organic material from the surface (FDA, 2013b). Rinsing with water alone is not effective enough in reducing microbial populations, especially on produce with rough, uneven surfaces or on produce with a waxy cuticle (Ukuku, 2006; Keskinen and Annous, 2011; FDA, 2013b). However, the initial removal of surface debris increases the efficacy of following sanitizing steps by increasing surface contact and reducing the organic material that may interfere with disinfectants.

2.3.3 Chlorine

Chlorine is the most commonly used disinfectant in the produce industry because of its low cost and effectiveness against a broad spectrum of undesirable organisms. Hypochlorous acid (HOCl), the active form of chlorine, is often referred to as free chlorine, reactive chlorine, and available chlorine. Chlorine is an effective disinfectant because HOCl is a powerful oxidizer and the bactericidal mechanism of HOCl works through several modes of action. Hypochlorous acid has been shown to denature DNA, enzymes, and proteins, disrupt adenosine triphosphate production and other membrane-associated activity. It also damages the cell membranes of both Gram positive and Gram negative organisms, increasing cell membrane permeability and causing cellular leakage (Barrette et al., 1989; Prütz, 1996; McDonnell and Russell, 1999; Virto et al., 2005). Because chlorine is an effective and broad-spectrum disinfectant, it is widely used in the produce industry to control microbial populations.

Chlorine is added to produce wash water as either a gas (Cl_2) or as a solid hypochlorite salt (Suslow, 1997). The FDA recommends a chlorine concentration of 50 to 200 ppm to sanitize produce surfaces (FDA, 2013b), but in industry chlorine concentration is usually maintained below recommended levels because fresh produce processors wash the product several times, increasing the total produce-chlorine contact time. Lower levels of chlorine are also less costly, create a more comfortable environment for workers, and leave less of a residual odor on the product (W. Brown, personal communication, September 12, 2014).

The pH of the wash water can affect the antimicrobial activity of chlorine. The active form of chlorine, HOCl, is a weak acid with a pKa of 7.52 (Brown et al., 2009). Chlorinated wash water is maintained at a pH of 6.5 to 7.5; this pH range increases the proportion of HOCl molecules and minimizes corrosion of equipment while maintaining acceptable antimicrobial activity (Suslow, 1997; FDA, 2013b). The presence of organic material in the wash water also affects efficacy; organic matter interacts with free chlorine, reducing the amount of HOCl available to react with microorganisms present on the produce (Suslow, 1997; Virto et al., 2005).

The wash water in a produce processing facility is a dynamic environment, and all of the factors affecting the antimicrobial activity of chlorine make the microbial reduction caused by chlorine insufficient to ensure the safety of fruits and vegetables. Studies have shown that washing leafy greens with chlorine causes close to or less than a 1 log reduction in *L. monocytogenes* (Beuchat and Brackets, 1990; Zhang and Farber, 1996), *Salmonella* (Weissinger et al., 2000; Neal et al., 2012), and *E. coli* O157:H7 (Behrsing et al., 2000; Keskinen et al., 2009; Keskinen and Annous, 2011; Neal et al., 2012).

2.3.4 Surfactants

Chlorine must come into direct contact with the target organism in order to have any bactericidal effect. Some produce have a naturally occurring hydrophobic, waxy coating; this coating can prevent chlorine-pathogen contact, thus reducing antimicrobial efficacy (Suslow, 1997; FDA, 2013b). Chlorine may also have less penetration into surface cracks and crevices, preventing contact with microorganisms in these areas. Surfactants, which are amphiphilic molecules that reduce the surface tension of the liquid in which they are dissolved, decrease surface hydrophobicity, increasing access of the chlorine to pathogens.

The combination of chlorine and surfactants in produce wash water has been investigated. A study by Predmore and Li (2011) investigated the effect of surfactant addition to chlorinated wash water for the removal of norovirus from various fruits and vegetables. The surfactants sodium dodecyl sulfate, NP-40, Triton X-100, and polysorbates (including Tween 20, Tween 65, and Tween 80) were added individually at 50 ppm to a 200 pm chlorine solution. The combination of chlorine and a surfactant resulted in an additional 1-2 log reduction on strawberries, a 1.5 log reduction on raspberries, a 1 log reduction on cabbage, and a 1.6 log reduction on lettuce over the reduction in norovirus levels when treated with chlorine alone (Predmore and Li, 2011). The addition of the surfactant sucrose monolaurate to a 200 ppm chlorine solution resulted in an approximately 1 log CFU/leaf of spinach greater reduction in *E. coli* O157:H7 than the reduction seen with chlorine alone (Xiao et al., 2011). A chlorine solution (at approximately 1,000 ppm and a pH of 6.5) with 0.1% Tween 80 improved

Salmonella enterica reduction on whole cantaloupe by 0.65 log CFU/g compared to chlorine alone (Bastos et al., 2005).

In contrast, the combination of chlorine and the surfactants Tergitol or Orenco Peel 40 did not decrease *L. monocytogenes* populations when compared to chlorine alone on lettuce or cabbage (Zhang and Farber, 1996). The surfactants dodecylbenzenesulfonic acid or sodium 2-ethyl hexyl sulfate also failed to increase the efficacy of a 200 ppm chlorine solution against *E. coli* O157:H7 on romaine lettuce (Keskinen and Annous, 2011). The effect surfactant addition has on chlorine efficacy is dependent on surfactant type and concentration, target pathogen, and type of produce. The variation in efficacy of the combined methods demonstrates the need for a new approach.

2.3.5 Other Disinfectants

2.3.5.1 Ozone

Ozone (O_3) can be applied as an aqueous solution or as a gas and is used in the disinfection of drinking water. Ozone as a sanitizer has many advantages: it is generated on-site, reducing the amount of chemicals stored in the processing facility, and it decomposes into oxygen, leaving no chemical residue on produce (Rice, 2011). Ozone use is not common in produce sanitation because its powerful oxidizing capabilities can be deleterious to the sensory characteristics of produce, antimicrobial efficacy rapidly decreases in the presence of organic matter, it can corrode equipment, and it can pose a safety risk for workers (Suslow, 1997; Ölmez and Kretzschmar, 2009; fda, 2013b). An aqueous ozone dip was not more effective than chlorine in reducing *Listeria innocua*,

Salmonella, or *E. coli* O157:H7 on spinach (Neal et al., 2012; Karaca and Velioglu, 2014).

2.3.5.2 Peroxyacetic Acid

Peroxyacetic acid, also called peracetic acid, is a mixture of hydrogen peroxide (H_2O_2) and acetic acid (CH_3COOH). It is sporicidal, bactericidal, virucidal, and fungicidal at low concentrations ($< 0.3\%$) and is approved by the FDA for use in the sanitation of fruits and vegetables at a concentration not exceeding 80 ppm (McDonnell and Russell, 1999; FDA, 2014a). The advantages of peroxyacetic acid include low reactivity with organic matter in wash water, it does not require pH control, it can be used at a lower concentration than chlorine, and it degrades into harmless acetic acid, oxygen, and water (University of Georgia, 2011). However, peroxyacetic acid is more expensive than chlorine and has not been shown to be more effective in reducing the microbial load of produce (Ölmez and Kretzschmar, 2009; University of Georgia, 2011; Neal et al., 2012).

2.4 Biocontrol

2.4.1 Antifungal and Antimicrobial Modes of Action

Due to the increasing number of foodborne disease outbreaks involving fresh produce, combined with the growing concern over the use of chemical fungicides in the control of plant pathogens, an alternative means of suppressing the growth and spread of

fungal phytopathogens and foodborne pathogens are of interest to the agricultural

industry. Biocontrol, which is the use of bioprotective organisms (such as nonpathogenic

bacteria) to control undesirable organisms, offers an alternative means of microbial

management. Several species of bacteria, including species of Lactic Acid Bacteria

(LAB), *Bacillus*, *Serratia*, *Ochrobactrum*, and *Oceanobacillus*, have demonstrated

antifungal and antimicrobial activity on fresh produce. However, because the antifungal

and antimicrobial activity of bioprotective organisms is a function of not only species and

strain, but also of the food system, the efficacy of each biocontrol agent must be

confirmed in each new food matrix.

The antifungal and antimicrobial effect of the biocontrol agent can be exerted in

two ways: specific suppression and general suppression. In specific suppression, the

biocontrol agent inhibits a specific known pathogen; the bioprotective organism is chosen

based on a known interaction with the target pathogen. General suppression occurs when

the microbial population creates an environment generally unsuitable for pathogenic

organisms (Singh and Vyas, 2009).

The mechanisms employed by biocontrol agents for the suppression or inhibition

of undesirable microorganisms include antibiosis, predation of the target organism,

stimulation of induced systemic resistance in the host plant, and competitive inhibition. In

antibiosis, the bioprotective organism produces some kind of antibiotic compound that

has a detrimental effect on the target organism. Predation of the target organism refers to

the bioprotective organism directly feeding on the target organism, or producing enzymes

or some other compound that kills the undesirable organism, allowing the biocontrol

agent to feed on the dead target pathogen. Induced systemic resistance occurs when the

exposure of the host plant to bioprotective organisms triggers an intrinsic response that increases its resistance to infection. Competitive inhibition is the suppression of undesirable organisms through the introduction of a microbial population that competes for nutrients and space in the environment (Singh and Vyas, 2009). Biocontrol agents can employ one or more modes of action to suppress the growth of phytopathogenic fungi and bacterial pathogens.

2.4.2 Biocontrol Agents

2.4.2.1 Lactic Acid Bacteria

Lactic Acid Bacteria (LAB) are a group of Gram positive, non-spore forming, rod and cocci shaped bacteria which ferment carbohydrates and alcohols to produce lactic acid and other metabolites (Stiles and Holzapfel, 1997). Lactic Acid Bacteria include the families *Lactobacillaceae*, *Aerococcaceae*, *Carnobacteriaceae*, *Enterococcaceae*, *Leuconostocaceae*, and *Streptococcaceae* (De Vos et al., 2009); the genera most commonly associated with foods are *Carnobacterium*, *Enterococcus*, *Lactobacillus*, *Lactococcus*, *Leuconostoc*, *Oenococcus*, *Pediococcus*, *Streptococcus*, *Teragenococcus*, *Vagococcus*, and *Weissella* (Stiles and Holzapfel, 1997).

Lactic Acid Bacteria have long been used in the biopreservation of foods— cheese, yogurt, salami and sauerkraut are produced through fermentation by these organisms. During fermentation, growth of undesirable organisms, both spoilage and pathogenic, is inhibited by LAB through the production of organic acids (which reduces pH) and antimicrobial compounds such as bacteriocins. Bacteriocins are small, heat-stable, ribosomally synthesized proteins that exhibit antimicrobial activity against

organisms closely related to the producer. Antimicrobial activity of bacteriocins produced

by LAB is typically limited to Gram positive organisms, including *L. monocytogenes*

(Delves-Broughton, 2005; Rodríguez et al., 2005; Mills et al., 2011). Antimicrobial

activity against Gram negative pathogens is generally not as effective, but can be

enhanced with the addition of membrane-disrupting compounds such as weak acids and

chelators (Alakomi et al., 2000; Lu et al., 2011). Bacteriocin production has been

identified in several genera of LAB (Table 2.3).

Table 2.3. Bacteriocins produced by Lactic Acid Bacteria species (adapted from Sullivan et al., 2002)

Bacteriocin	Inhibition spectrum[1]
Lactococcus spp.	
Nisin	Broad-spectrum
Lacticin 3147	Broad-spectrum
Lacticin 481	Medium-spectrum
Lactococcin A, B, and M	Narrow-spectrum
Lactobacillus spp.	
Lactocin 27	Narrow-spectrum
Sakacin A	Narrow-spectrum
Sakacin B	Narrow-spectrum
Plantaricin C	Broad-spectrum
Pediococcus spp.	
Pediocin A	Broad-spectrum
Pediocin AcH (PA-1)	Broad-spectrum
Leuconostoc spp.	
Leucocin A-UAL187	Broad-spectrum
Enterococcus spp.	
Enterocin A	Narrow-spectrum
Carnobacterium spp.	
Carnocin U149	Broad-spectrum
Piscicolin 126	Broad-spectrum
Divercin V41	Broad-spectrum

[1]Broad-spectrum: inhibits most strains of a wide range of species of Gram positive bacteria.
Medium-spectrum: inhibits some to most strains of producer and several other species of bacteria.
Narrow-spectrum: inhibition of some strains within a species and no activity against others.

Antibiosis caused by organic acids and bacteriocins alone is not enough to control both Gram positive and Gram negative pathogens. The addition of another antimicrobial mechanism of biocontrol, such as competitive inhibition, could enhance microbial control of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7. A LAB strain capable of both survival and bacteriocin production at refrigeration temperatures may be more effective in controlling pathogen proliferation on produce during storage. Because many LAB are Generally Recognized as Safe (GRAS) for human consumption (Crowley et al., 2013), their use as biocontrol agents for the management of fungal phytopathogens and foodborne pathogens has been investigated.

2.4.2.1.1 Antifungal Activity on Produce

Lactic Acid Bacteria have shown antifungal activity against phytopathogenic fungi. The antifungal activity of LAB species has been attributed to low molecular weight peptides (Schnürer and Magnusson, 2005), fatty acids (Ryan et al., 2011), cyclic dipeptides (Strom et al., 2002; Magnusson et al., 2003; Ryan et al., 2011), and 3-phenyllactic acid and other carboxylic acids (Strom et al., 2002; Ryan et al., 2011; Mu et al., 2012; Cortés-Zavaleta et al., 2014; Ryu et al., 2014). Strains of *Lactobacillus plantarum* and *Pediococcus pentosaceus* have demonstrated broad spectrum fungal inhibition *in vitro*, including the suppression of *Fusarium culmorum* (Crowley et al., 2013), *Fusarium graminearum,* and *Bot. cinerea* (Sathe et al., 2007). *P. pentosaceus* ATCC 25745, isolated from maize, inhibited five strains each of *Fusarium verticillioides* and *Fusarium proliferatum.* Two antifungal compounds were isolated; both were of low molecular weight (500-1,400 Da and 1,400-1,800 Da), heat stable, most effective at pH

below 5, and maintained antifungal activity after exposure to proteases (Dalie et al.,

2010). *In vivo* evaluation of *Lb. plantarum* showed its ability to suppress *Bot. cinerea* and

F. graminearum on cucumbers (Sathe et al., 2007).

2.4.2.1.2 Antimicrobial Activity on Produce

2.4.2.1.2.1 Lactic Acid Bacteria Isolates on Produce

Many bacteriocin-producing LAB species have demonstrated antimicrobial

activity against Gram positive organisms, and thus antimicrobial activity against

foodborne pathogens on different types of produce has been investigated. *Lactobacillus*

rhamnosus GG has shown inhibitory activity against *L. monocytogenes* on apples; there

was an approximately 1 log difference in *L. monocytogenes* levels between apple wedges

inoculated with *Lb. rhamnosus* GG and untreated apple wedges when incubated at 5 or

10°C for 28 d. *Lb. rhamnosus* GG did not have an effect on *Salmonella* populations under

the same conditions (Alegre et al., 2011). A study by Trias et al. (2008) determined the

antimicrobial activity of two strains of *Lactococcus lactis* and three strains of

Leuconostoc mesenteroides isolated from several fruit and vegetable sources on apples

and lettuce; four of the five strains reduced *L. monocytogenes* to undetectable levels after

2 d incubation at 25°C; one strain of *Lc. lactis* did not significantly affect *L.*

monocytogenes populations. These five strains were less effective in suppressing

Salmonella enterica ser. Typhimurium; inhibition on apples and lettuce ranged from no

inhibition to approximately 2 log (Trias et al., 2008a).

The food matrix has an effect of the efficacy of the bioprotective agent. In the

previously mentioned study by Trias et al. (2008) the same five LAB strains were

evaluated for antimicrobial activity against *E. coli* ATCC 11775 and *S. enterica* ser.

Typhimurium on apples and lettuce. Of the five LAB strains tested, one strain of *Ln.*

mesenteroides caused a significant difference of less than 1 log in *E. coli* levels ($P < 0.05$)

on apples compared to untreated apples; the other LAB strains did not cause a significant

difference in *E. coli* levels. On lettuce, four of the five LAB strains caused a significant

difference of less than 1 log in *E. coli* levels ($P < 0.05$); one strain of *Lc. lactis* did not

significantly inhibit *E. coli*. Regardless of produce type, inhibition of *E. coli* was less than

1 log (Trias et al., 2008a).

2.4.2.1.2.2 Bacteriocins on Produce

The antimicrobial activity of LAB metabolites against the foodborne pathogens *L.*

monocytogenes, *Salmonella*, and *E. coli* O157:H7 has been well studied. The bacteriocins

produced by some strains of LAB have been assessed for their antimicrobial activity

against *L. monocytogenes* on produce. Mundticin, a bacteriocin synthesized by

Enterococcus mundtii, was able to suppress *L. monocytogenes* on mungbean sprouts;

there was an approximately 2 log CFU/g difference in *L. monocytogenes* levels between

mundticin-treated sprouts and untreated sprouts after 13 d incubation at 8°C (Bennik et

al., 1999). Iceberg lettuce sprayed with a bacteriocin from *Lc. lactis* RUC9 or Nisaplin®

(a commercially available nisin product) had approximately 2.5 and 2 log CFU/g less *L.*

monocytogenes, respectively, than untreated lettuce after 7 d incubation at 4°C (Randazzo

et al., 2009). Dips containing the bacteriocins coagulin or nisin Z caused a 3.2 to 3.5 log

CFU/g reduction in *L. monocytogenes* (initially 4.8 ± 0.1 log CFU/g) on iceberg lettuce.

However, after 7 d incubation at 4°C, there was no statistical difference in *L.*

monocytogenes between bacteriocin-treated and untreated lettuce (Allende et al., 2007).

Bacteriocin treatments successfully decreased *L. monocytogenes* populations upon initial

contact, but antimicrobial activity was not present at the end of 7 d.

Purified bacteriocins alone do not typically inhibit Gram negative organisms, but

antimicrobial activity can be enhanced with the addition other antimicrobial compounds.

A study by Cobo Molinos et al. (2008) investigated the antimicrobial activity of the

combination of the bacteriocin enterocin AS-48, produced by *Enterococcus faecalis* A-

48-32, with weak acids, chlorine, and the chelator EDTA against *S. enterica* on soybean

sprouts. Enterocin AS-48 alone did not result in a significant decrease in *S. enterica*

levels. However, combining enterocin AS-48 with 1.5% (w/v) lactic acid, 0.1% (w/v)

polyphosphoric acid, 100 ppm chlorine, or 100 mM EDTA all resulted in approximately

4 log reductions in *S. enterica* (initially approximately 5 log CFU/g) compared to

enterocin AS-48 alone. After 48 h incubation at 15°C, *S. enterica* populations were

significantly lower ($P < 0.05$) in the enterocin AS-48 combination treatments than in

enterocin AS-48 only treatments; the most effective combination was with lactic acid,

followed by EDTA, chlorine, and polyphosphoric acid. The combination of enterocin

AS-48 and 0.4% (w/v) polyphosphoric acid was also applied to *E. coli* O157:H7 CECT

4972-inoculated soybeans; *E. coli* O157:H7 was undetectable after 48 h at 6°C and after

24 h at 15°C, while soybeans treated with only enterocin AS-48 had approximately 5.8

and 6.1 log CFU/g *E. coli* after 48 h incubation at 6 and 15°C, respectively.

2.4.2.2 *Bacillus* Species

Bacillus are rod-shaped, Gram positive, aerobic or facultatively anaerobic bacteria. These endospore-formers are widespread throughout the environment, with soil being their primary habitat (De Vos et al., 2009). Several species of *Bacillus*, including *Bacillus subtilis*, *Bacillus amyloliquefaciens*, and *Bacillus licheniformis*, are extensively utilized in the industrial production of enzymes such as proteases, amylases, and lipases because of their ability to secrete these compounds in high quantity (Westers et al., 2004; Maarten van Dijl and Hecker, 2013). There is much diversity within the genus and a wide variety of secondary metabolites are synthesized, including lipopeptides, polyketides, and bacteriocins and bacteriocin-like compounds.

Lipopeptides are biosurfactants consisting of a cyclic peptide chain attached to a fatty acid; these compounds are produced through non-ribosomal synthesis. Lipopeptides are separated into three families based on their molecular structure: surfactin, iturin, and fengycin. These potent biosurfactants exert strong antifungal activity and some antimicrobial activity through membrane disruption (Arguelles-Arias et al., 2009; Sansinenea and Ortiz, 2011; Mongkolthanaruk, 2012; Christie, 2014).

Lipopeptides belonging to the surfactin family are powerful biosurfactants and exhibit antifungal, antimicrobial and antiviral activity. Two novel lipopeptides belonging to the surfactin family produced by *B. amyloliquefaciens* BO5A inhibited the growth of several fungal phytopathogens *in vitro*, including *Fusarium oxysporum*, *Aspergillus niger*, and *Trichoderma harzianum* (Romano et al., 2013). Surfactin has also shown antimicrobial and antiviral activity; it has demonstrated suppression of *L. monocytogenes*

(Sabaté and Audisio, 2013), *S. enterica* ser. Enteritidis (Huang et al., 2011), and both

enveloped and nonenveloped viruses (Kracht et al., 1999; Huang et al., 2006).

Iturin family lipopeptides are strongly antifungal. Iturin A suppressed the growth

of *Podosphaera fusca* (Romero et al., 2007) and *F. oxysporum* (Chen et al., 2014), and

decreased the disease incidence of the fungal phytopathogens *Alternaria citri*,

Colletotrichum gloeosporioides, and *Penicillium crustosum* on Valencia oranges

(Arrebola et al., 2010). The iturin bacillomycin D has demonstrated broad spectrum

fungal inhibition *in vitro*, including the inhibition of the phytopathogens *F. oxysporum*

(Moyne et al., 2001; Yuan et al., 2012), *Aspergillus flavus*, and *C. gloeosporioides*

(Moyne et al., 2001). Mycosubtilin, also belonging to the iturin family, has antifungal

activity; it decreased Lettuce Downy Mildew, caused by *Bremia lactucae*, by 82.7%

when applied at 100 mg/L to growing lettuce (Deravel et al., 2014).

Lipopeptides belonging to the fengycin family are antifungal, with good

inhibition of filamentous fungi (Tao et al., 2011). Fengycin suppressed the growth of

Rhizopus stolonifer (Tao et al., 2011; Tang et al., 2014), *F. oxysporum* f. sp. *spinaciae*

(Zhao et al., 2014) and *Bot. cinerea in vitro* (Romero et al., 2007). Fengycin also

inhibited the fungal phytopathogens *Pyricularia oryzae*, *Curvularia lunata*, *Alternaria*

kikuchiana, and *Rhizoctonia solani* (Vanittanakom and Leoffler, 1986).

In addition to exerting fungal inhibition, lipopeptide-producing species of *Bacillus*

can also act as antimicrobial agents. *B. amyloliquefaciens* S20, a Iturin A-producing

strain, inhibited the bacterial phytopathogen *Ralstonia solanacearum*, the causative agent

of Brown Rot, Bacterial Wilt, or Southern Wilt in many different crops, *in vitro* (Chen et

al., 2014). Broad spectrum inhibition of Gram positive and Gram negative foodborne

pathogens, including *L. monocytogenes*, *B. cereus*, *Salmonella*, and *E. coli* 81 *nr* 149

SKN 541 has been demonstrated by strains of *B. amyloliquefaciens* ssp. *plantarum*

producing surfactins, iturins, and other lipopeptides (Compaoré et al., 2013).

Proteinaceous antimicrobial compounds produced by some *Bacillus* species

include polyketides and bacteriocins and bacteriocin-like compounds. Polyketides are

synthesized by polyketide synthases, while bacteriocins and bacteriocin-like compounds

are ribosomally synthesized. Some species of *Bacillus* produce the polyketides difficidin

and bacillaene (Chen et al., 2006, 2009; Arguelles-Arias et al., 2009; Mongkolthanaruk,

2012). Difficidin has broad-spectrum antimicrobial activity against Gram positive and

Gram negative foodborne pathogens including *Staphylococcus aureus*, *E. coli*, *S. enterica*

ser. Typhimurium, *Clostridium perfringes*, and *Clostridium difficile* (Zimmerman et al.,

1987), as well as the ability to inhibit *Erwinia amylovora*, the causative agent of Fire

Blight in orchard trees (Chen et al., 2009).

In addition to polyketides, several different bacteriocins and bacteriocin-like

compounds are produced by *Bacillus* species. Bacteriocins from *Bacillus* follow a

classification scheme similar to that of LAB bacteriocins: Class I) post-translationally

modified peptides, Class II) unmodified peptides, and Class III) large proteins (Abriouel

et al., 2011). Subtilin, a Class I bacteriocin, is similar in structure to nisin and exhibits

antimicrobial activity against a broad spectrum of Gram positive organisms, including

Listeria species, by causing cell leakage (Parisot et al., 2008; Lee and Kim, 2011).

Mersacidin, also a Class I bacteriocin, has shown inhibition of methicillin-resistant

Staphylococccus aureus by inhibiting peptidoglycan synthesis (Brötz et al., 1995). The

pediocin-like Class II bacteriocin coagulin has a narrow spectrum of inhibition against

Gram positive bacteria, including *L. monocytogenes* (Hyronimus et al., 1998; Le Marrec et al., 2000). Cerein is a Class III bacteriocin that inhibits *L. monocytogenes*, but does not exert a strong effect on the Gram negative pathogens *S. enterica* ser. Enteritidis or *E. coli* (Bizani et al., 2005). Thuricin, another Class III bacteriocin, inhibits Gram positive organisms, especially those closely related to *Bacillus thuringiensis* (Favret and Yousten, 1989; Gray et al., 2006).

Members of the *Bacillus* genus synthesize a number of compounds that have antifungal, antimicrobial, and biosurfactant activity, making *Bacillus* potential biocontrol agents. Antifungal and antimicrobial compounds produced by species of this genus can potentially control pathogenic organisms on produce, and the addition of *Bacillus*-synthesized biosurfactants to a produce wash system may increase the efficacy of an antimicrobial agent.

2.4.2.2.1 Antifungal Activity on Produce

Some *B. subtilis* and *Bacillus megaterium* strains have demonstrated fungal inhibition to varying degrees. In a study conducted by Donmez et al. (2011), several *Bacillus* species were screened for inhibitory activity against *Bot. cinerea*. Of the organisms screened, four strains of *B. subtilis* and one strain of *B. megaterium* showed strong inhibition of *Bot. cinerea in vitro*. Two of the *B. subtilis* isolates, *B. subtilis* MFD-20 and *B. subtilis* MFD-Ü2, inhibited *Bot. cinerea* when inoculated directly onto strawberries; *B. megaterium* GC subgroup B CD-8 did not suppress *Bot. cinerea* (Donmez et al., 2011). *B. subtilis* has also shown broad-spectrum fungal suppression. *B.*

subtilis B25, isolated from banana rhizosphere, inhibited several fungal species, including *Bot. cinerea* and *F. oxysporum* f. sp. *cubense* (Tan et al., 2013).

2.4.2.2.2 Antimicrobial Activity on Produce

Bacillus species that suppress foodborne pathogens have also been identified. *Bacillus licheniformis* VPS50.2 inhibited *L. monocytogenes in vitro*; a novel bacteriocin, licheniocin, was identified as the antimicrobial compound (Berić et al., 2013). *B. thuringiensis* ssp. *kurstaki* produces the bacteriocin thuricin Bn1, which exhibits anitlisterial activity *in vitro* (Ugras et al., 2013). *Bacillus* YD1 also demonstrated inhibitory activity on bell peppers; log reductions of 3.4, 3.6, and 3.3 of *L. monocytogenes*, *S. enterica* ser. Mbandaka, and *E. coli* O157:H7, respectively, were observed after 2 d incubation at 20°C (Liao, 2009).

2.4.2.3 Other Bioprotective Species

2.4.2.3.1 *Serratia plymuthica*

Serratia plymuthica is a member of the *Enterobacteriaceae* family; it is a Gram negative, facultatively anaerobic, rod-shaped bacterium that occurs in the natural environment (soil, water, plant surfaces) (Brenner et al., 2005a). Strains of *S. plymuthica* produce bioactive compounds. The antifungal compound haterumalide produced by *S. plymuthica* A 153 demonstrated broad-spectrum activity that suppressed the spore germination of *Sclerotinia sclerotiorum*, the fungus responsible for White Mold on legumes and several fruits and vegetables, as well as inhibition of several other filamentous fungi *in vitro* (Levenfors et al., 2004). *S. plymuthica* IC14, producing the

antifungal compound pyrrolnitrin in addition to proteases and chitinolytic enzymes, reduced Leaf Mold caused by *Bot. cinerea* and *S. sclerotiorum* on cucumber seedlings by 62.7% and 69.4%, respectively (Kamensky et al., 2003).

2.4.2.3.2 *Ochrobactrum anthropi*

Ochrobactrum anthropi is a rod shaped, Gram negative, obligatorily aerobic bacteria that can be isolated from soil (Brenner et al., 2005b). This species has demonstrated broad-spectrum antifungal activity *in vitro*. *O. anthropi* inhibited the fungal phytopathogens *Scelorotium* sp. (Chaiharn et al., 2009), *Phellinus noxius*, *Poria hypolaterita* (Chakraborty et al., 2009), *F. oxysporum*, and *R. solani* (Sowndhararajan et al., 2013). Antifungal activity was most likely the result of cellulase, β 1,3 glucanase, and chitinase production (Chaiharn et al., 2009).

The bioprotective activity of *O. anthropi* has also been evaluated on tea plants. When applied to the soil of tea plants, *O. anthropi* TRS-2 promoted plant growth (based on an increase in plant height, number of branches, and number of leaves) and reduced the incidence of Brown Rot, caused by *P. noxius* (Chakraborty et al., 2009). *O. anthropi* BMO-111 also reduced Blister Blight disease, a fungal plant disease caused by *Exobasidium vexans* (Sowndhararajan et al., 2013).

2.4.2.3.3 *Oceanobacillus* Species

Oceanobacillus species are Gram positive, endospore-forming bacteria that are alkaliphilic and aerobic or facultatively anaerobic. Members of this genus are halotolerant or halophilic, with optimal growth occurring at NaCl concentrations of 3-10% (w/v);

some species of *Oceanobacillus* are able to grow in up to 20% NaCl (De Vos et al., 2009). *Oceanobacillus* sp. BRI 10, isolated from Antarctic sea water, produced a biosurfactant composed of carbohydrates, lipid, and amino acids. The biosurfactant was inhibitory of *E. coli* NCIM 2065, but showed no antimicrobial activity against Gram positive *S. aureus* or *Streptococcus faecalis* (Jadhav et al., 2013). Biosurfactant production has also been identified in *Oceanobacillus picturae*, and its lysate demonstrated antifungal activity; it was inhibitory of *Fusarium* species *in vitro* (Pakpitcharoen et al., 2008).

2.5 Commercially Available Biocontrol Products

2.5.1 Antifungal Biocontrol Products

Biocontrol products are commercially available for the suppression of fungal phytopathogens. Serenade® Optimum, manufactured by Bayer CropScience, utilizes *B. subtilis* QST 713 to control *Bot. cinerea* and *Colletotrichum*, among other fungal phytopathogens, on berries, bulb vegetables, tomatoes, and stone fruit, and *Sclerotinia* on leafy greens (Bayer CropScience LP, 2013a). Sonata®, also manufactured by Bayer CropScience, contains *Bacillus pumilus* QST 2808. It is used for the suppression of Powdery Mildew on a variety of crops, including berries, leafy vegetables, fruiting vegetables, cucumbers, melon, and squash (Bayer CropScience LP, 2013b). DoubleNickel LC is another commercially available biocontrol product that can be applied to the soil or to the plant; *B. amyloliquefaciens* strain D747 is the active

ingredient. It is used for the suppression of a variety of fungal phytopathogens including *Bot. cinerea*, *Fusarium*, *Rhizoctonia*, and *Colletotrichum* (Certis USA LLC, 2012).

2.5.2 Antimicrobial Biocontrol Product

Lactic Acid Bacteria blends are available commercially for the control of foodborne pathogens in foods. LactiGuard™, a commercially available LAB product produced by Nutrition Physiology Company, LLC, is used to control *Listeria*, *Salmonella*, *E. coli*, *Campylobacter*, and other pathogenic bacteria on raw whole and ground meat, meat and poultry carcasses, and ready-to-eat meat products (Nutrition Physiology Company, 2012). LactiGuard™ significantly suppressed the growth of *L. monocytogenes* in frankfurters (Koo et al., 2012), *Salmonella* in turkey and beef (Echeverry et al., 2010; Dow et al., 2011), and *E. coli* O157:H7 in beef (Echeverry et al., 2010). Because of the ability of LactiGuard™ to suppress Gram positive and Gram negative pathogen growth during storage, research into its application has been expanded to fresh produce.

The antimicrobial activity of LactiGuard™ against *E. coli* O157:H7 and *S. enterica* on spinach has recently been investigated. LactiGuard™ (applied at 7-8 log CFU/g) reduced both pathogen populations by 1-2 log CFU/g after 6 d storage at 4-7°C, although the magnitude of the difference was affected by pathogen inoculation level. After 6 d storage at 4-7°C, *E. coli* O157:H7 populations for all inoculation levels were between 5 and 6.3 log CFU/g on untreated spinach. LactiGuard™-treated spinach had lower levels of *E. coli* O157:H7; spinach inoculated with *E. coli* O157:H7 at 2, 4, and 6 log CFU/g had a 2.1, 0.7, and 1.43 log CFU/g difference in *E. coli* O157:H7 levels

between LactiGuard™-treated and untreated spinach (Brown et al., 2011; Cálix-Lara et al., 2014).

S. enterica at 2 and 4 log CFU/g inoculation levels followed a pattern similar to *E. coli* O157:H7. After 6 d storage at 7°C, *S. enterica* populations on untreated spinach were between 4 and 5 log CFU/g for both inoculation levels. There was a 0.7 and 1.9 log CFU/g difference in *S. enterica* between LactiGuard™-treated and untreated spinach, respectively (Cálix-Lara et al., 2014).

Although LactiGuard™ was able to suppress the growth of *E. coli* O157:H7 and *S. enterica* inoculated at low and high levels for 6 d at refrigeration temperatures, it did not maintain its antimicrobial activity over a longer storage period. At both pathogen inoculation levels, *E. coli* O157:H7 and *S. enterica* populations on untreated spinach were approximately 6 and 5 log CFU/g, respectively, after 12 d incubation at 7°C. Spinach inoculated with 2 log CFU/g pathogen had 0.3 log and 0.7 log less *E. coli* O157:H7 and *S. enterica*, respectively, on LactiGuard™-treated spinach than on untreated spinach. There was no statistical difference between LactiGuard™-treated and untreated spinach in *E. coli* O157:H7 or *S. enterica* populations at a 4 log CFU/g inoculation level (Cálix-Lara et al., 2014).

2.6 Conclusion

The consumption of fresh fruits and vegetables is increasing in the United States, and maintaining crop and consumer health is important in sustaining the growing

agricultural industry. With the increase in consumer demand for more natural products and the phasing out of chemical fumigants and pesticides, the need for an alternative or additional means of the microbial control of phytopathogens affecting crop health is needed. The incidence of foodborne disease outbreaks involving *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on fresh and fresh-cut produce has also demonstrated the need for a novel approach to pathogen management to ensure consumer safety. Biocontrol offers a possible supplement to conventional treatments in the management of plant and foodborne pathogens on produce.

A biologically-based approach to enhancing the safety of minimally processed fruits and vegetables is a novel way to exert control over undesirable microorganisms. Several species of bacteria produce compounds with antifungal and antimicrobial activity. The production of bioactive compounds such as bacteriocins, lipopeptides, and enzymes has been identified in many species of LAB, *Bacillus*, *Serratia*, *Ochrobactrum,* and *Oceanobacillus* and suppression of plant and foodborne pathogens has been observed both *in vitro* and on fresh produce. Application of these bioprotective species to fresh and fresh-cut produce has yielded promising but mixed results; the effect of these biocontrol bacteria on fungal phytopathogens, *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 is not only dependent on the bioprotective agent and the pathogen, but also on food matrix. Therefore, antifungal and antimicrobial efficacy must be evaluated for each bioprotective agent-pathogen-matrix combination.

3. ANTIFUNGAL SCREENING OF BIOPROTECTIVE ISOLATES AGAINST *BOTRYTIS CINEREA*, *FUSARIUM PALLIDOROSEUM*, AND *FUSARIUM MONILIFORME*

3.1 Introduction

Crop loss due to phytopathogenic microorganisms has a dramatic impact on the agricultural industry. *Botrytis cinerea* and *Fusarium* species are fungal phytopathogens that cause substantial losses across a wide variety of crops both before and after harvest. In the field, *Bot. cinerea*, the fungi responsible for the plant diseases gray mold and Botrytis rot, affects over 200 crop species, ranging from ornamentals to fruits and vegetables like lettuce, grapes, and strawberries (Elmer and Reglinski, 2006; Williamson et al., 2007). Fusarium Wilt and Fusarium Rot, caused by various *Fusarium* species, also affect a wide array of crops pre-harvest including lettuce, soybeans, strawberries, tomatoes, peppers, potatoes, and oranges (Miller et al., 1996; Díaz Arias et al., 2013; UC IPM, 2014a). Postharvest, *Bot. cinerea* and *Fusarium* species continue to contribute to commodity loss. *Bot. cinerea* causes the rapid decay of fresh produce because of its ability invade damaged, weak, or rapidly senescing tissue (Swartzberg et al., 2007), decreasing shelf life. *Fusarium* species are also problematic during postharvest storage, particularly in cereal grains and animal feeds (D'Mello et al., 1999). The mycotoxins fumonisin B_1 and B_2, trichothecenes, and zearalenone are produced by species of *Fusarium*, can cause disease in humans and animals (Peraica et al., 1999; Zinedine et al.,

2007). While there are many steps taken before and after harvest to mitigate the impact of these fungi, they are still problematic to the agricultural industry.

Management of fungal phytopathogen before harvest includes clearing fields of plant detritus, selecting locations without a history of fungal disease, using chemical fungicides (Williamson et al., 2007b; UC IPM, 2014a). Postharvest, the microbial load of fruits and vegetables is reduced by mechanical scrubbing and washing in disinfectants such as chlorine, and fungal growth is slowed by storing produce at low temperatures (Suslow, 1997; FDA, 2013b). Fumigation with fungicides and sulfur dioxide and irradiation are also used to control spoilage and phytopathogenic fungi after harvest (Smilanick et al., 1990; U.S. EPA, 2002, 2004; FDA, 2014b). The postharvest control of fungi in cereals and grains includes quick and efficient drying and maintaining a moisture content of less than 14%, storage in hygienic silos, and treatment with salts of propionic and sorbic acids (Magan and Aldred, 2007). Although the use of chemicals to control fungal phytopathogens before and after harvest is widespread, increases in fungicide resistance and health and environmental concerns associated with the use of harsh chemicals have created the need for an alternative means of treatment.

The use of bioprotective bacteria in an integrated pest management plan or as a postharvest treatment could help to protect crops against fungi and extend the shelf life of fresh produce. Several bacterial species across a range of genera produce compounds active against phytopathogenic fungi. Synthesis of the antifungal lipopeptides have been identified in species of *Bacillus*, including *B. amyloliquefaciens* (Romero et al., 2007; Arrebola et al., 2010; Zhao et al., 2014), *B. subtilis* (Vanittanakom and Leoffler, 1986; Moyne et al., 2001), and *B. thuringiensis* (Kim et al., 2004; Roy et al., 2013). Species of

LAB also produce antifungal compounds; these include organic acids, phenyllactic acid, 3-hydroxy fatty acids, and cyclic dipeptides (Strom et al., 2002; Magnusson et al., 2003; Ryan et al., 2011). The antifungal activity of bioprotective bacteria *in vitro* has translated into fungal suppression when applied both in the field and postharvest.

Bioprotective bacteria have shown antifungal efficacy when applied to crops before harvest. Species of *Bacillus* have reduced the incidence of *Fusarium* disease in maize and tomato plants when applied pre-harvest (Bressan and Figueiredo, 2010; Prabhukarthikeyan et al., 2014) and decreased fruit rot caused by *Bot. cinerea* on strawberry plants (Ilhan and Karabulut, 2013). Biocontrol agents have also suppressed fungal growth on fruits and vegetables when applied postharvest. Various species of *Bacillus* have been shown to reduce *Bot. cinerea* infection on pears, strawberries and tomatoes (Mari et al., 1996a; b; Donmez et al., 2011). *Lb. plantarum* suppressed *Bot. cinerea* and *F. graminearum* on cucumbers (Sathe et al., 2007), and *P. pentosaceus* suppressed the growth of *Penicillium expansum* on pears (Crowley et al., 2013). Fungal suppression by various bacterial species demonstrates their potential use as biocontrol agents for the control of fungal phytopathogens both before and after harvest. Several species of *Bacillus* are commercially available as pre-harvest biocontrol treatments for various fruits and vegetables; Serenade® Optimum utilizes *B. subtilis* (Bayer CropScience LP, 2013a), Sonata® contains *B. pumilus* (Bayer CropScience LP, 2013b), and DoubleNickel LC uses *B. amyloliquefaciens* as the active ingredient (Certis USA LLC, 2012).

The purpose of this study was to screen 22 bacterial isolates, which included *Bacillus* and LAB species, for antifungal activity against *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme*.

3.2 Materials and Methods

3.2.1 Antifungal Screening

3.2.1.1 Fungal Pathogens

All fungi were obtained from American Type Culture Collection (ATCC; Manassas, VA). Working cultures of *Botrytis cinerea* ATCC 46522, *Fusarium pallidoroseum* ATCC 48152, and *Fusarium moniliforme* ATCC 60846 (Table 3.1) were maintained on Potato Dextrose Agar (PDA, Becton, Dickson and Company; Sparks, MD) at 4°C and as fungal-colonized PDA plugs suspended in Potato Dextrose Broth (PDB, Becton, Dickson and Company; Sparks, MD) with 25% glycerol at -70°C for long term storage.

3.2.1.2 Bioprotective Isolates

All bioprotective isolates were provided by BiOWiSH Technologies (Cincinnati, OH). Working cultures of LAB isolates were maintained on de Man, Rogosa, Sharpe agar (MRS; Oxnoid; Basingstoke, Hampshire, UK) at 4°C and all other bioprotective isolates on Tryptic Soy Agar (TSA; Remel, Lenexa, KS) or TSA with 5% NaCl (w/w) at room temperature. For long term storage, isolates were kept in the appropriate growth media

(Table 3.1) with 15% glycerol at -70°C with the exception of *Bacillus clausii* and

Bacillus firmus—these two isolates were stored as spore crops at 6°C. *Bacillus subtilis*

fermentate were stored at 6°C. Prior to use in screening, LAB isolates were inoculated

into 10 mL of MRS Broth and incubated at 35°C for 18-24 h. All other isolates were

inoculated into 10 mL of Tryptic Soy Broth (TSB; Remel, Lenexa, KS) or TSB with 5%

NaCl (w/w) and incubated with agitation on an orbital shaker at either 30 or 35°C,

depending on the isolate, for 18-24 h.

3.2.1.3 Antifungal Screening

A loopful of prepared bioprotective cultures was spot-inoculated in two locations

on the surface of solidified media; the type of screening media was determined by the

growth requirements of the isolate (Table 3.1). Using a cork borer, a 9 mm plug was cut

from the perimeter of a working culture of fungal pathogen and placed in the center of the

isolate-inoculated plate. Plates were wrapped in Parafilm® and incubated at 24°C for 10

d. The diameter of the fungal colony between the two isolate inoculation locations was

measured daily, beginning on Day 3. A fungus-inoculated plate of each screening media

was used as a control. Percent fungal inhibition was determined using the following

equation:

$$\frac{\text{(control fungus diameter)-(isolate fungus diameter)}}{\text{(control fungus diameter)}} \times 100\% = \% \text{ inhibition}$$

Equation 1. Percent fungal inhibition

Table 3.1. Bioprotective isolates and fungal phytopathogens

Isolate	Growth medium	Incubation temperature	Screening medium
Bioprotective isolate			
Bacillus amyloliquefaciens BA1	TSB	35°C	PDA
Bacillus amyloliquefaciens BA2	TSB	35°C	PDA
Bacillus amyloliquefaciens BA3	TSB	35°C	PDA
Bacillus amyloliquefaciens BA4	TSB	35°C	PDA
Bacillus amyloliquefaciens BA5	TSB	35°C	PDA
Bacillus clausii	TSB	30°C	PDA
Bacillus coagulans	TSB	35°C	PDA
Bacillus firmus	TSB	30°C	TSA
Bacillus licheniformis	TSB	35°C	PDA
Bacillus megaterium	TSB	35°C	PDA
Bacillus pumilus	TSB	35°C	PDA
Bacillus sphaericus	TSB	30°C	PDA
Bacillus thiaminolyticus	TSB	30°C	TSA
Bacillus thuringiensis BT1	TSB	35°C	PDA
Bacillus thuringiensis BT2	TSB	35°C	PDA
Lactobacillus amylovorus	MRS	35°C	MRS
Lactobacillus plantarum	MRS	35°C	MRS
Oceanobacillus sojae	TSB w/ 5% NaCl (w/w)	35°C	TSA
Ochrobactrum anthropi	TSB	35°C	PDA
Pediococcus acidilactici	MRS	35°C	MRS
Pediococcus pentosaceus	MRS	35°C	MRS
Serratia plymuthica	TSB	30°C	PDA
Fungal phytopathogen			
Botrytis cinerea	PDA	24°C	N/A[1]
Fusarium pallidoroseum	PDA	24°C	N/A
Fusarium moniliforme	PDA	24°C	N/A

[1]N/A: not applicable

3.2.2 Biosurfactant Screening

3.2.2.1 Bioprotective Isolates

Bioprotective isolates were inoculated as previously described and incubated for 7 d; all isolates but LAB were incubated with agitation on an orbital shaker. After incubation, cultures were centrifuged at 3,000 RPM for 15 min and the supernatant was used in the biosurfactant screening.

3.2.2.2 Drop-Collapse Test

The drop collapse test, which was used to detect biosurfactant production, was adapted from Turgrul and Cansunar (2005). Briefly, the surface of a petri dish was coated with mineral oil and allowed to sit at room temperature for at least 1 h. A 20 µL aliquot of bioprotective culture supernatant was placed onto the oiled surface at a 45° angle and the diameter of the drop was measured after 2 min.

3.2.2.3 Statistical Analysis

The drop-collapse test was completed twice in duplicate. Data were analyzed using Analysis of Variance (ANOVA); analysis was completed with Minitab 16 (Minitab Inc., State College, PA). Dunnett's test was used to identify isolate drop diameters that were significantly larger than the diameter of the media control, which indicated biosurfactant production by the bioprotective isolates. An isolate was considered to be a biosurfactant producer if the diameter of the supernatant drop was significantly larger than the diameter of the media control ($\alpha = 0.05$).

3.3 Results and Discussion

3.3.1 Antifungal Activity

3.3.1.1 *Bacillus* Species

Bacillus species have been investigated as possible biocontrol agents because of their ability to synthesize antifungal compounds, including lipopeptides and other antibiotics. Several *Bacillus* species have demonstrated antifungal activity against fungal phytopathogens, including *Bot. cinerea* and/or *Fusarium* species, *in vitro* (Agarry et al., 2005; Tendulkar et al., 2007; Saidi et al., 2009; Gomaa, 2012; Wang et al., 2014). However, antifungal capability is a function of bacterial strain and fungal pathogen. In this study, eight of the *Bacillus* isolates suppressed the growth of *Bot. cinerea*, *F. pallidoroseum*, and *F. moniliforme* (Table 3.2, Figure 3.1, Appendix B); *Bacillus megaterium* was the most effective against all three fungi, followed by *Bacillus coagulans* and several strains of *B. amyloliquefaciens*. Fungal inhibition by these isolates ranged from 51-62% for *Bot. cinerea*, 60-69% for *F. pallidoroseum*, and 40-61% for *F. moniliforme* (Table 3.2). Three *Bacillus* isolates inhibited *Bot. cinerea* but did not suppress the growth of the *Fusarium* species tested. This screening identified eleven *Bacillus* isolates capable of fungal suppression *in vitro*; additional evaluation is required to determine the antifungal ability of these isolates when applied to produce.

Inhibition of fungal phytopathogens by *Bacillus* species has not only been observed *in vitro*, but also *in planta*. The application of *Bacillus* species pre-harvest reduced the incidence and severity of diseases caused by *F. oxysporum* (Saidi et al., 2009), *Alternaria solani*, and *Phytophthora infestans* (Chowdappa et al., 2013) on tomato

plants and *Bot. cinerea* fruit rot on strawberry plants (Ilhan and Karabulut, 2013).

Postharvest application of *Bacillus* species has also caused fungal suppression; decay was

reduced on pears, tomatoes, bananas, and oranges (Mari et al., 1996a; b; Alvindia and

Natsuaki, 2009; Arrebola et al., 2010). Further testing of the isolates used in this study,

including challenge studies on various types of produce, is needed to determine their

possible use as biocontrol agents.

Table 3.2. Fungal inhibition of bioprotective isolates against *Botrytis cinerea*, *Fusarium moniliforme,* and *Fusarium pallidoroseum* after 10 d incubation at 24°C

Bioprotective isolate	% inhibition		
	Botrytis cinerea	*Fusarium pallidoroseum*	*Fusarium moniliforme*
B. amyloliquefaciens BA1	52%	62%	53%
B. amyloliquefaciens BA2	56%	63%	60%
B. amyloliquefaciens BA3	58%	67%	61%
B. amyloliquefaciens BA4	60%	67%	60%
B. amyloliquefaciens BA5	61%	69%	61%
B. clausii	30%	N/I[1]	N/I
B. coagulans	62%	66%	59%
B. firmus	49%	N/I	N/I
B. licheniformis	N/I	N/I	N/I
B. megaterium	62%	68%	61%
B. pumilus	N/I	N/I	N/I
B. sphaericus	N/I	N/I	N/I
B. thiaminolyticus	50%	N/I	N/I
B. thuringiensis BT1	N/I	N/I	N/I
B. thuringiensis BT2	56%	68%	58%
Lb. amylovorus	N/I	N/I	N/I
Lb. plantarum	36%	N/I	N/I
O. sojae	N/I	N/I	N/I
O. anthropi	N/I	N/I	N/I
P. acidilactici	56%	N/I	N/I
P. pentosaceus	46%	N/I	N/I
S. plymuthica	51%	60%	40%

[1]N/I: no inhibition; fungal colony grew over/around bacterial isolate colony.
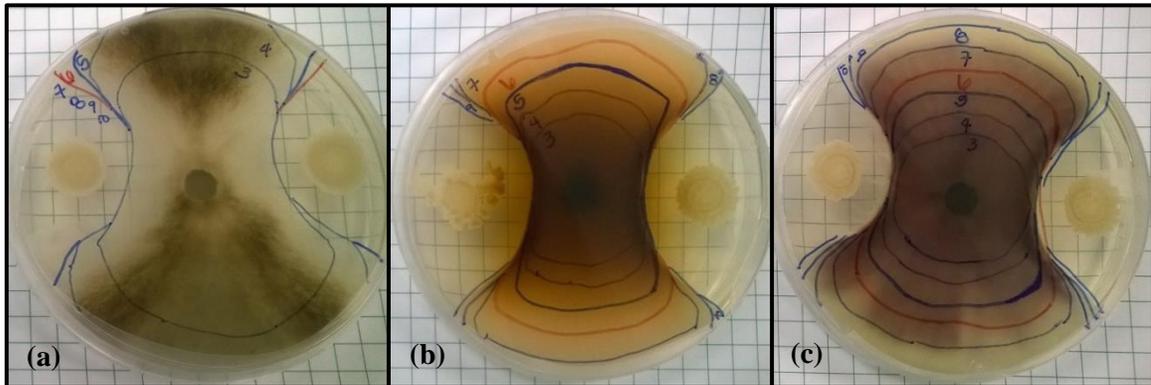
Figure 3.1. Antifungal screening of *Bacillus amyloliquefaciens* BA4 against *Botrytis cinerea* (a), *Fusarium pallidoroseum* (b), and *Fusarium moniliforme* (c). Markings indicate the border of the fungal colony each day of incubation, beginning on Day 3.

3.3.1.2 Lactic Acid Bacteria

Antifungal activity has also been detected from some LAB species; bioactive compounds produced by LAB include organic acids, phenyllactic acid, 3-hydroxy fatty acids, bacteriocins and bacteriocin-like substances, and cyclic dipeptides (Strom et al., 2002; Magnusson et al., 2003; Voulgari et al., 2010; Ryan et al., 2011; Gerez et al., 2013; Cortés-Zavaleta et al., 2014). Three of the four LAB isolates used in this study inhibited *Bot. cinerea*, but not *F. pallidoroseum* or *F. moniliforme* (Appendix B). *P. acidilactici* demonstrated the strongest antifungal activity, followed by *P. pentosaceus* and *Lb. plantarum*; inhibition was 56%, 46%, and 36% respectively (Table 3.2). Although these isolates were only effective against *Bot. cinerea*, other LAB species have demonstrated broad spectrum fungal inhibition when used as biocontrol agents on produce, but again efficacy varies between LAB and fungus species.

Pre- and postharvest application of LAB to agricultural crops has shown mixed results. *P. acidilactici* and *P. pentosaceus* decreased *Fusarium* infection on wheat seeds when germinated on PDA, but failed to significantly reduce disease incidence during greenhouse trials (Suproniene et al., 2014). In contrast, *Lactobacillus* species

45

administered to tomato plants as a seed treatment or soil drench have promoted plant growth and mitigated the impact of *F. oxysporum* in potted trials (Hamed et al., 2011). When applied as a postharvest treatment, LAB have reduced the decay caused by *Bot. cinerea* and *F. graminearum* on cucumbers (Sathe et al., 2007) and decay by *P. expansum* on apples (Trias et al., 2008b) and pears (Crowley et al., 2013). Again, additional investigation of the three antifungal LAB isolates identified in this study is needed to evaluate their use as biocontrol agents for the control of phytopathogenic fungi on produce.

3.3.1.3 *Serratia plymuthica*

Serratia plymuthica is another species of bacteria that has demonstrated suppression of fungal phytopathogens. Synthesis of antifungal compounds such as pyrrolnitrin, haterumalide, siderophores, chitinolytic enzymes, and proteases has been detected in strains of *S. plymuthica* (Kamensky et al., 2003; Levenfors et al., 2004). In this study, *S. plymuthica* suppressed the growth of *Bot. cinerea*, *F. pallidoroseum*, and *F. moniliforme* (Table 3.2), making it a possible candidate for additional evaluation. The use of other strains of *S. plymuthica* as a biocontrol agent both pre- and postharvest has been investigated. Foliar application of *S. plymuthica* reduced disease caused by *Bot. cinerea* and *Sclerotinia sclerotiorum* in cucumber plants (Kamensky et al., 2003) and postharvest treatment of potatoes decreased the severity of dry rot caused by *Fusarium sambucinum* (Gould et al., 2008).

3.3.1.4 Isolates with No Antifungal Activity

Antifungal activity is not only a function of bioprotective isolate and fungus species, it can also be affected by growth conditions. Culture conditions such as media composition, pH, and incubation period and temperature can affect the production of antifungal compounds (Mandal et al., 2008; Shih et al., 2008). In this study, four *Bacillus* isolates, *Lactobacillus amylovorus*, *Oceanobacillus sojae*, and *Ochrobactrum anthropi* did not suppress *Bot. cinerea*, *F. pallidoroseum*, or *F. moniliforme*. Although these seven isolates did not inhibit any of the fungi against which they were challenged, this does not mean these isolates are incapable of producing antifungal compounds. The culture media and testing conditions used in this study may not have been optimal for the synthesis of bioactive compounds; further investigation is required to determine if these seven isolates truly do not inhibit *Bot. cinerea*, *F. pallidoroseum*, or *F. moniliforme*.

3.3.2 Biosurfactant Activity

3.3.2.1 *Bacillus* Species, *Oceanobacillus sojae*, and *Ochrobactrum anthropi*

In addition to reducing the surface tension of water, biosurfactant lipopeptides produced by bacteria have demonstrated antifungal activity (Kalinovskaya et al., 2002; Huszcza and Burczyk, 2006; Arguelles-Arias et al., 2009; Troyano Pueyo et al., 2009; Romano et al., 2013; Chen et al., 2014). These bioactive lipopeptides, including surfactin, mycosubtilin, fengycin, and iturin, affect cell membranes, causing membrane disruption and cell leakage (Makovitzki and Shai, 2005). In this study, biosurfactant production was identified in seven *Bacillus* isolates (Figure 3.2). *B. megaterium*, *B. coagulans*, *B. thuringiensis* BT2 and several *B. amyloliquefaciens* isolates demonstrated both strong

biosurfactant activity and suppression of *Bot. cinerea*, *F. pallidoroseum*, and *F. moniliforme*, implying the ability to synthesize antifungal lipopeptides. The production of antifungal lipopeptides has been identified in other strains of the species of *Bacillus* used in this study, including *B. amyloliquefaciens* (Arrebola et al., 2010; Romano et al., 2013; Zhao et al., 2014), *B. coagulans* (Huszcza and Burczyk, 2006), *B. megaterium* (Troyano Pueyo et al., 2009), and *B. thuringiensis* (Hathout et al., 2000; Kim et al., 2004; Roy et al., 2013). *B. pumilus* and *O. sojae* demonstrated weak biosurfactant activity but no fungal suppression, suggesting that the biosurfactants secreted either did not have antifungal capabilities against the three fungi against which they were challenged or were not produced in large enough quantities to suppress fungal growth.

 Bacillus species also produce other compounds with antifungal activity including antibiotics (Schneider et al., 2007; Saidi et al., 2009; Arguelles-Arias et al., 2009; Yuan et al., 2012), proteinaceous substances (Tan et al., 2013; Wang et al., 2014), siderophores (Arguelles-Arias et al., 2009; Chaiharn et al., 2009), and chitinolytic enzymes (Aktuganov et al., 2003; Bressan and Figueiredo, 2010; Gomaa, 2012). In this study, five *Bacillus* isolates demonstrated antifungal activity, but biosurfactant secretion was not detected; this indicates either the production of antifungal lipopeptides at low levels or that antifungal activity was not due to synthesized lipopeptides, but some other bioactive compound. Biosurfactant production and antifungal activity was not detected in four *Bacillus* isolates and *O. anthropi*. The lack of both fungal inhibition and biosurfactant activity suggests that these strains did not secrete antifungal lipopeptides.
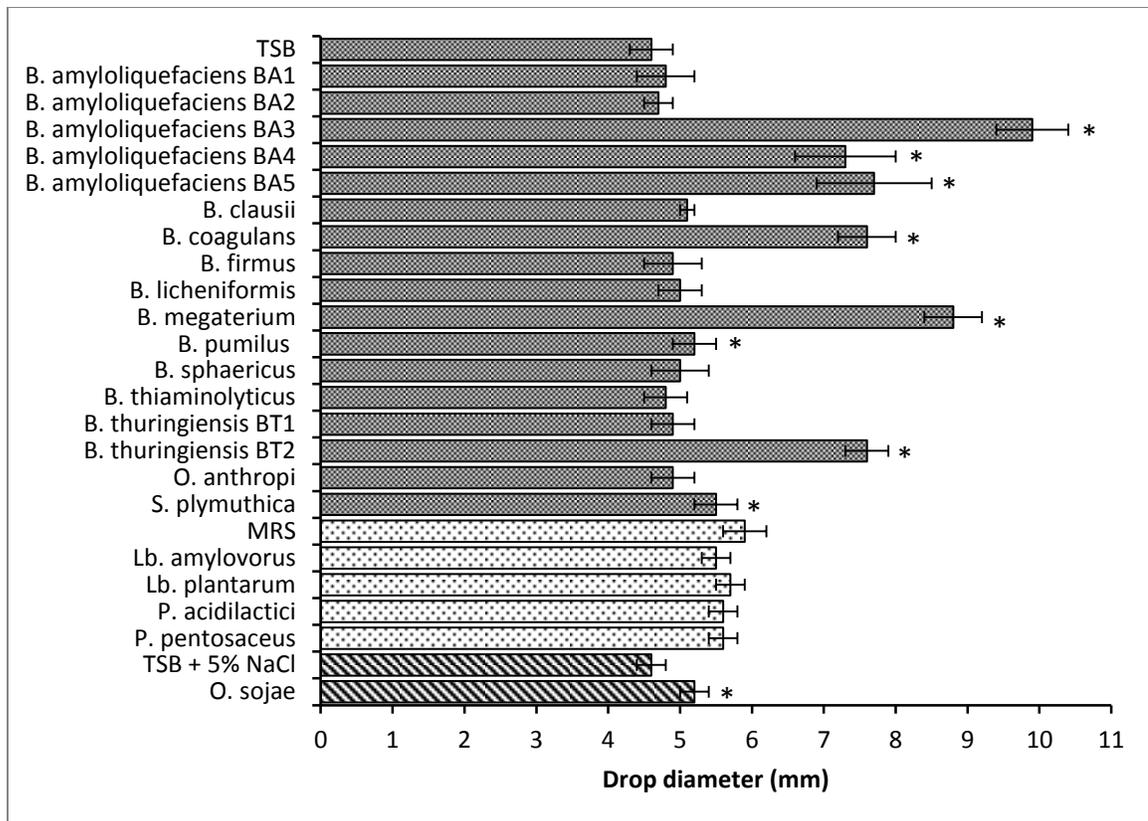
Figure 3.2. Biosurfactant activity of bioprotective isolates. Error bars indicate standard deviation. Media used are designated by bars with corresponding patterns. Asterisks indicate drop diameters significantly larger than that of the media control ($\alpha = 0.05$).

3.3.2.2 Lactic Acid Bacteria Isolates

Biosurfactant production was not detected in the four LAB isolates used in this study (Figure 3.2), but this does not mean these isolates are incapable of producing biosurfactants. Biosurfactants produced by bacteria can be either be excreted or remain cell-bound. Gudiña et al. (2011), found that *Lactobacillus coryniformis*, *Lactobacillus paracasei*, *Lb. plantarum*, and *Ln. mesenteroides* produced cell-bound biosurfactants at higher levels than excreted ones. Cell-bound biosurfactant production has been observed in several species of *Lactobacillus* (Gomaa, 2013a), including *Lb. plantarum* (Velraeds et al., 1996). The drop-collapse test used in this study to determine biosurfactant production was only able to detect excreted biosurfactants and not those which are cell-bound; LAB

49

isolates should be rescreened for the production of biosurfactants with the addition of an extraction step.

3.3.2.3 *Serratia plymuthica*

The production of antifungal compounds including pyrrolnitrin, haterumalide, siderophores, chitinolytic enzymes, and proteases has been identified in strains of *S. plymuthica*. The *S. plymuthica* isolate used in this study demonstrated weak biosurfactant activity (Figure 3.2) and suppressed the growth of *Bot. cinerea*, *F. pallidoroseum*, and *F. moniliforme* (Table 3.2). This suggests that antifungal activity was due to the production of antifungal lipopeptides at low levels, the synthesis of some other type of bioactive compound, or a combination of lipopeptides and antibiotics.

3.4 Conclusion

Of the 22 isolates tested, nine isolates—eight *Bacillus* isolates and *S. plymuthica*—suppressed the growth of *Bot. cinerea*, *F. pallidoroseum*, and *F. moniliforme* and six isolates—three *Bacillus* and three LAB—inhibited only *Bot. cinerea*. Biosurfactant production was detected in many of the *Bacillus* species which showed antifungal activity, suggesting the production of antifungal lipopeptides. Characterization of antifungal compounds produced by the bioprotective isolates used in this study and further investigation into their antifungal efficacy on produce both before and after harvest is needed to evaluate their potential use as biocontrol agents in the agricultural

industry. Based on the results of this screening, six isolates—*B. amyloliquefaciens* strains

BA3, BA4, and BA5, *B. coagulans*, *B. megaterium*, and *B. thuringiensis* BT2—are

recommended for further evaluation.

4. ANTIMICROBIAL SCREENING OF BIOPROTECTIVE ISOLATES AGAINST

LISTERIA MONOCYTOGENES, *SALMONELLA*, AND *ESCHERICHIA COLI* O157:H7

AND ANTIMICROBIAL EFFICACY ON ICEBERG LETTUCE AND GALA APPLES

4.1 Introduction

Foodborne illness affects an estimated 48 million Americans annually. Between 2006 and 2008, approximately 22% of foodborne illness outbreaks were produce-associated (Gould et al., 2013b). Major outbreaks in produce involving *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 have resulted in hundreds of illnesses and several deaths. In 2014 an outbreak of *L. monocytogenes* on prepackaged caramel apples produced by Bidart Bros. Apples caused 35 illnesses and 7 deaths (CDC, 2015) and *L. monocytogenes* on cantaloupes from Jensen Farms resulted in 147 cases of listeriosis and 34 deaths in 2011 (CDC, 2012a). An outbreak of *Salmonella enterica* ser. Saintpaul in peppers and tomatoes from Mexico in 2008 caused 1,442 illnesses and 2 deaths in the United States (Jungk et al., 2008). More recently, outbreaks of *Salmonella* have been linked to cucumbers, mangos, cantaloupe, and papayas (CDC, 2011b, 2012c; d, 2013a). Shiga-toxin producing *E. coli* outbreaks have been associated with sprouts, ready-to-eat salads, and Romaine lettuce (CDC, 2012e, 2013b, 2014), but one of the largest produce-associated *E. coli* O157:H7 outbreaks occurred in 2006 in spinach, which sickened 199 people and caused 3 deaths (CDC, 2006). Because of the devastating effects

of foodborne illness outbreaks, multiple steps are taken to ensure the safety of agricultural commodities.

The postharvest processing of fresh produce can be challenging because of the nature of the product. Many fruits and vegetables are consumed raw, and thus the use of high temperatures to reduce microbial load is not an option. Fresh produce manufacturers must use a series of less harsh treatments to control microbial populations and growth during postharvest processing, transport, and storage. Storage at low temperatures throughout postharvest processing and the distribution chain is the most common method used for slowing microbial growth; minimum growth temperatures for *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 are -0.4, 5.2, and 6.5°C, respectively (FDA, 2011b). The FDA requires ready-to-eat fruits and vegetables to be stored at or below 5°C (FDA, 2013a), however *L. monocytogenes* can grow at and below this low temperature and temperature abuse or deviations during processing, storage, and transport can also lead to the growth of *Salmonella* and *E. coli* O157:H7.

In addition to temperature control, produce is often washed in a disinfectant to reduce microbial populations. Chlorine is often used because it is cheap with broad-spectrum antimicrobial activity; studies have shown that washing leafy greens with chlorine causes close to or less than a 1 log reduction in *L. monocytogenes* (Beuchat and Brackets, 1990; Zhang and Farber, 1996), *Salmonella* (Weissinger et al., 2000; Neal et al., 2012), and *E. coli* O157:H7 (Behrsing et al., 2000; Keskinen et al., 2009; Keskinen and Annous, 2011; Neal et al., 2012). Peroxyacetic acid is another disinfectant used by the produce industry, however it is more expensive than chlorine and has not been shown to be more effective in reducing the microbial load of produce (Ölmez and Kretzschmar,

2009; University of Georgia, 2011; Neal et al., 2012). The methods used by the agricultural industry to reduce and control the microbial populations on produce have been shown to be insufficient, as evidenced by an increase in foodborne illness linked to produce, and therefore new or additional steps in postharvest processing are needed to enhance the safety of fruits and vegetables.

Biocontrol, which is the use of bioprotective organisms (such as non-pathogenic bacteria) to control undesirable organisms, offers an additional means of microbial management. The mechanisms employed by biocontrol agents for the suppression of undesirable microorganisms include antibiosis, predation of the target organism, and competitive inhibition (Singh and Vyas, 2009). Isolated compounds produced by biocontrol agents can be used to suppress the target organism (antibiosis), but when the whole organism is used one or more modes of action may be engaged to suppress the growth of the target organism, which may result in more effective inhibition.

Various species of bacteria, such as LAB, have been evaluated for the potential use as biocontrol agents on fresh produce. In addition to organic acids, some species of LAB produce bacteriocins, which are small, proteinaceous compounds active against organisms closely related to the producer; this typically includes Gram positive organisms, such as *L. monocytogenes* (O'Sullivan et al., 2002; Delves-Broughton, 2005; Rodríguez et al., 2005; Mills et al., 2011). Antimicrobial activity against Gram negative pathogens is generally not as strong, but can be enhanced with the addition of membrane-disrupting compounds such as weak acids and chelators (Alakomi et al., 2000; Lu et al., 2011).

Species of *Bacillus* also synthesize antimicrobial compounds, including bacteriocins and bacteriocin-like substances, lipopeptides, and other antibiotics. Bacteriocins produced by *Bacillus* species are similar in function to those produced by LAB. Lipopeptides are biosurfactants consisting of a cyclic peptide chain attached to a fatty acid; these compounds exert antimicrobial activity through membrane disruption (Arguelles-Arias et al., 2009; Sansinenea and Ortiz, 2011; Mongkolthanaruk, 2012; Christie, 2014). Broad-spectrum activity by *Bacillus*-synthesized lipopeptides includes the inhibition of *L. monocytogenes* (Sabaté and Audisio, 2013; Compaoré et al., 2013), *Salmonella* (Huang et al., 2011; Compaoré et al., 2013), *E. coli* (Compaoré et al., 2013), and both enveloped and non-enveloped viruses (Kracht et al., 1999; Huang et al., 2006). Other antibiotics produced by *Bacillus* species include macrolactin, difficidin, and bacillaene (Chen et al., 2006; Schneider et al., 2007; Sohn et al., 2008; Arguelles-Arias et al., 2009). The identification of antimicrobial compounds synthesized by LAB and *Bacillus* species has prompted investigation into their use as biocontrol agents on fresh produce.

Antimicrobial activity on fruits and vegetables is dependent on biocontrol agent strain and target pathogen, as well as produce type. Inhibition by LAB of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on produce can range from a 2 log reduction to complete inhibition on lettuce, apple wedges, and spinach (Trias et al., 2008a; Alegre et al., 2011; Cálix-Lara et al., 2014) and a 3 log reduction by *Bacillus* species on bell peppers (Liao, 2009). Antimicrobial efficacy of LAB and *Bacillus* strains on fruits and vegetables has led to further investigation into their use as an additional hurdle for foodborne pathogens on fresh produce.

55

The purpose of this study was 1) to screen 22 bacterial isolates for antimicrobial activity against *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7, 2) to evaluate antimicrobial efficacy of the three most inhibitory LAB isolates against *L. monocytogenes* on Iceberg lettuce, and 3) to determine the antimicrobial efficacy of DF1, a dry product containing the three LAB isolates, against *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on Gala apples.

4.2 Materials and Methods

4.2.1 Antimicrobial Screening

4.2.1.1 Bacterial Pathogen Preparation

Five strains each of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 (Table 4.1) were used in this study. Bacterial cultures were maintained on TSA slants at room temperature and in TSB with 15% glycerol at -70°C for long term storage. Cultures were prepared for screening by placing a loopful of culture into 10 mL of TSB and incubating for 24 h at 35°C. After incubation, strains were combined by organism to form five-strain cocktails of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7.

Table 4.1. Bacterial pathogens

Pathogen	Strain number	Source[1]
Listeria monocytogenes	NFPA 6301	NFL
Listeria monocytogenes	NFPA 6306	NFL
Listeria monocytogenes	NRRL B-33000	ARS
Listeria monocytogenes	NRRL B-33233	ARS
Listeria monocytogenes	FSL J1-177	ILSI NA
Salmonella enteriditis ser. Enteriditis	NFPA 7100	NFL
Salmonella enteriditis ser. Oranienburg	NFPA 7201	NFL
Salmonella enteriditis ser. Tennessee	FSL R8-5221	ILSI NA
Salmonella enteriditis ser. Montevideo	ATCC BAA 710	NFL
Salmonella enteriditis ser. Antaum	ATCC BAA 1592	NFL
Escherichia coli O157:H7	NFPA 4200	NFL
Escherichia coli O157:H7	NFPA 4213	NFL
Escherichia coli O157:H7	NFPA 4212	NFL
Escherichia coli O157:H7	NFPA 4216	NFL
Escherichia coli O157:H7	NFPA 4217	NFL

[1]ARS: Agricultural Research Service (College Park, MD); ILSI NA: International Life Sciences Institute, North America (Cornell University; Ithaca, NY); NFL: National Food Lab (Livermore, CA).

4.2.1.2 Bioprotective Isolate and *Bacillus subtilis* Fermentate Preparation

All bioprotective isolates were provided by BiOWiSH Technologies (Cincinnati, OH). Working cultures of LAB isolates were stored on MRS agar at 4°C. Working cultures of all other isolates were stored on TSA at room temperature; *Oceanobacillus sojae* was maintained on TSA with 5% NaCl (w/w), and the remaining isolates were maintained on TSA. All isolates were kept in the proper growth media (Table 4.2) with 15% glycerol at -70°C for long term storage except *B. clausii* and *B. firmus*—these two isolates were stored as spore crops at 6°C. *B. subtilis* fermentate were stored at 6°C. Prior to use, isolates were inoculated into 10 mL of the appropriate growth media and incubated at either 30 or 35°C, depending on the isolate, for 18-24 h (Table 4.2). All isolates but LAB were incubated with agitation on an orbital shaker.

Table 4.2. Bioprotective isolates

Isolate	Growth medium	Incubation temperature
Bacillus amyloliquefaciens BA1	TSB	35°C
Bacillus amyloliquefaciens BA2	TSB	35°C
Bacillus amyloliquefaciens BA3	TSB	35°C
Bacillus amyloliquefaciens BA4	TSB	35°C
Bacillus amyloliquefaciens BA5	TSB	35°C
Bacillus clausii	TSB	30°C
Bacillus coagulans	TSB	35°C
Bacillus firmus	TSB	30°C
Bacillus licheniformis	TSB	35°C
Bacillus megaterium	TSB	35°C
Bacillus pumilus	TSB	35°C
Bacillus sphaericus	TSB	30°C
Bacillus thiaminolyticus	TSB	30°C
Bacillus thuringiensis BT1	TSB	35°C
Bacillus thuringiensis BT2	TSB	35°C
Lactobacillus amylovorus	MRS broth	35°C
Lactobacillus plantarum	MRS broth	35°C
Oceanobacillus sojae	TSB w/ 5% NaCl	35°C
Ochrobactrum anthropi	TSB	35°C
Pediococcus acidilactici	MRS broth	35°C
Pediococcus pentosaceus	MRS broth	35°C
Serratia plymuthica	TSB	30°C

4.2.1.3 Lactic Acid Bacteria Cocktail Preparation

The LAB cocktails LM1, NP1B, and FGA, provided by BiOWiSH Technologies (Cincinnati, OH) contained *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus* combined with other components (Table 4.3). The LAB cocktails were stored in heat-sealed bags at room temperature. Prior to screening, the cocktails were rehydrated in sterile DI water (target concentration of 7 log CFU/mL), then diluted to a target concentration of 6 log CFU/mL with either sterile DI water or MRS broth. Cocktails rehydrated in DI water were immediately screened for antimicrobial activity and cocktails in MRS broth were incubated at 35°C for 18-24 h then screened.

Table 4.3. Lactic Acid Bacteria cocktail formulation components

Formulation	Log CFU/g	Other components
LM1	9.60	Anhydrous dextrose
NP1B	10.23	MRS fermentation media
FGA	9.11	Rice flour, whey protein, inulin, salt, calcium carbonate, magnesium sulfate, sodium phosphate, ferrous sulfate
DF1	10.31	Soy peptone, yeast extract, soy protein, dextrose

4.2.1.4 Antimicrobial Screening of Bioprotective Isolates

Lactic Acid Bacteria isolates and cocktails were screened using a seeded-overlay method; all other isolates were screened using a pour-plate method. Antimicrobial activity was determined by the presence of a zone of inhibition. Each organism was screened twice in triplicate.

Seeded Overlay Method. A loopful of prepared LAB culture or cocktail was spotted onto MRS agar and incubated at 35°C for 24 h. The plates were then overlaid with TSA seeded with pathogen cocktail (6 log CFU/mL). Plates were incubated at 35°C for 24 h then the zone of inhibition was measured from the border of the bacterial colony to the perimeter of the clearing.

Pour Plate method. A pathogen-seeded plate was prepared by placing 1 mL of pathogen cocktail (6 log CFU/mL) into a petri dish, then adding 15-20 mL molten TSA tempered to 50°C. When the TSA had solidified, a loopful of prepared bioprotective isolate culture was spotted onto the agar in three separate locations. All plates were incubated at 35°C for 24 h then the zone of inhibition was measured from the border of the bacterial colony to the perimeter of the clearing.

4.2.2 Effect of pH on Antimicrobial Activity of Lactic Acid Bacteria Isolates

4.2.2.1 Bacterial Pathogen Preparation

Cocktails of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 were prepared as described in Section 4.2.1.1. Cocktails were centrifuged at 3,000 RPM for 15 min then resuspended in fresh TSB. Pathogen cocktail concentrations were adjusted to 4 log CFU/mL with TSB.

4.2.2.2 Lactic Acid Bacteria Isolate Cell-Free Supernatant and Neutralized Cell-Free Supernatant Preparation

Lb. plantarum, *P. acidilactici*, and *P. pentosaceus* were individually inoculated into 10 mL of MRS broth and incubated at 35°C for 48 h. After incubation, LAB cultures were centrifuged, then the supernatant was filter-sterilized using 0.22 µm syringe filters (Thermo Fisher Scientific, Inc.; Waltham, MA) to make cell-free supernatant (CFS). The pH of a portion of CFS was adjusted to 6.5-7.0 with 1 M NaOH (Thermo Fisher Scientific, Inc.; Waltham, MA) then filter-sterilized again to make neutralized cell-free supernatant (NCFS).

4.2.2.3 Effect of pH on the Antimicrobial Activity of Lactic Acid Bacteria Cell-Free Supernatant

A Bio-Rad XMark™ spectrophotometer (Hercules, CA) was used to measure the optical density (OD) of pathogen cocktails in the presence of LAB isolate CFS and NCFS. In a 96 well microplate, 100 µL of pathogen cocktail was combined with 100 µL of either LAB isolate CFS or NCFS. Pathogen cocktail mixed with MRS broth was used

as a control. Samples were incubated at 25°C and OD was measured at 600 nm every hour for 48 h.

4.2.2.4 Statistical Analysis

The experiment was conducted three times in duplicate. All statistical analyses were conducted with MiniTab 16 (Minitab Inc., State College, PA) software. Data were log-transformed, then analyzed using Analysis of Variance (ANOVA); Tukey's test was used to identify significant differences between treatments ($\alpha = 0.05$).

4.2.3 Application on Produce: Effect of Lactic Acid Bacteria Cocktail on *Listeria monocytogenes* on Iceberg Lettuce

4.2.3.1 Iceberg Lettuce Preparation

Whole heads of Iceberg lettuce were purchased from a local grocery store (Albertsons; San Luis Obispo, CA) and stored at refrigeration temperatures for no more than 24 h prior to use. In a biosafety cabinet, the four outermost leaves of the lettuce head were removed and the head was cut into quarters with a flame-sterilized chef's knife. The lettuce leaves were separated, portioned into15 g samples and arranged in a single layer, outer surface facing up, in a biosafety cabinet.

4.2.3.2 *Listeria monocytogenes* Inoculation of Iceberg Lettuce

L. monocytogenes cocktail was prepared as described in Section 4.2.1.1. *L. monocytogenes* cocktail was washed twice and resuspended in 0.1% peptone water (PW, Becton, Dickinson and Company; Sparks, MD). *L. monocytogenes* inoculum was

adjusted to 4.7 log CFU/mL with 0.1% PW. Each 15 g Iceberg lettuce sample was inoculated with 15 spots of 20 µL of prepared *L. monocytogenes* inoculum (resulting in 3 log CFU/g lettuce) and allowed to dry at room temperature for 1 h in a biosafety cabinet.

4.2.3.3 Lactic Acid Bacteria Cocktail Treatment of Iceberg Lettuce

Lb. plantarum, *P. acidilactici*, and *P. pentosaceus* were each individually inoculated into 240 mL MRS broth and incubated at 35°C for 18-24 h. After incubation, LAB cultures were washed twice and resuspended in 80 mL of 0.1% PW, then combined in an ethanol-sterilized spray bottle to form the LAB cocktail. Each 15 g lettuce sample was sprayed with approximately 1 mL LAB cocktail and allowed to dry for 1 h. Lettuce samples were then placed in Whirl-Pak® bags and stored at 4°C for 24 h, then 10°C for 13 d (14 d incubation total). Untreated lettuce was used as a control.

4.2.3.4 Bacterial Enumeration

The experiment was conducted twice. *L. monocytogenes* and LAB cocktail populations were enumerated in duplicate on Days 0, 1, 3, 7, 10, and 14. To prepare Iceberg lettuce samples, 135 g of 0.1% PW was added to the lettuce sample and then homogenized in a stomacher for 60 s. The sample was plated on Modified Oxford Agar with antimicrobic supplement (MOX; Becton, Dickson and Company; Sparks, MD) to recover *L. monocytogenes* and MRS agar to recover LAB cocktail. Serial dilutions were prepared as needed with 0.1% PW. All plates were incubated at 35°C for 48 h.

4.2.3.5 Statistical Analysis

The experiment was conducted twice in duplicate. All statistical analyses were conducted with MiniTab 16 (Minitab Inc., State College, PA) software. Data were log-transformed, then analyzed using Analysis of Variance (ANOVA); Tukey's test was used to identify significant differences between treatments ($\alpha = 0.05$).

4.2.4 Application on Produce: Effect of DF1 on Iceberg Lettuce

4.2.4.1 DF1 Solution Preparation

DF1, a powder that contained *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus* and various dried media (Table 4.3), was provided by BiOWiSH Technologies (Cincinnati, OH). DF1 was stored at refrigeration temperatures until use. Immediately before application, 6 g DF1 was mixed with 20 g DI water (23% DF1 solution, by weight). The DF1 solution was transferred to an ethanol-sterilized plastic spray bottle for lettuce application.

4.2.4.2 DF1 Treatment of Iceberg Lettuce

Whole heads of Iceberg lettuce were purchased from a local grocery store (Albertsons; San Luis Obispo, CA) and stored at refrigeration temperatures for no more than 24 h prior to use. Before DF1 treatment, the four outermost leaves of the lettuce head were removed and the head was cut into 5x5 cm pieces with a flame-sterilized chef's knife in a biosafety cabinet. Lettuce pieces were arranged in a single layer in a biosafety cabinet and sprayed with DF1 solution until evenly coated. Lettuce was then dried in a residential salad spinner (OXO; New York, NY), divided into 15 g samples and

placed into Whirl-Pak® bags. Lettuce samples were incubated at 10°C for 2 d and visually evaluated daily.

4.2.4.3 Bacterial Enumeration

Lactic Acid Bacteria populations were enumerated on Days 0 and 2. Lettuce samples were prepared as described in Section 4.2.3.4. Samples were plated on MRS agar to recover LAB; all plates were incubated at 35°C for 48 h.

4.2.5 Application on Produce: Effect of DF1 on *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 on Gala Apples

4.2.5.1 Bacterial Pathogen Preparation

Cocktails of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 were prepared as described in Section 4.2.1.1. Cocktails were washed twice and resuspended with 0.1% PW.

4.2.5.2 Pathogen Inoculation of Gala Apples

Organic unwaxed Gala apples were purchased from a local grocery store (Albertsons; San Luis Obispo, CA) and held at refrigeration temperatures for no more than 48 h prior to use. Apples were spot-inoculated with 20 µL of pathogen cocktail (8-9 log CFU/mL) and allowed to sit in a biosafety cabinet for 2 h at room temperature. The pathogen-inoculated apples were then stored at 4°C overnight to allow for bacterial attachment.

4.2.5.3 DF1 Treatment of Gala Apples

Immediately before application, a 20% DF1 solution was prepared by combining 25 g DF1 with 100 g DI water. The DF1 solution was then transferred to an ethanol-sterilized spray bottle. The pathogen-inoculated apples were arranged in a single layer in a biosafety cabinet and each sprayed with approximately 3 mL of DF1 at the pathogen inoculation sites. DI water was sprayed onto apples as a control. The apples remained in the biosafety cabinet for 2 h at room temperature to dry, then were incubated at 20°C for 5 d.

4.2.5.4 Bacterial Enumeration

Bacterial populations were enumerated in duplicate on Days 0, 1, 2, 3, and 5. To prepare the apple sample, a plug 12 mm in diameter was cut from the apple at the pathogen inoculation site with a cork borer. The plug was placed into a Whirl-Pak® bag containing 9 mL 0.1% PW and homogenized by hand for 1 min, then serially diluted with 0.1% PW for plating. The native microflora and LAB populations were enumerated using TSA and MRS agar, respectively. An overlay method adapted from Al-Holy et al. (2008) was used for the recovery of stressed or injured *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7. Briefly, apple samples were pour-plated in TSA and incubated at 35°C for 2-3 h to allow stressed and injured cells to repair themselves. Plates were then overlaid with selective/differential media and incubated at 35°C for 22-46 h, depending on the requirements of the media. The selective/differential media used to enumerate pathogen populations were MOX for *L. monocytogenes*, Xylose Lysine Deoxycholate Agar (XLD;

Oxnoid; Basingstoke, Hampshire, UK) for *Salmonella*, and Sorbitol MacConkey Agar (SMAC; Oxnoid; Basingstoke, Hampshire, UK) for *E. coli* O157:H7.

4.3 Results and Discussion

4.3.1 Antimicrobial Screening

4.3.1.1 Appearance of Zones of Inhibition During Antimicrobial Screening

The size and appearance of the zone of inhibition produced in the antimicrobial screening could be correlated to the efficacy of the antimicrobial compounds produced by the bioprotective isolate. When the LAB isolates and cocktails were screened, the clearings produced were large and visually free of pathogen growth (Figure 4.1, Appendix C), indicating strong inhibition of *L. monocytogenes*, *Salmonella*, and *E. coli* O15:H7. This suggests that the antimicrobial compounds produced by the LAB isolates were strongly inhibitory to Gram positive and Gram negative pathogens.

However, during the antimicrobial screening of the non-LAB isolates, the appearance of the zones of inhibition were small compared to those produced by the LAB isolates and differed between Gram positive and Gram negative pathogens. Clearings were visually free of *L. monocytogenes* colonies across all inhibitory isolates and *B. subtilis* fermentate, but those clearings produced when testing against *Salmonella* and *E. coli* O157:H7 were hazy and contained pathogen colonies; the colonies were smaller and less dense within the clearing (Figure 4.1, Appendix C). The difference in the appearance of the clearings of Gram positive *L. monocytogenes* and Gram negative *Salmonella* and

E. coli O157:H7 suggests that the presence of an outer membrane reduces the efficacy of

the antimicrobial compounds produced by the bioprotective isolates.

The difference in size between the zones of inhibition of the LAB isolates and

non-LAB isolates could have been the result of the difference in testing protocols. When

screening the LAB isolates, cultures were incubated for 24 h on MRS agar plates before

pathogen inoculation, providing time for the isolates grow and produce antimicrobial

substances before the introduction of the pathogen. In the screening of non-LAB isolates,

isolate and pathogen cultures were inoculated on the same day. The non-LAB isolates

were not able to grow for 24 h unchallenged as the LAB isolates were, and the presence

of the pathogen during isolate growth may have affected antibiotic production.
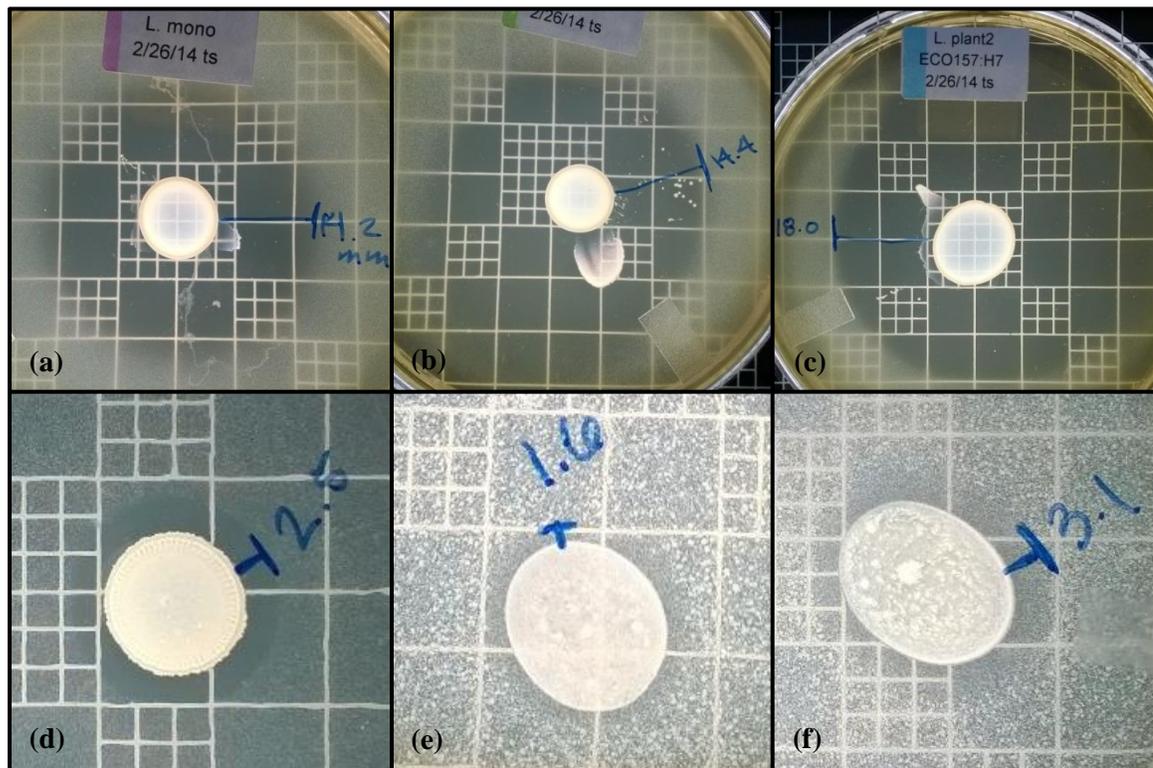
Figure 4.1. Antimicrobial screening of (top row) *Lactobacillus plantarum* and (bottom row) *Bacillus amyloliquefaciens* BA3 against (from left to right) *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7. Handwritten numbers indicate zone size in mm.

4.3.1.2 Lactic Acid Bacteria Isolates and Cocktails

Several species of LAB synthesize antimicrobial compounds, including organic acids, bacteriocins, and other antibiotics (Bhunia et al., 1988; Diep et al., 2006; Mandal et al., 2008; Xie et al., 2011). Lactic acid—a major metabolite of all LAB species—exerts microbial inhibition by reducing environmental pH and disrupting bacterial membranes (Alakomi et al., 2000). Bacteriocins produced by LAB typically inhibit Gram positive organisms, but efficacy against Gram negative organisms can be increased with the addition of membrane disruptors (Cobo Molinos et al., 2008). Therefore, a bacteriocin-producing strain of LAB could potentially inhibit both Gram positive and Gram negative organisms. In this study, three of the four LAB isolates and all three LAB cocktails suppressed *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7. *Lb. plantarum* isolate demonstrated the strongest inhibition of all three pathogens (Figure 4.2, Appendix C). NP1B produced the largest clearings of the LAB cocktails when rehydrated in either DI water or MRS broth (Table 4.4, Appendix C). Inhibition of both Gram positive and Gram negative pathogens suggests that the organic acids produced by the LAB contributed to their antimicrobial efficacy. The role of organic acids in the antimicrobial ability of these LAB isolates was further investigated in this study, but more testing is needed to evaluate bacteriocin production by *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus* isolates used in this study. Based on the results of the screening, *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus* were selected for further evaluation.
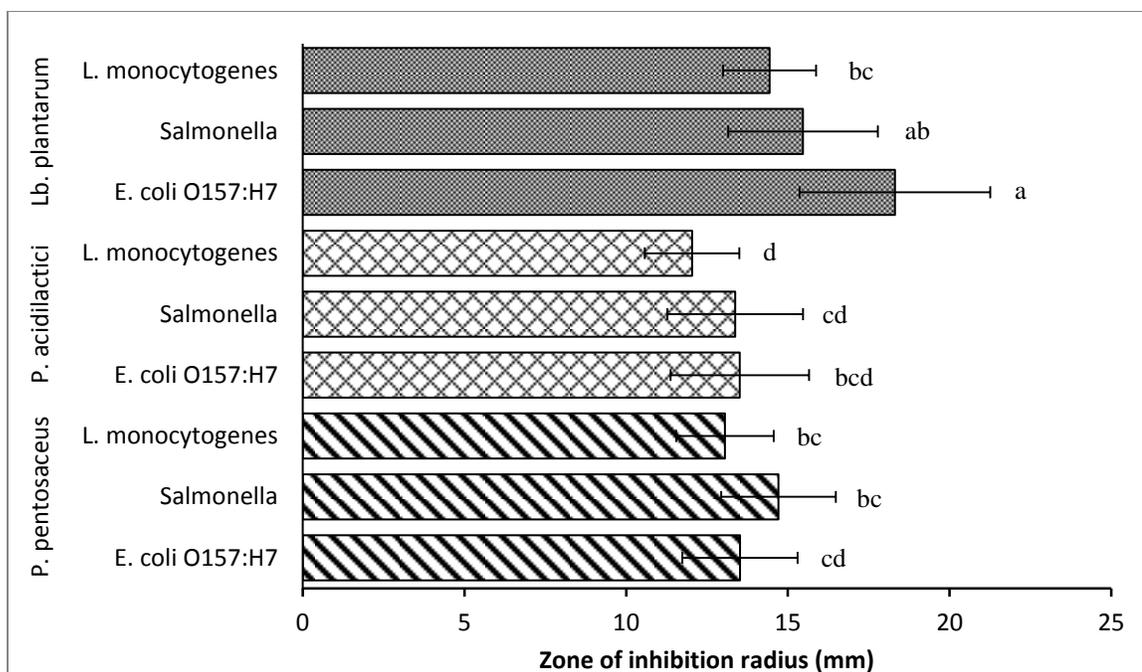
Figure 4.2. Antimicrobial activity of Lactic Acid Bacteria isolates. LAB isolates are differentiated by pattern. Error bars indicate standard error of the mean. Those results not sharing a letter are statistically different ($\alpha = 0.05$).

Table 4.4. Antimicrobial activity of Lactic Acid Bacteria cocktails

Cocktail	Bacterial pathogens		
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
FGA-DI	+	+	+
FGA-MRS	++	++	++
LM1-DI	+	+	+
LM1-MRS	++	++	++
NP1B-DI	+	+	+
NP1B-MRS	+++	++++	++++

(+): 0.1-12.0 mm zone; (++): 12.1-14.0 mm zone; (+++): 14.1-16.0 mm zone; (++++): ≥ 16.1 mm zone. DI: DI water sample; MRS: MRS sample.

4.3.1.3 *Bacillus* Isolates, *Serratia plymuthica*, and *Bacillus subtilis* Fermentate

Several bacteria produce various types of antimicrobial compounds and lipopeptides are among the bioactive compounds produced by some species of *Bacillus*. *Bacillus*-synthesized lipopeptides have shown antimicrobial activity against *L. monocytogenes*, *Salmonella*, and *E. coli* (Huang et al., 2011; Gomaa, 2013b; Płaza et al.,

69

2013; Sabaté and Audisio, 2013). Lipopeptide production in the *Bacillus* isolates and *S. plymuthica* used in this study was discussed in Chapter III; all isolates that produced biosurfactants—with the exception of *O. sojae*—also demonstrated antimicrobial activity. *B. amyloliquefaciens* BA3 and *B. megaterium*, the two isolates that demonstrated strong biosurfactant activity, also inhibited *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 (Table 4.5). Pathogen inhibition and biosurfactant activity implies the production of antimicrobial lipopeptides by some of the *Bacillus* isolates used in this study.

In addition to lipopeptide synthesis, some *Bacillus* species are able to produce bacteriocins and bacteriocin-like compounds and other antibiotics. Similar to bacteriocins produced by LAB, inhibition by *Bacillus*-synthesized bacteriocins is typically seen in Gram positive organisms (Martirani et al., 2002; Gray et al., 2006), including *L. monocytogenes* (Lisboa et al., 2006; Kamoun et al., 2011; Berić et al., 2013), with enhanced activity against Gram negative organisms with the addition of a membrane disruptor (Bizani et al., 2005). Other antibiotics synthesized by some *Bacillus* species include polyketides such as macrolactin, bacillaene, and difficidin (Zimmerman et al., 1987; Chen et al., 2006; Schneider et al., 2007; Sohn et al., 2008; Yuan et al., 2012). In this study, antimicrobial activity was observed from *B. amyloliquefaciens* BA1 and BA2 and *B. licheniformis*, but biosurfactant production was not detected; this suggests the synthesis of antimicrobial compounds other than lipopeptides by these isolates.

Table 4.5. Antimicrobial activity of isolates and fermentates

Organism	Bacterial Pathogens		
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
Isolates			
B. amyloliquefaciens BA1	+++	++	++
B. amyloliquefaciens BA2	++++	N/I	+
B. amyloliquefaciens BA3	++	++	+++
B. amyloliquefaciens BA4	++	+	++
B. amyloliquefaciens BA5	+	N/I	+
B. clausii	N/I	N/I	N/I
B. coagulans	++	++	++
B. firmus	N/I	N/I	N/I
B. licheniformis	++	N/I	N/I
B. megaterium	+++	++	++
B. pumilus	+	N/I	N/I
B. sphaericus	N/I	N/I	N/I
B. thiaminolyticus	N/I	N/I	N/I
B. thuringiensis BT1	N/I	N/I	N/I
B. thuringiensis BT2	+++	+	++
O. anthropi	N/I	N/I	N/I
O. sojae	N/I	N/I	N/I
S. plymuthica	+	N/I	+
Fermentates			
B. subtilis fermentate	+	+	+

N/I: no inhibition; (+): 0.1-1.0 mm zone; (++): 1.1-2.0 mm zone; (+++): 2.1-3.0 mm zone; (++++): ≥ 3.0 mm zone.

4.3.2 pH Neutralization

4.3.2.1 Optical Density

In this experiment, the OD, which is a measure of turbidity, was used to observe the growth of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 in the presence of *Lb. plantarum*, *P. acidilactici*, or *P. pentosaceus* CFS (with a pH of 3.81-4.27, depending on LAB isolate) or NCFS (pH of 6.5 -7.0) over a 48 h period. An increase in OD correlated to an increase in microbial population, and so the impact of the organic acids produced by the isolates on pathogen growth was seen by comparing the OD of CFS, NCFS, and

pathogen controls. Although other antimicrobial compounds may have been produced by the LAB isolates, their detection was beyond the capabilities of the testing protocol; this will be addressed further in this discussion.

The experiment was conducted three times and, in some trials, the results were not as expected. The pathogen control should have an OD higher than CFS and NCFS because the MRS used in the controls to take the place of CFS and NCFS was fresh and thus had a higher nutrient content than CFS and NCFS, both of which had been used to grow LAB for 48 h. In some trials, the OD of the NCFS was significantly higher than that of the pathogen control ($\alpha = 0.05$), indicating some type of contamination (Appendix D). Those trials where the OD of NCFS was significantly higher than the pathogen control were not included in the final statistical analysis.

4.3.2.2 The Role of Organic Acids Produced by Lactic Acid Bactria Isolates in the Inhibition of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7

Lactic acid, a major metabolite of LAB, has antimicrobial potential. Organic acids exert antimicrobial activity by decreasing extracellular pH and acidifying the internal environment of the cell, disrupting of metabolic reactions and stressing intracellular homeostasis (Brul and Coote, 1999). The pH growth limits for *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 are 4.4, 3.7, and 4.0 respectively (FDA, 2011b). In this study, the CFS of *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus*, with a pH of 3.81 ± 0.05, 4.27 ± 0.13, and 4.10 ± 0.13 respectively, greatly inhibited the growth of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7; OD did not increase over the 48 h incubation period (Figures 4.3, 4.4, and 4.5). The pH of the CFS of all three LAB isolates

were near or below the lower growth limits of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 and capable of suppressing the growth of these pathogens. When neutralized, the antimicrobial activity of the CFS of the three LAB isolates was greatly diminished. After 48 h incubation, OD of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 cultured in the NCFS of *Lb. plantarum*, *P. acidilactici*, or *P. pentosaceus* were significantly higher than when grown in CFS ($\alpha = 0.05$), illustrating the role of lactic acid in the inhibition of pathogen growth.

The impact of organic acids and low pH was clearly demonstrated in this experiment, but pathogen suppression could also be the result of antimicrobial compounds other than organic acids. The synthesis of bacteriocins and other antibiotics by LAB species was discussed in Section 4.3.1.2. Neutralizing the CFS of the LAB isolates removes the effect of organic acids on pathogen growth, and an OD from NCFS lower than the pathogen control OD would suggest the presence of other antimicrobial compounds. However, the protocol used in this experiment was not sufficient to identify the production of other antimicrobial compounds by the LAB isolates.

To prepare the pathogen controls, fresh MRS was combined with the pathogen inoculum. The NCFS used had been incubating the LAB isolates for 48 h, and thus would have had lower levels of the nutrients needed to sustain growth. Therefore, lower OD from NCFS could have been the result of lower nutrient levels in the media or the presence of antimicrobial compounds. In this study, The NCFS of *P. pentosaceus* caused a small but significant reduction of 0.025 in the OD of *L. monocytogenes* compared to the pathogen control ($\alpha = 0.05$) (Figure 4.5); *Lb. plantarum* and *P. acidilactici* NCFS did not affect *L. monocytogenes* OD. The OD of *Salmonella* was significantly lower in the

73

presence of the NCFS of all three LAB, with the NCFS of *P. pentosaceus* causing the

largest difference of 0.453 (Figure 4.4). Only the NCFS of *P. acidilactici* caused a

significant decrease in the OD of *E. coli* O157:H7 ($\alpha = 0.05$); OD was 0.243 lower than

the control (Figure 4.4). Because of the limitations of the testing protocol used in this

experiment, the results obtained could not conclusively determine the production of

antimicrobial compounds other than lactic acid by *Lb. plantarum*, *P. acidilactici*, or *P.*

pentosaceus, and further analysis of the isolates and their metabolites is needed to

identify the production of bacteriocins or other antibiotics.
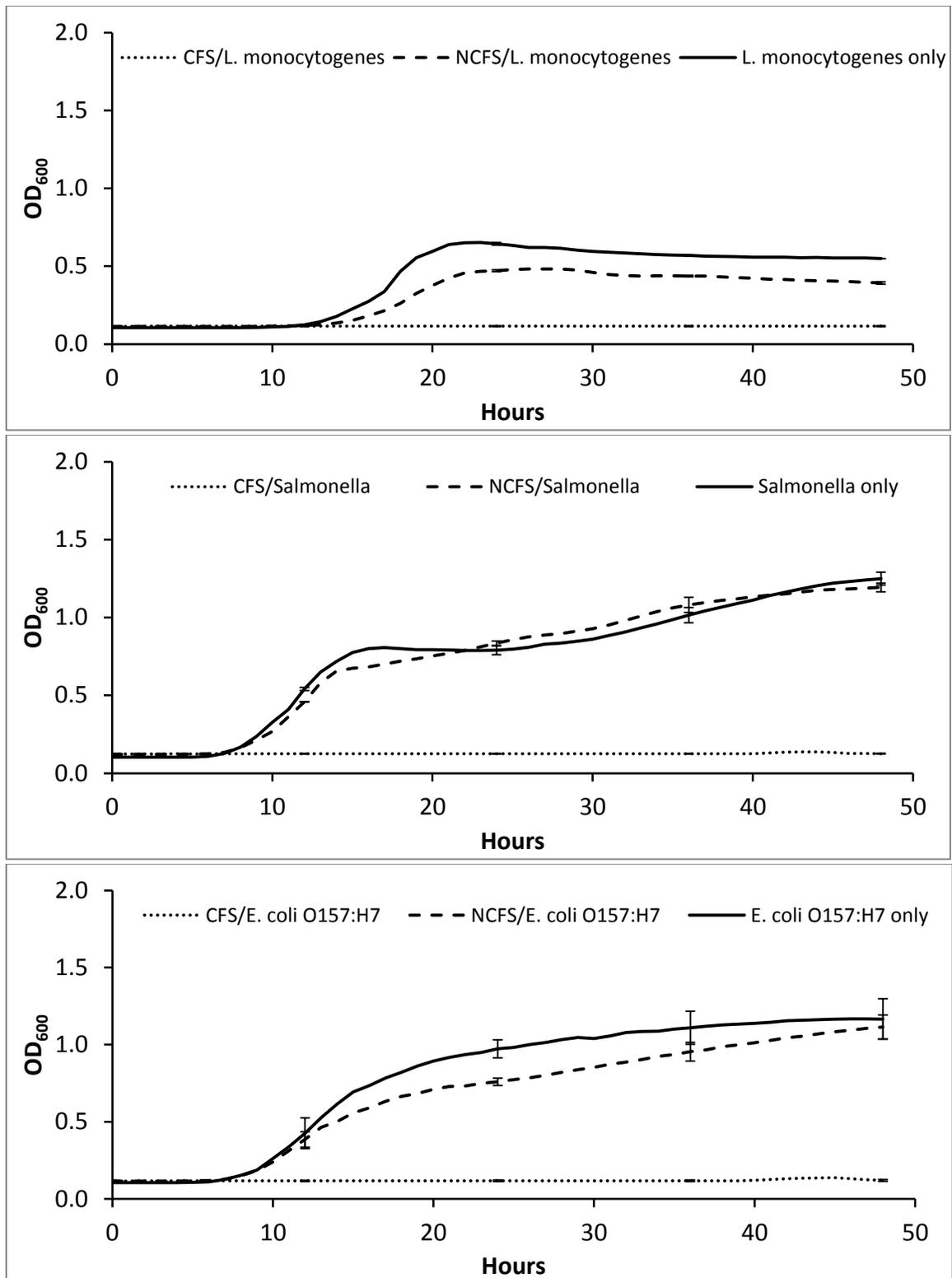
Figure 4.3. Optical density of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 grown in *Lactobacillus plantarum* CFS and NCFS. Error bars indicate standard error of the mean.
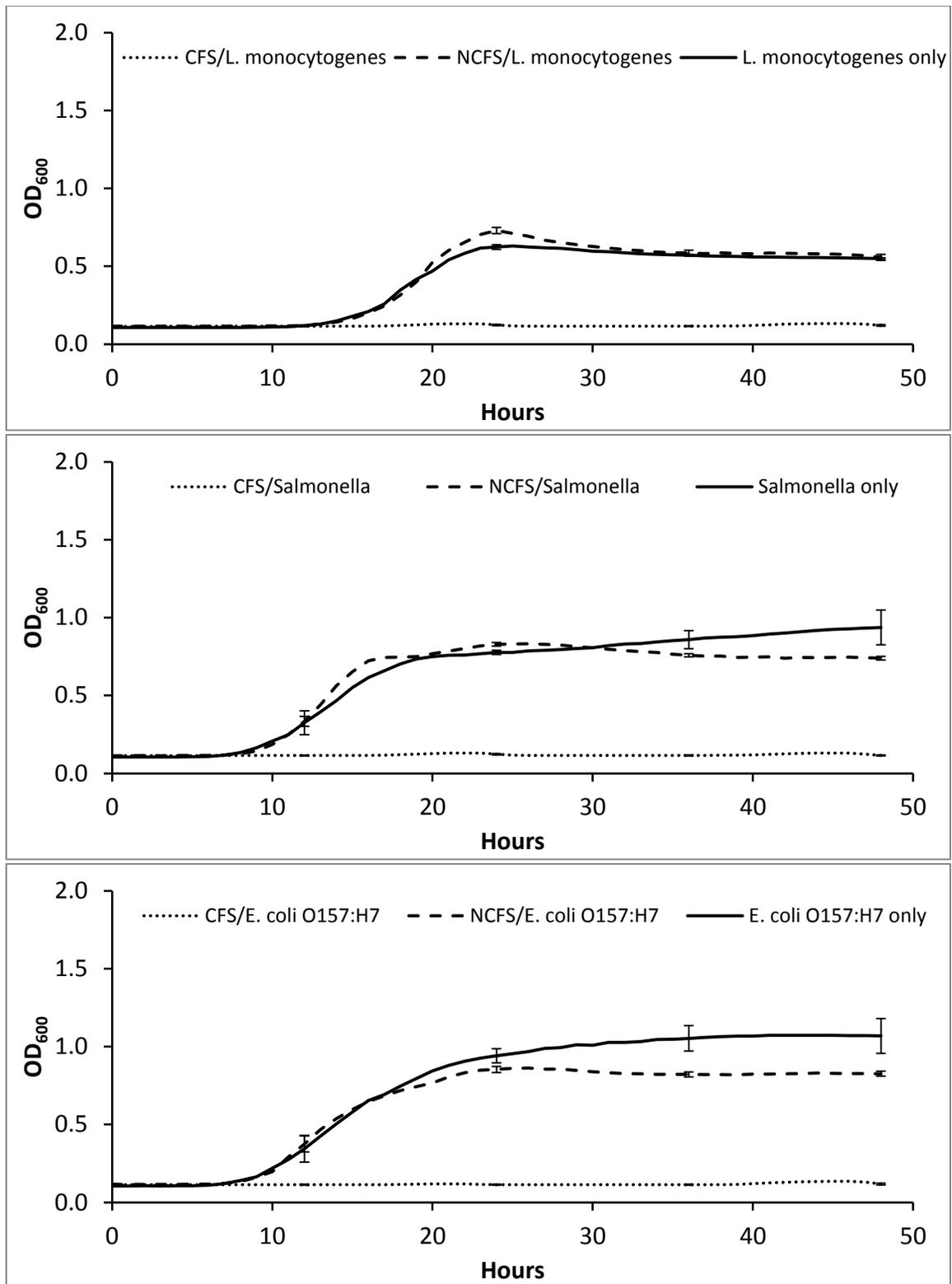
Figure 4.4. Optical density of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 grown in *Pediococcus acidilactici* CFS and NCFS. Error bars indicate standard error of the mean.
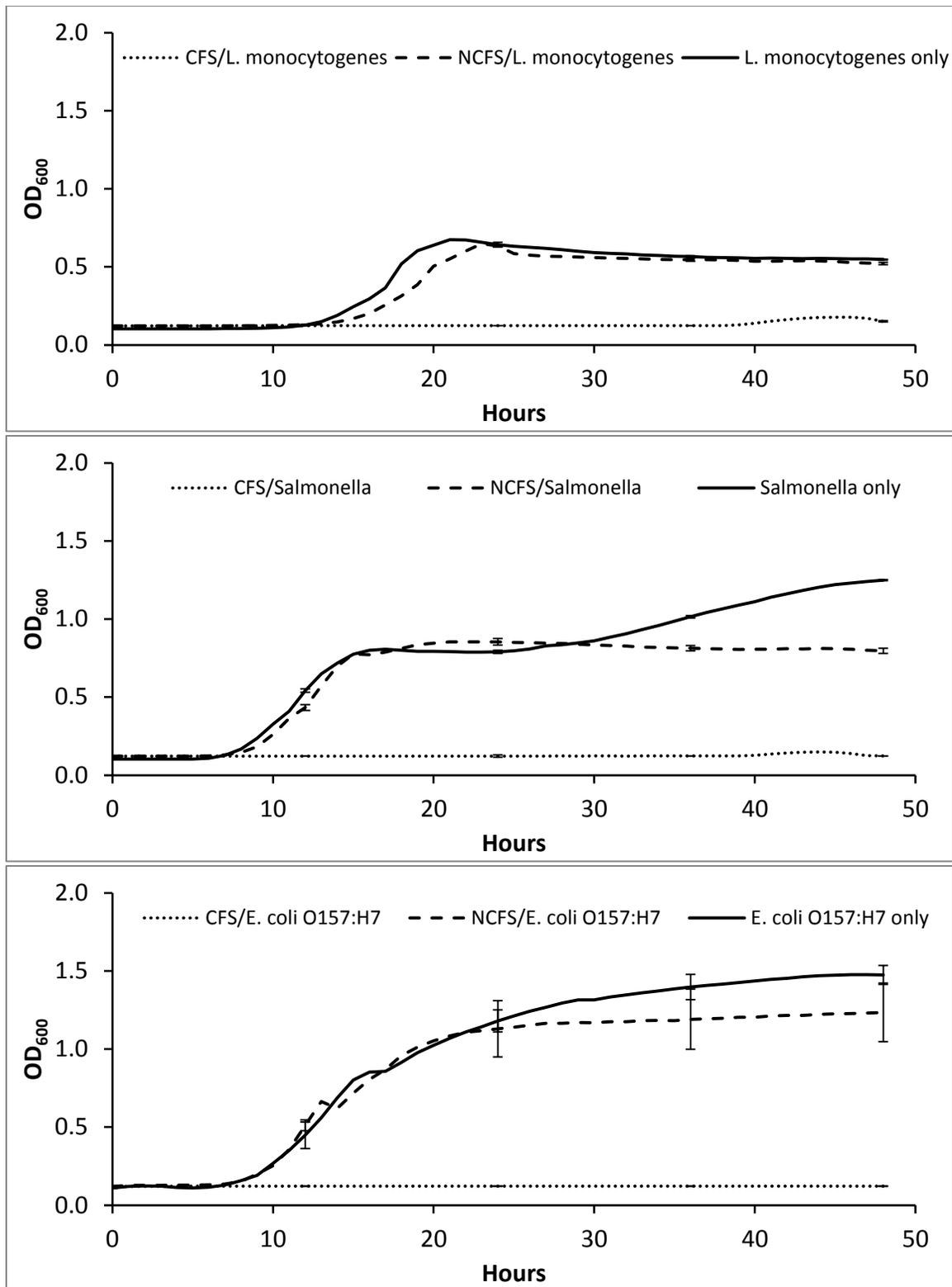
Figure 4.5. Optical density of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 grown in *Pediococcus pentosaceus* CFS and NCFS. Error bars indicate standard error of the mean.

4.3.3 Application on Produce

4.3.3.1 Effect of Lactic Acid Bacteria Cocktail on *Listeria monocytogenes* on Iceberg Lettuce

Lactic Acid Bacteria have long been used in the production of fermented foods such as cheese, yogurt, and sauerkraut. Their use as biocontrol agents on fresh produce is of interest to the food industry because of their GRAS status and ability to synthesize antimicrobial compounds. This preliminary experiment was used to evaluate the capability of the LAB isolates used in this study to translate *in vitro* pathogen inhibition to the suppression of pathogen growth on produce stored at refrigeration temperatures and to assess LAB survival.

When applied to Iceberg lettuce, the LAB cocktail suppressed the growth of *L. monocytogenes*; levels were significantly lower on LAB-treated lettuce ($\alpha = 0.05$) and remained lower throughout the incubation period (Figure 4.6a). After 14 d, *L. monocytogenes* populations were 1.84 log lower on LAB-treated lettuce. These results were as expected; efficacy against *L. monocytogenes* varies between produce type and LAB species. Application of bioprotective LAB species has resulted in a 1 log reduction to complete inhibition of *L. monocytogenes* on apple wedges (Trias et al., 2008a; Alegre et al., 2011) and a half log reduction to complete inhibition on Iceberg lettuce (Allende et al., 2007; Trias et al., 2008a). The LAB used in this study suppressed the growth of *L. monocytogenes* and LAB levels remained unchanged throughout the 14 d incubation period (Figure 4.6b), therefore testing with these isolates continued.
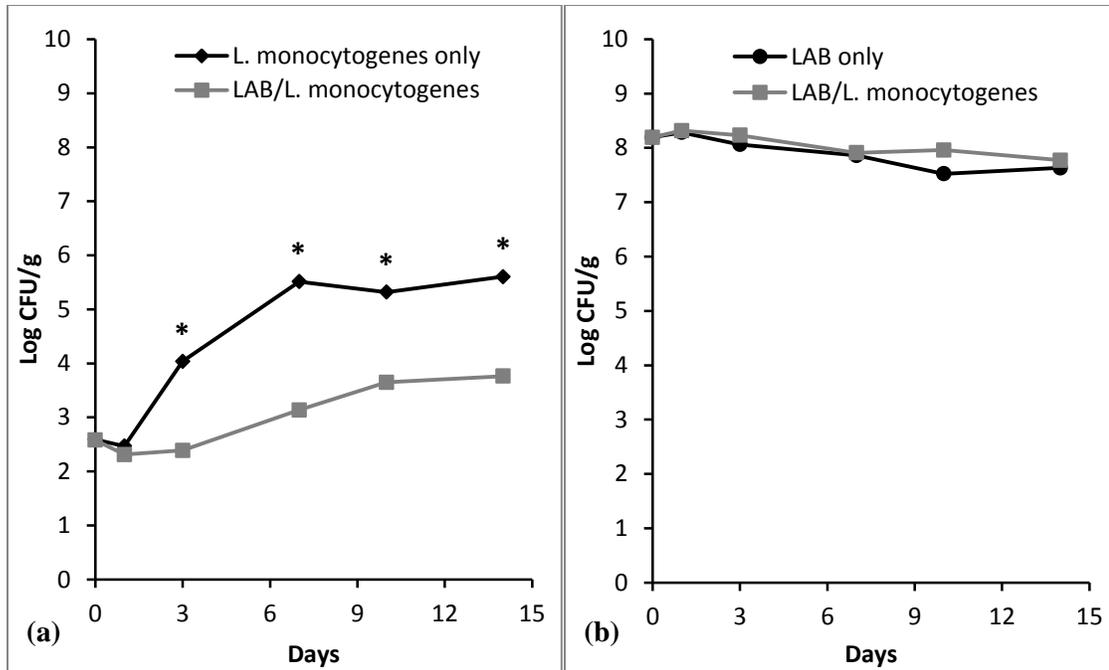
Figure 4.6. *Listeria monocytogenes* (a) and Lactic Acid Bacteria populations (b) on Iceberg lettuce treated with Lactic Acid Bacteria cocktail after 14 d incubation at 10°C. Asterisks indicate microbial populations which are significantly different on each sample day ($\alpha = 0.05$).

4.3.3.2 The Effect of DF1 Treatment on the Appearance of Iceberg Lettuce

In order to be an effective antimicrobial treatment for food products, the method must not only control microbial populations, but also not have a deleterious effect of the product's shelf life or sensory characteristics. The DF1 formulation was selected for testing on produce because it contained the three LAB isolates used in the previous Iceberg lettuce experiment. The purpose of this experiment was to evaluate the effects of DF1, a powder containing *Lb. plantarum*, *P. acidilactici*, *P. pentosaceus*, and other media (Table 4.3), on the sensory characteristics on chopped Iceberg lettuce during refrigerated storage and to determine LAB survival under those conditions.

Figure 4.7. Iceberg lettuce treated with DF1 after 2 d incubation at 10°C.

As in the previous experiment on Iceberg lettuce, LAB population levels did not change over the 2 d incubation period. However, DF1 solution was brown and cloudy when mixed with DI water; it left a faint brown tint on the lettuce after spin-drying. After 2 d incubation at 10°C, DF1-treated lettuce had substantial browning (Figure 4.7). No browning was observed in untreated lettuce or lettuce treated with LAB cocktail in the previous experiment with Iceberg lettuce after 2 d, indicating that media components in DF1 were most likely the cause the browning. Damaging the lettuce leaves by cutting them could have allowed for the uptake of DF1 into the vascular system of the lettuce, also contributing to the rapid color change. Because the application of DF1 left a brown tint on lettuce and caused browning after only 2 d incubation, it was deemed unacceptable for use on light-colored, chopped produce. Gala apples were chosen for further evaluation of DF1 because of their dark color and because apples are commonly stored and consumed as whole fruit.

4.3.3.3 Effect of DF1 on *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 on Gala Apples

4.3.3.3.1 The Fate of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 on the Surface of Gala Apples

The recent outbreak of *L. monocytogenes* in caramel apples has focused a spotlight onto the survival of pathogenic bacteria on apples. In this experiment, *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 levels were monitored over a total of 6 d; the pathogen-inoculated apples were incubated at 4°C for the first 24 h, then at 20°C for the remaining 5 d. *L. monocytogenes* and *Salmonella* did not survive well on the surface of Gala apples over the 6 d incubation period. The populations of *L. monocytogenes* and *Salmonella* decreased by 2 and 3 log, respectively, when incubated at 4°C overnight, then rapidly decreased over 5 d at 20°C to below detection levels. *E. coli* O157:H7 was more robust than *L. monocytogenes* or *Salmonella*. After a decrease of approximately 3 log after 24 h, *E. coli* O157:H7 levels remained around 3 log CFU/plug for the duration of the incubation period (Table 4.6). The rapid decrease in pathogen populations was most likely caused by the harsh conditions on the surface of the apple, where water and nutrients were not available in abundance. Apple peels also contain antimicrobial compounds such as polyphenols. Polyphenols extracted from apple peels have demonstrated inhibition of *E. coli* (Alberto et al., 2006; Fratianni et al., 2007), *S. aureus*, and *L. monocytogenes* (Alberto et al., 2006). The rapid decline in pathogen population was most likely the result of the severe surface conditions and antimicrobial compounds within the apple's peel.

Table 4.6. *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 on Gala apples treated with DF1 or DI water

Treatment	Day (log CFU/plug)[1]					
	Inoculation	0	1	2	3	5
L. monocytogenes only	6.91/6.99 (6.95)	4.80/5.06 (4.95)	3.29/2.60 (3.07)[2]	3.05/3.01 (3.03)	3.64/2.98 (3.43)	0.70/< 1 (0.40)[2]
L. monocytogenes/ H_2O	--	2.70/< 3 (2.40)[2]	1.18/1.18 (1.18)[2]	< 1/< 1 (< 1)	1.00/< 1 (0.70)[2]	0.70/0.70 (0.70)[2]
L. monocytogenes/ DF1	--	5.29/5.41 (5.36)	5.10/< 1 (4.80)	4.82/5.09 (4.98)	4.63/4.44 (4.55)	4.73/5.26 (5.07)
Salmonella only	6.02/6.14 (6.09)	3.30/2.70 (3.05)[2]	0.70/< 1 (0.40)[2]	< 1/< 1 (< 1)	0.70/< 1 (0.40)[2]	< 1/< 1 (< 1)
Salmonella/H_2O	--	< 3/< 3 (< 3)	< 1/< 1 (< 1)	< 1/< 1 (< 1)	< 1/< 1 (< 1)	< 1/< 1 (< 1)
Salmonella/DF1	--	2.70/< 3 (2.40)[2]	3.13/< 1 (2.83)	2.71/< 1 (2.41)	< 1/< 1 (< 1)	3.95/< 1 (3.35)
E. coli O157:H7 only	6.44/6.81 (6.66)	3.54/3.81 (3.70)[2]	3.25/1.65 (2.96)	3.01/3.31 (3.23)	3.05/1.30 (2.75)	2.79/3.24 (3.07)
E. coli O157:H7/ H_2O	--	< 3/< 3 (< 3)	1.00/< 1 (0.70)[2]	< 1/< 1 (< 1)	< 1/< 1 (< 1)	2.43/< 1 2.13
E. coli O157:H7/ DF1	--	< 3/< 3 (< 3)	2.51/3.26 (3.03)	2.64/< 1 (2.34)	1.18/1.90 (1.68)[2]	4.86/< 1 (4.56)

(--): not applicable.
[1]Counts for each of the samples tested; average of the two counts in parentheses.
[2]Estimated counts.

4.3.3.3.2 The Effect of DF1 on *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 on Gala Apples

The use of LAB on various fruits and vegetables for the suppression of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 has yielded mixed results; inhibition has ranged from 1 log to complete depending on LAB isolate, pathogen, and produce type (Trias et al., 2008a; Gragg and Brashears, 2010; Alegre et al., 2011; Cálix-Lara et

al., 2014). In this study, the DF1 formulation was chosen for evaluation because it contained *Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus*—the three LAB strains that demonstrated antimicrobial activity during *in vitro* screening. However, the application of DF1 allowed for the survival of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on Gala apples; no treatment at all and treating the apples with DI water were more effective in reducing pathogen populations. After 5 d incubation at 20°C, *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 levels on DF1-treated apples were approximately 4, 2, and 2 log higher than levels on apples treated with DI water, respectively(Table 4.6). The LAB from DF1 also survived well; levels on DF1-treated apples remained consistent throughout incubation (Figure 4.8). The media components and dextrose in DF1 could have provided nutrients to the pathogens and LAB, aiding in their survival on the harsh surface of the apple.
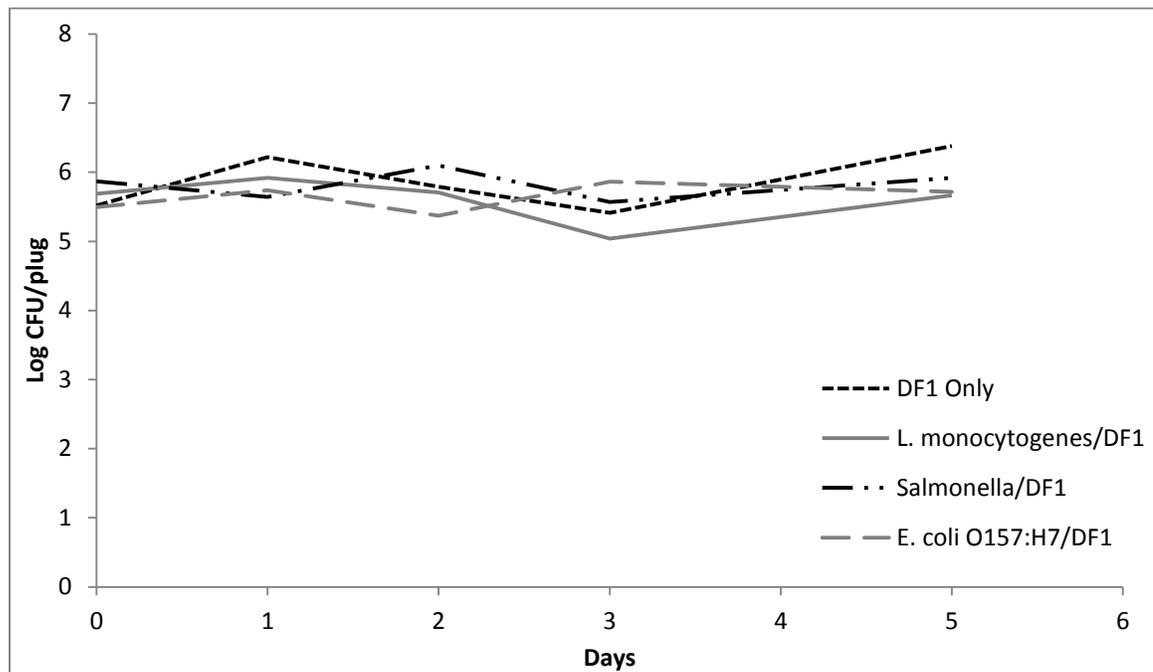
Figure 4.8. LAB population on DF1-treated Gala apples incubated at 20°C for 5 d.

4.3.3.3.3 The Effect of DF1 on the Appearance of Gala Apples

      In addition to aiding in pathogen survival, DF1 also negatively affected the appearance and feel of the apples. When DF1 was sprayed onto the apples it beaded up, leaving a brown, tacky residue on the surface (Figure 4.9). The tackiness was most likely the result of the dextrose present in the formulation. Based on the results of this experiment, DF1 is not the appropriate formulation for the biocontrol of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on fresh produce because its application promotes the growth of these pathogens and is detrimental to the sensory characteristics of the product.

Figure 4.9. Gala apple treated with DF1.

4.4 Conclusion

The antimicrobial screening identified three LAB isolates and six *Bacillus* isolates capable of inhibiting the growth of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 *in vitro*. The organic acids produced by the LAB isolates significantly contributed to the suppression of all three bacterial pathogens. Based on the results of the screening, the three LAB isolates—*Lb. plantarum*, *P. acidilactici*, and *P. pentosaceus*—were selected for evaluation on fresh produce.

The LAB isolates were cocktailed and activity against *L. monocytogenes* was evaluated on Iceberg lettuce. The LAB cocktail suppressed, but did not completely inhibit, the growth of *L. monocytogenes* over 14 d. However, the antimicrobial efficacy of the LAB cocktail on Iceberg lettuce prompted investigation into the use of DF1, a formulation containing the three LAB isolates and media components, for the inhibition of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on produce.

DF1 treatment of chopped Iceberg lettuce caused rapid browning after 2 d, demonstrating that DF1 may not be appropriate for use on cut produce. Therefore, apples were chosen for further testing because the fruit did not need to be cut before treatment. When applied to apples, DF1 did not reduce *L. monocytogenes*, *Salmonella*, or *E. coli* O157:H7 on apples, but in fact aided in the survival of these pathogens. DF1 was also detrimental to the appearance and feel of the apples. Based on the results of these experiments, DF1 is not the optimal formulation for biocontrol of *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7 on fresh produce.

5. FUTURE RESEARCH

This research identified several bacterial isolates with potential agricultural application. Of the 22 isolates investigated, nine demonstrated strong fungal suppression and nine showed strong antimicrobial activity. Investigation into these isolates is needed to assess their possible use in the agricultural industry.

Future research should include the characterization of the bioactive compounds produced by the inhibitory isolates to gain a better understanding of their antifungal and antimicrobial abilities. This information would allow for the optimization of product formulation and application; this could include alternative media and growth conditions and their effect on bioactive compound production. After optimization, challenge studies on produce should be conducted to assess the commercial viability of the product; produce quality, application methods, and pathogen efficacy must all be evaluated.

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APPENDICES

A.1 Antifungal Screening Inoculation Template
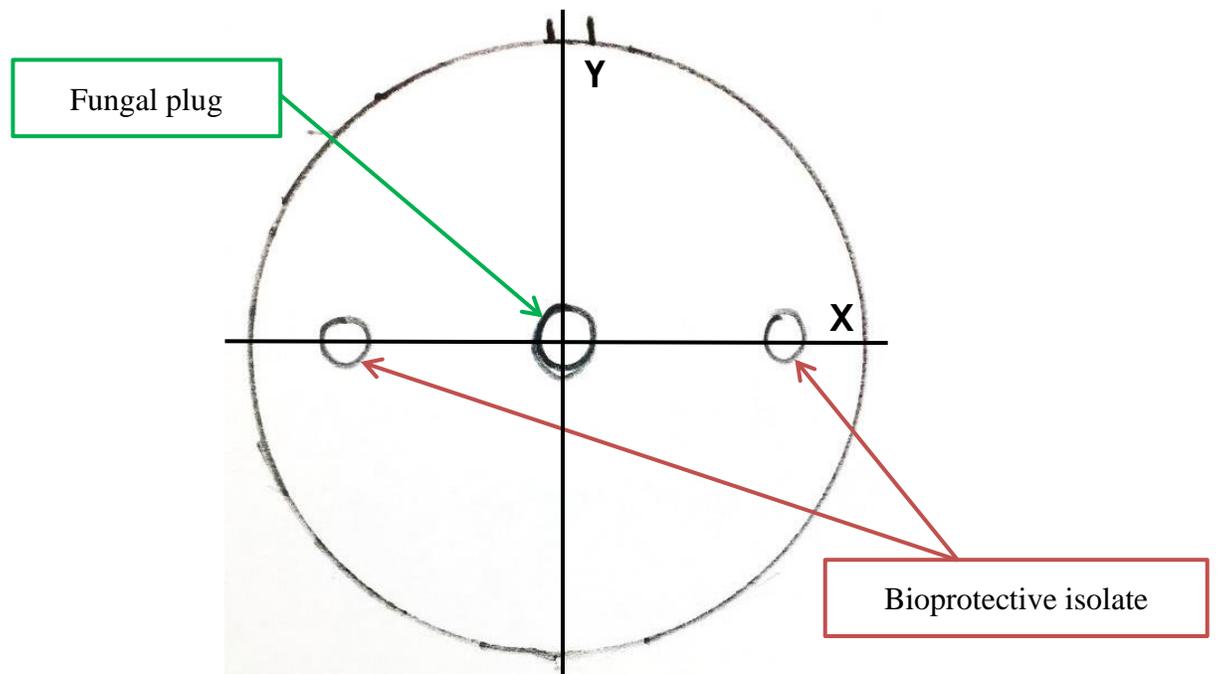
Figure A1. Antifungal screening inoculation template.

A.2 Average Diameters of *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme* Colonies in the Presence of

Bioprotective Isolates

Table A1. Average diameters (in mm) of *Botrytis cinerea*, *Fusarium pallidoroseum*, or *Fusarium moniliforme* colonies in the presence of bioprotective isolates (X axis/Y axis)

Isolate	Day							
	3	4	5	6	7	8	9	10
B. amyloliquefaciens BA1								
Bot. cinerea	40.4/68.1	40.4/84.3	40.4/84.3	40.4/84.3	40.4/84.3	40.4/84.3	40.4/84.3	40.4/84.3
F. pallidoroseum	30.2/34.0	31.5/42.1	32.2/52.7	32.2/62.9	32.2/72.9	32.2/77.5	32.2/84.3	32.2/84.3
F. moniliforme	37.4/30.8	39.4/41.1	39.4/52.5	39.4/63.7	39.4/75.5	39.4/83.0	39.4/84.3	39.4/84.3
B. amyloliquefaciens BA2								
Bot. cinerea	35.9/69.3	35.9/84.3	35.9/84.3	37.3/84.3	37.3/84.3	37.3/84.3	37.3/84.3	37.3/84.3
F. pallidoroseum	30.4/32.4	30.9/41.9	31.4/52.5	31.4/62.0	31.4/71.7	31.4/78.8	31.4/84.3	31.4/84.3
F. moniliforme	32.0/31.7	32.9/42.7	32.9/50.8	33.9/64.3	33.9/75.6	33.9/84.3	33.9/84.3	33.9/84.3
B. amyloliquefaciens BA3								
Bot. cinerea	31.1/66.8	31.1/84.3	31.1/84.3	35.8/84.3	35.8/84.3	35.8/84.3	35.8/84.3	35.8/84.3
F. pallidoroseum	26.5/35.1	27.6/45.8	28.2/56.2	28.2/67.4	28.2/78.8	28.2/83.4	28.2/83.4	28.2/83.4
F. moniliforme	29.8/30.2	32.041.9	32.0/52.5	32.9/66.5	32.9/74.2	32.9/79.5	32.9/84.3	32.9/84.3
B. amyloliquefaciens BA4								
Bot. cinerea	33.3/70.6	33.3/84.3	33.3/84.3	33.3/84.3	33.3/84.3	33.3/84.3	33.3/84.3	33.3/84.3
F. pallidoroseum	27.6/34.5	27.6/44.9	27.6/56.2	27.6/67.9	27.6/80.6	27.6/84.3	27.6/84.3	27.6/84.3
F. moniliforme	31.4/31.1	32.7/42.0	32.7/50.9	33.4/62.6	33.4/72.8	33.4/83.3	33.4/84.3	33.4/84.3
B. amyloliquefaciens BA5								
Bot. cinerea	33.2/84.3	31.4/67.5	31.4/84.3	31.4/84.3	33.2/84.3	33.2/84.3	33.2/84.3	33.2/84.3
F. pallidoroseum	24.8/32.2	24.8/39.3	24.8/45.7	26.1/55.7	26.1/62.6	26.1/69.4	26.1/74.9	26.1/76.8
F. moniliforme	30.5/33.2	32.6/42.6	32.6/52.0	32.6/65.3	32.6/75.6	32.6/82.4	32.6/84.3	32.6/84.3

Table A1 continued…

Isolate	Day							
	3	4	5	6	7	8	9	10
B. clausii								
Bot. cinerea	45.6/48.4	50.7/60.0	55.2/74.1	58.8/84.3	58.8/84.3	58.8/84.3	58.8/81.8	58.8/76.8
F. pallidoroseum	45.8/47.7	52.4/57.9	69.5/72.4	71.6/79.9	79.8/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. moniliforme	40.2/40.0	48.9/54.4	48.9/76.0	48.9/83.5	58.5/84.3	65.8/84.3	70.9/84.3	70.9/84.3
B. coagulans								
Bot. cinerea	32.4/66.0	32.4/84.3	32.4/84.3	32.4/84.3	32.4/84.3	32.4/84.3	32.4/84.3	32.4/84.3
F. pallidoroseum	25.7/31.5	27.3/36.0	27.3/39.1	27.3/45.1	28.5/51.5	28.5/58.4	28.5/64.3	28.5/67.8
F. moniliforme	31.0/33.9	32.3/43.7	32.3/52.7	32.3/67.0	32.3/75.7	32.3/84.3	33.3/84.3	34.6/84.3
B. firmus								
Bot. cinerea	39.9/50.2	42.8/61.6	42.8/79.2	42.8/84.3	42.8/84.3	42.8/84.3	42.8/82.9	42.8/81.9
F. pallidoroseum	47.5/51.6	52.3/59.6	57.8/72.2	62.0/80.6	65.0/80.6	65.0/80.6	65.0/80.6	65.0/80.6
F. moniliforme	43.8/45.3	46.8/55.4	46.8/68.3	46.8/78.1	51.8/81.5	51.8/84.3	51.8/84.3	51.8/84.3
B. licheniformis								
Bot. cinerea	65.8/64.3	74.8/81.8	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	33.5/33.0	45.6/43.8	51.9/55.8	54.7/65.9	58.7/81.3	70.4/84.3	78.8/84.3	80.1/84.3
F. moniliforme	29.3/37.9	38.9/47.8	48.4/57.0	54.5/64.4	58.7/72.0	64.4/79.9	68.3/84.3	70.4/84.3
B. megaterium								
Bot. cinerea	32.1/66.8	32.1/84.3	32.1/84.3	32.1/84.3	32.1/84.3	32.1/84.3	32.1/84.3	32.1/84.3
F. pallidoroseum	26.5/34.2	27.1/43.3	27.1/52.7	27.1/63.8	27.1/74.2	27.1/83.2	27.1/84.3	27.1/84.3
F. moniliforme	30.0/30.6	31.6/40.7	31.6/50.5	32.5/62.3	32.5/74.1	32.5/84.3	32.5/84.3	32.5/84.3
B. pumilus								
Bot. cinerea	58.1/63.1	70.8/77.8	79.5/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	33.0/32.5	44.0/42.8	51.7/51.2	56.4/62.6	63.3/71.2	81.8/84.3	83.5/84.3	84.3/84.3
F. moniliforme	32.3/29.0	41.7/38.8	50.7/51.6	54.4/63.8	69.7/74.0	83.2/81.2	84.3/84.3	84.3/84.3
B. sphaericus								
Bot. cinerea	60.2/62.1	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	28.7/27.6	40.2/40.7	52.6/53.8	61.8/59.4	75.6/66.8	84.3/79.9	84.3/84.3	84.3/84.3
F. moniliforme	27.6/28.7	40.7/40.2	53.8/52.6	59.4/61.8	66.8/75.6	79.9/84.3	84.3/84.3	84.3/84.3

Table A1 continued…

Isolate	Day							
	3	4	5	6	7	8	9	10
B. thiaminolyticus								
Bot. cinerea	34.1/37.7	39.0/51.1	41.8/69.6	42.5/78.3	42.5/78.3	42.5/78.3	42.5/76.9	42.5/74.3
F. pallidoroseum	40.7/40.1	51.8/58.1	57.6/73.9	59.5/80.9	59.5/84.3	68.3/84.3	72.4/84.3	72.4/84.3
F. moniliforme	38.8/40.3	47.8/55.6	52.7/76.4	54.7/83.7	65.6/84.3	73.7/84.3	84.3/84.3	84.3/84.3
B. thuringiensis BT1								
Bot. cinerea	65.9/67.4	79.2/81.9	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	32.7/33.6	43.0/43.1	49.7/54.3	59.1/66.0	68.4/76.8	79.4/84.3	81.0/84.3	84.3/84.3
F. moniliforme	32.5/31.5	42.0/41.7	51.5/51.1	61.6/61.7	70.5/70.1	78.1/79.3	84.3/84.1	84.3/84.3
B. thuringiensis BT2								
Bot. cinerea	31.1/61.5	32.2/84.3	32.2/84.3	37.0/84.3	37.0/84.3	37.0/84.3	37.0/84.3	37.0/84.3
F. pallidoroseum	26.8/32.8	27.2/43.1	27.2/53.2	27.2/65.5	27.2/75.6	27.2/84.3	27.2/84.3	27.2/84.3
F. moniliforme	32.9/30.8	34.3/40.6	35.3/50.0	35.3/59.8	35.3/70.1	35.3/78.4	35.3/84.3	35.3/84.3
Lb. amylovorus								
Bot. cinerea	36.1/39.5	45.8/50.7	49.4/58.9	52.9/84.3	52.9/84.3	52.9/84.3	50.8/84.3	49.4/70.1
F. pallidoroseum	36.3/35.8	48.1/49.3	56.2/58.7	63.0/70.5	71.5/79.7	79.7/84.1	84.3/84.3	84.3/84.3
F. moniliforme	32.0/32.5	42.3/43.3	51.6/53.9	59.7/65.9	66.5/77.7	73.0/84.3	77.4/84.3	80.1/84.3
Lb. plantarum								
Bot. cinerea	30.2/36.0	36.1/48.5	39.1/59.5	53.7/84.3	50.0/84.3	48.0/84.3	45.3/83.6	42.5/69.6
F. pallidoroseum	31.6/31.8	40.6/44.4	48.1/53.2	54.5/60.5	58.7/73.5	69.0/80.9	75.5/84.3	79.6/84.3
F. moniliforme	27.7/31.6	32.5/41.0	36.8/49.3	44.0/60.7	48.6/68.7	61.5/80.4	68.9/84.3	73.7/84.3
O. sojae								
Bot. cinerea	43.3/43.1	51.5/50.5	52.2/60.2	84.3/84.3	84.3/84.3	82.3/83.8	77.4/77.4	63.5/67.2
F. pallidoroseum	45.4/46.3	50.5/61.3	62.6/78.1	65.2/83.1	70.1/84.2	73.0/84.3	73.0/84.3	73.0/84.3
F. moniliforme	41.1/40.6	52.7/55.0	65.3/75.0	67.0/82.1	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
O. anthropi								
Bot. cinerea	56.4/68.2	72.1/84.3	79.9/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	32.3/36.0	43.3/45.7	49.8/54.7	49.8/62.3	49.8/73.4	49.8/77.5	56.4/80.1	56.4/80.1
F. moniliforme	31.5/40.6	42.0/55.0	48.0/75.0	62.2/82.1	76.3/84.3	82.1/84.3	84.3/84.3	84.3/84.3

Isolate	Day							
	3	4	5	6	7	8	9	10
P. acidilactici								
Bot. cinerea	25.4/26.5	29.8/37.4	31.6/47.3	37.3/84.3	37.3/79.7	35.7/72.3	34.5/62.2	34.1/56.2
F. pallidoroseum	31.6/32.8	40.9/43.4	49.6/52.6	60.7/63.8	67.4/66.9	73.7/74.5	80.4/79.1	83.1/83.4
F. moniliforme	32.3/33.4	40.2/44.6	47.5/56.2	56.4/69.5	62.0/80.1	72.1/84.3	78.6/84.3	83.9/84.3
P. pentosaceus								
Bot. cinerea	27.2/30.9	30.5/43.1	33.5/48.3	45.6/84.3	44.1/79.3	41.4/75.7	38.0/66.9	37.0/58.7
F. pallidoroseum	29.8/32.2	35.5/39.2	39.4/45.2	45.5/52.1	48.6/55.6	49.5/55.6	49.5/55.6	49.5/55.6
F. moniliforme	30.7/34.7	37.5/48.0	45.5/59.2	54.9/72.1	65.4/82.2	73.6/84.3	79.2/84.3	84.3/84.3
S. plymuthica								
Bot. cinerea	40.5/56.4	41.1/79.9	41.1/84.3	41.1/84.3	41.1/84.3	41.1/84.3	41.1/84.3	41.1/84.3
F. pallidoroseum	20.8/21.7	30.5/32.8	33.1/44.4	34.0/53.9	34.0/66.3	34.0/76.2	34.0/84.3	34.0/84.3
F. moniliforme	31.5/29.4	39.3/40.8	44.7/55.2	49.9/67.4	50.5/79.2	50.5/84.3	50.5/84.3	50.5/84.3
PDA								
Bot. cinerea	59.4/61.2	75.9/77.7	84.3/83.7	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	24.0/24.3	34.5/34.9	45.4/46.5	55.6/55.4	69.5/68.7	79.0/77.6	84.3/84.3	84.3/84.3
F. moniliforme	31.7/30.4	41.8/42.2	54.3/54.8	65.1/66.0	84.3/78.0	84.3/84.3	84.3/84.3	84.3/84.3
TSA								
Bot. cinerea	40.5/40.3	60.2/61.5	80.7/80.2	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	47.1/45.7	62.6/59.8	77.3/75.3	82.2/79.7	84.3/82.1	84.3/82.1	84.3/82.1	84.3/82.1
F. moniliforme	42.5/42.9	55.1/56.7	73.5/74.0	83.4/82.3	84.3/84.3	84.3/84.3	84.3/84.3	84.3/84.3
MRS								
Bot. cinerea	37.0/36.1	54.6/54.1	62.5/62.1	74.7/84.3	81.5/84.3	84.3/84.3	84.3/84.3	84.3/84.3
F. pallidoroseum	25.4/27.3	34.2/36.4	40.2/42.5	45.3/47.1	49.5/52.2	57.2/58.6	67.9/67.9	77.8/78.6
F. moniliforme	25.4/27.3	34.2/36.4	40.2/42.5	45.3/47.1	49.5/52.2	57.2/58.6	67.9/67.9	77.8/78.6

APPENDIX B. PICTURES OF THE ANTIFUNGAL SCREENING OF

BIOPROTECTIVE ISOLATES AGAINST *BOTRYTIS CINEREA*, *FUSARIUM*
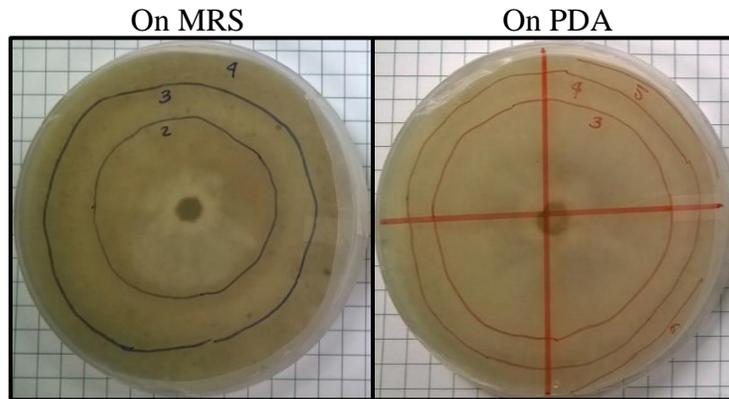
PALLIDOROSEUM, AND *FUSARIUM MONILIFORME*

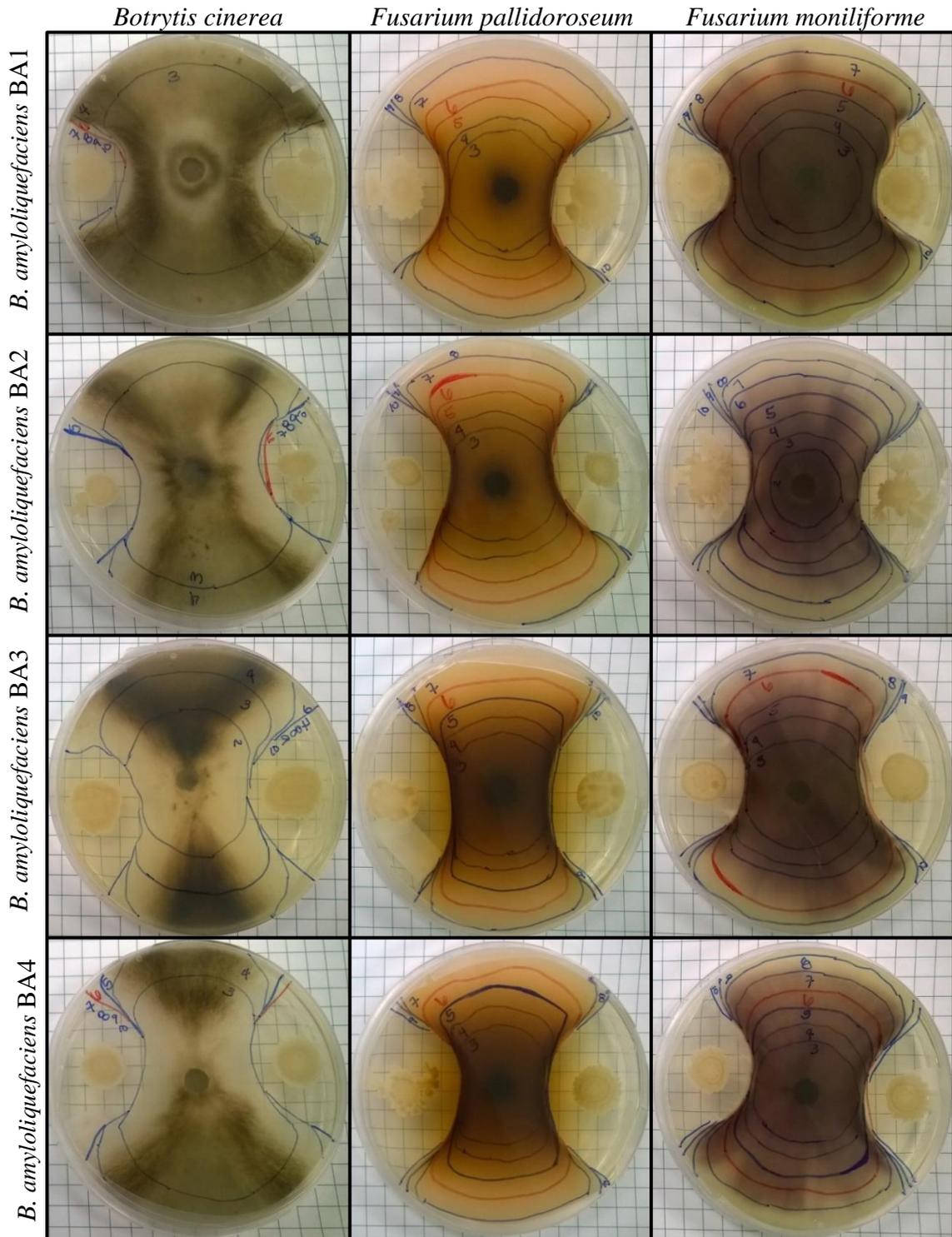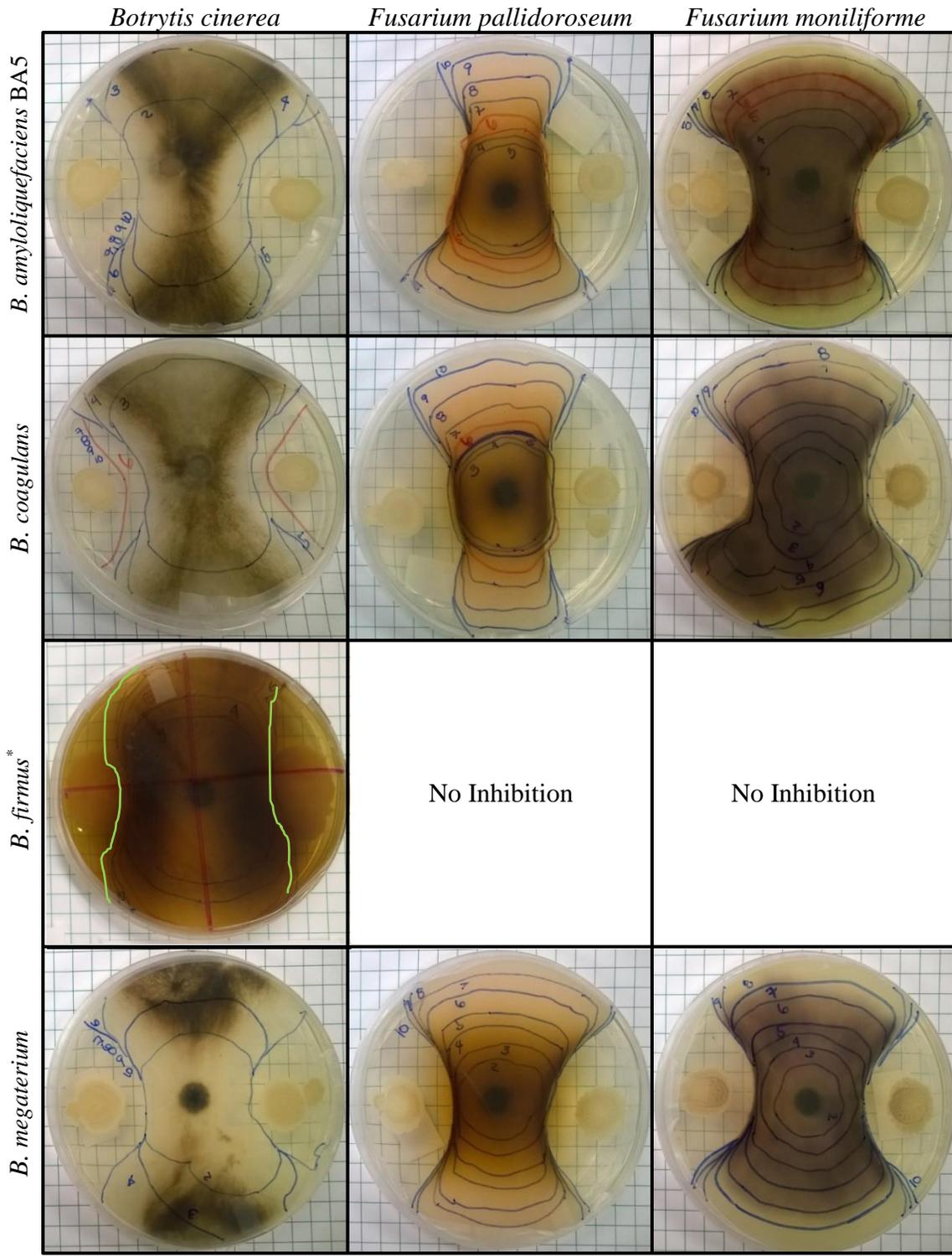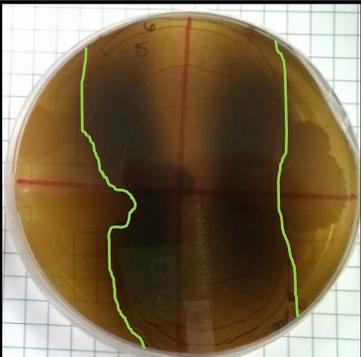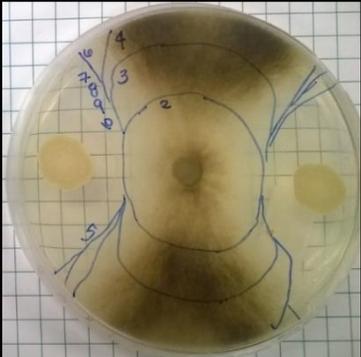This appendix contains the pictures from the antifungal screening of the bioprotective isolates against *Botrytis cinerea*, *Fusarium pallidoroseum*, and *Fusarium moniliforme*. Pictures were taken of control plates and the plates of those isolates that demonstrated fungal inhibition only; pictures were not taken of bioprotective isolate plates that showed no inhibition. Each isolate was screened twice in duplicate; the pictures included in this appendix most clearly illustrate the results of the screenings.

The rings drawn on the plates indicate the boarder of the fungal colony and the number corresponds with the numbers of days the plate had been incubated when the ring was drawn. On some plates, Day 2 is marked, but all measurements began on Day 3.

The vertical and horizontal lines drawn on some of the plates represent the X and Y axes on which measurements were taken (see Figure 1A for antifungal screening inoculation template). Some plates do not have the X and Y axes because the picture was taken before they were drawn.

B.1 Control Plates

Botrytis cinerea

On MRS	On PDA

Fusarium pallidoroseum

On PDA
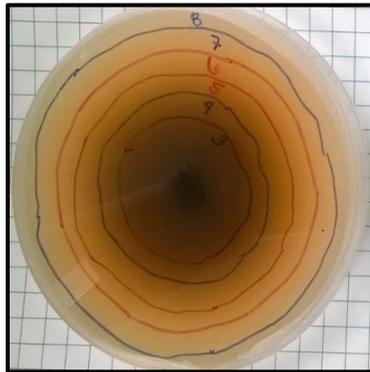
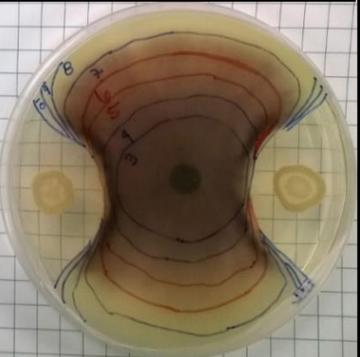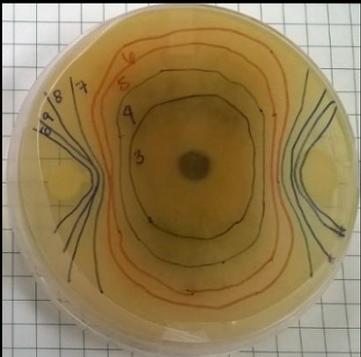### *Fusarium moniliforme*

On PDA
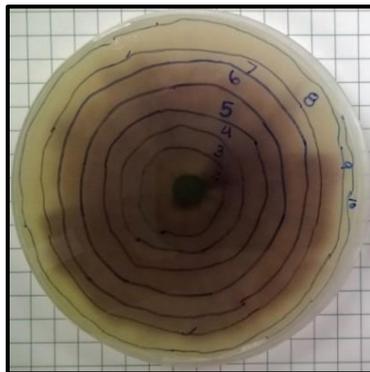
B.2 *Botrytis cinerea*, *Fusarium pallidoroseum*, or *Fusarium moniliforme* Grown in the

Presence of Bioprotective Isolates

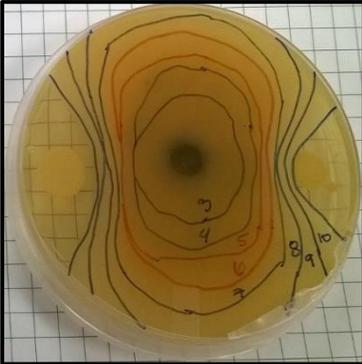|	*Botrytis cinerea*	*Fusarium pallidoroseum*	*Fusarium moniliforme*

B. amyloliquefaciens BA1

B. amyloliquefaciens BA2

B. amyloliquefaciens BA3

B. amyloliquefaciens BA4

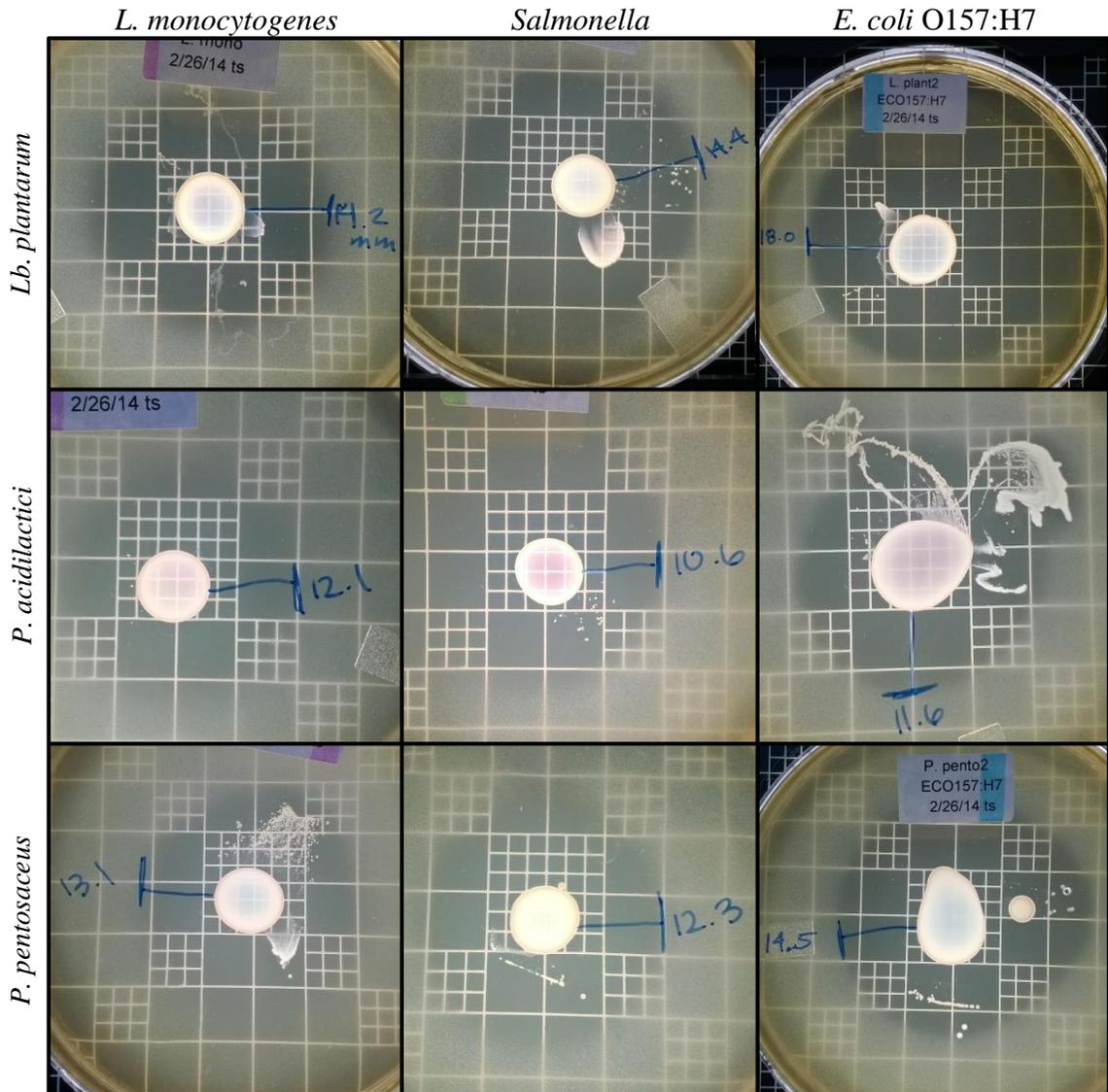|	*Botrytis cinerea*	*Fusarium pallidoroseum*	*Fusarium moniliforme*
B. amyloliquefaciens BA5			
B. coagulans			
B. firmus*		No Inhibition	No Inhibition
B. megaterium			

*Fungal colony border after 10 d incubation was added to the picture because the marks on the petri dish were difficult to see

	Botrytis cinerea	*Fusarium pallidoroseum*	*Fusarium moniliforme*
B. thiaminolyticus[*]		No Inhibition	No Inhibition
B. thuringiensis BT2			
Lb. plantarum		No Inhibition	No Inhibition
P. acidilactici		No Inhibition	No Inhibition
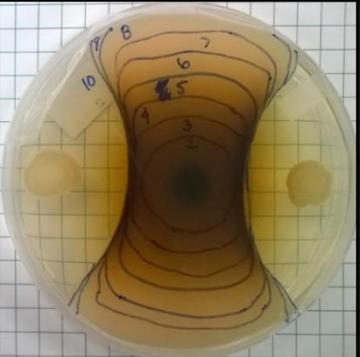
*Fungal colony border after 10 d incubation was added to the picture because the marks on the petri dish were difficult to see
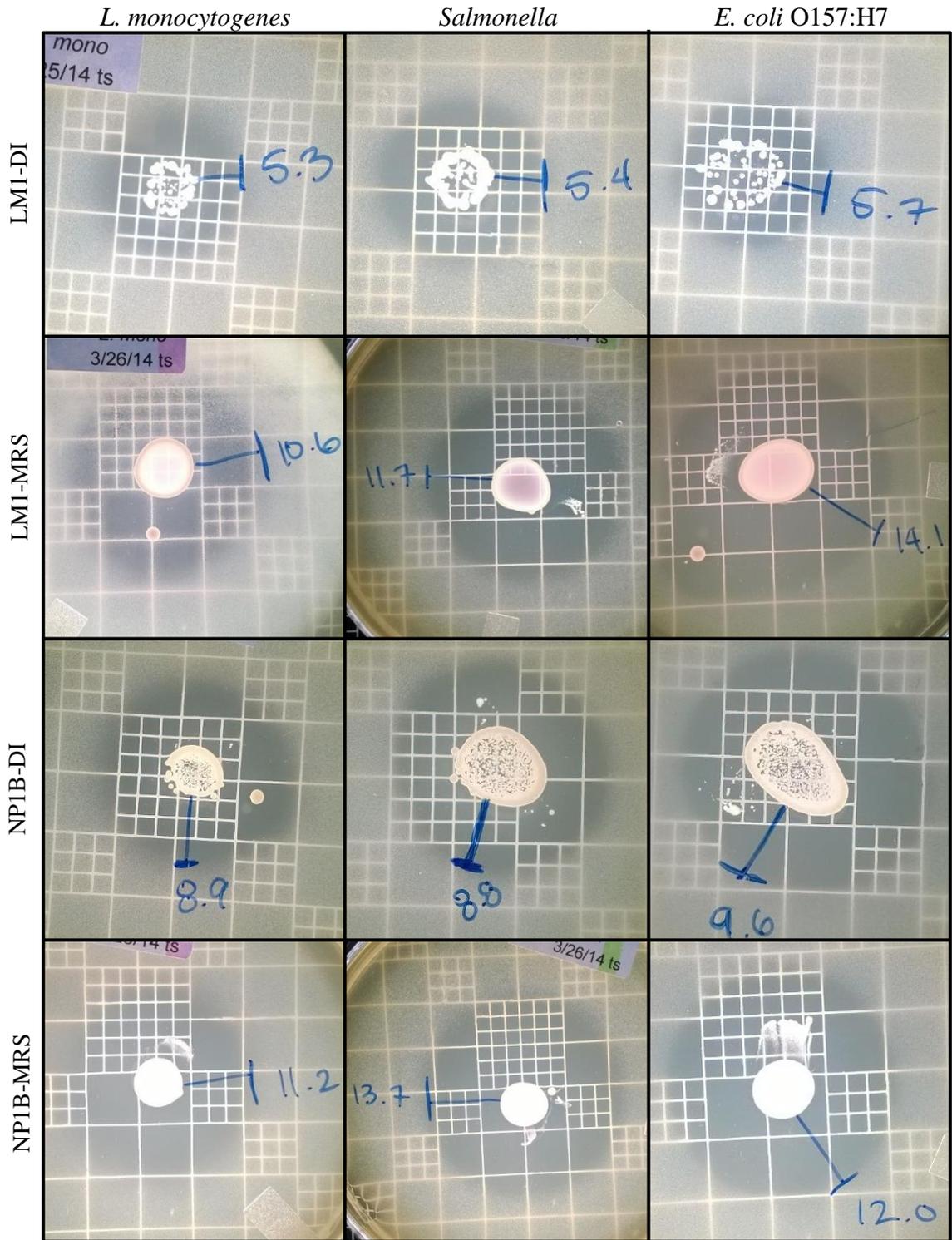
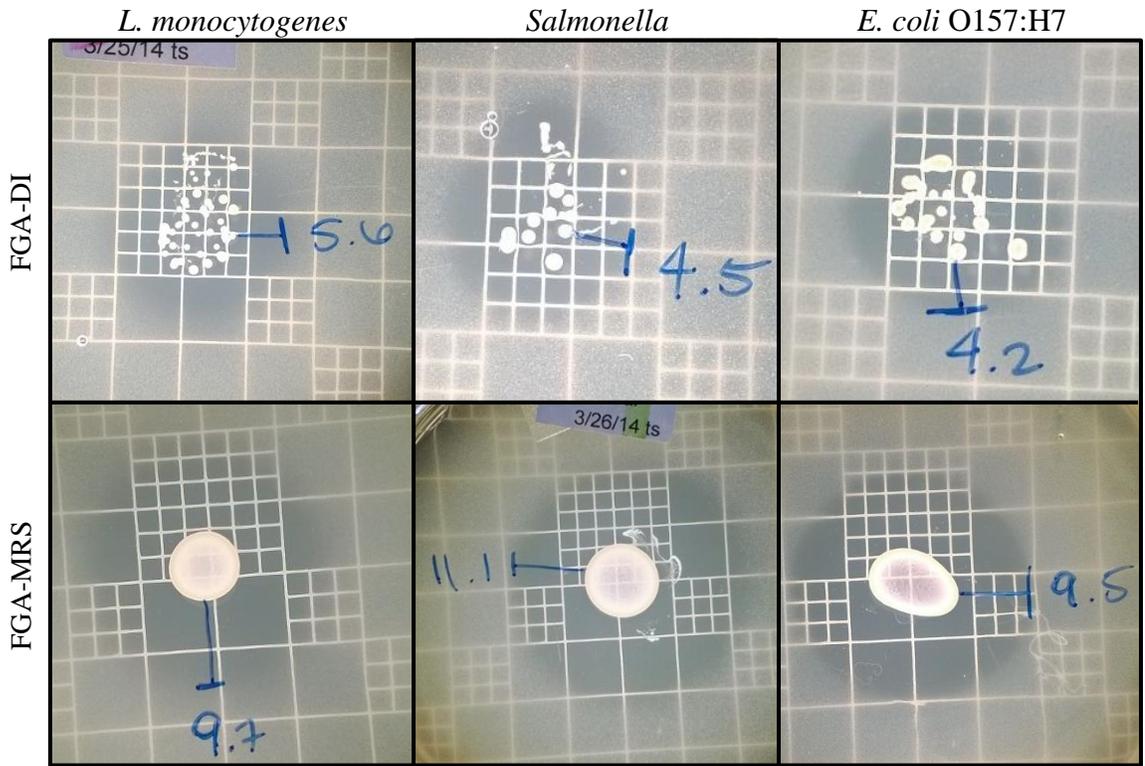|	*Botrytis cinerea*	*Fusarium pallidoroseum*	*Fusarium moniliforme*
P. pentosaceus		No Inhibition	No Inhibition
Serratia plymuthica			
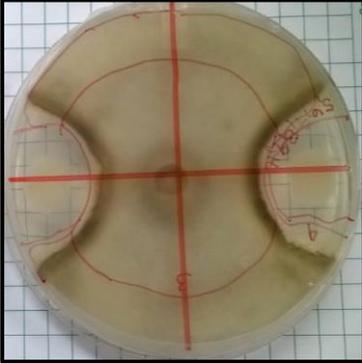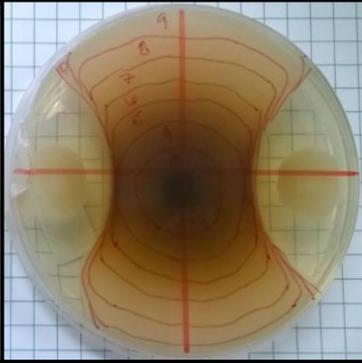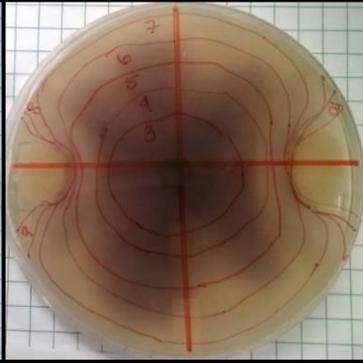
APPENDIX C. ANTIMICROBIAL SCREENING PICTURES

This appendix contains the pictures from the antimicrobial screening of the bioprotective isolates, Lactic Acid Bacteria cocktails, and *B. subtilis* fermentate against *L. monocytogenes*, *Salmonella*, and *E. coli* O157:H7. Pictures were taken those isolates that demonstrated bacterial inhibition only; pictures were not taken of bioprotective isolate plates that showed no inhibition. Each isolate was screened twice in triplicate; the pictures included in this appendix most clearly illustrate the results of the screenings. The handwritten numbers in the pictures indicate the size of the zone of inhibition in mm.
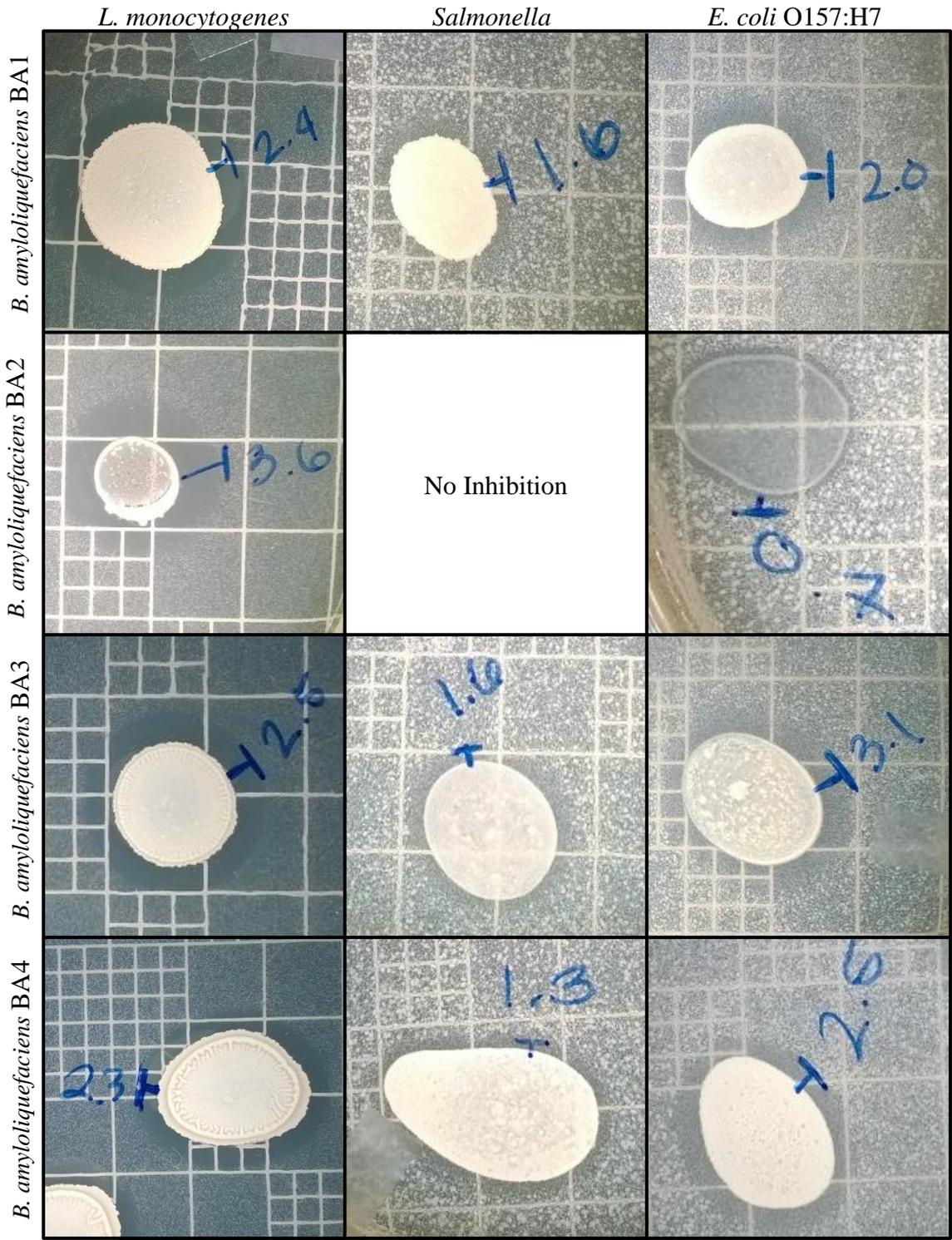
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
LM1-DI	5.3	5.4	5.7
LM1-MRS	10.6	11.71	14.1
NP1B-DI	8.9	8.8	9.6
NP1B-MRS	11.2	13.7	12.0

	L. monocytogenes	*Salmonella*	*E. coli* O157:H7

FGA-DI

FGA-MRS

C.2 Screening of Non-Lactic Acid Bacteria Isolates and *Bacillus subtilis* Fermentate

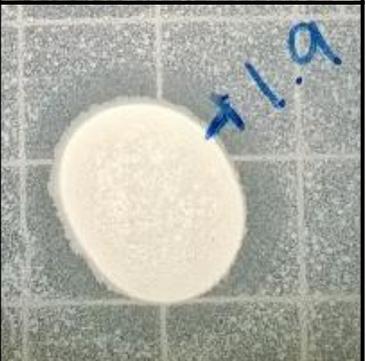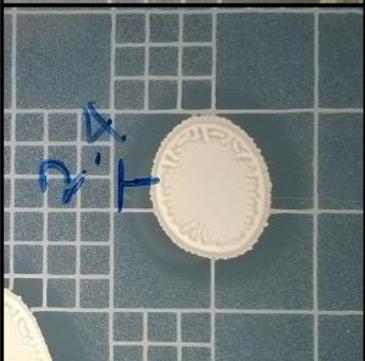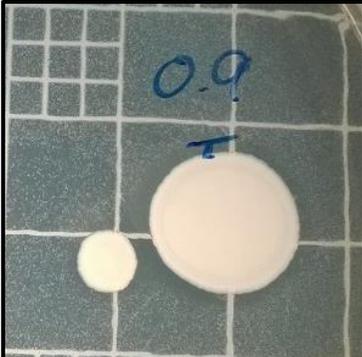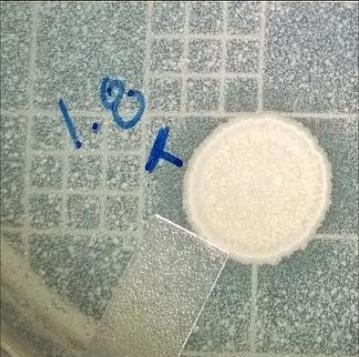Against *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7

	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
B. amyloliquefaciens BA1	2.4	1.6	2.0
B. amyloliquefaciens BA2	3.6	No Inhibition	0.7
B. amyloliquefaciens BA3	2.6	1.6	3.1
B. amyloliquefaciens BA4	2.3	1.3	2.6
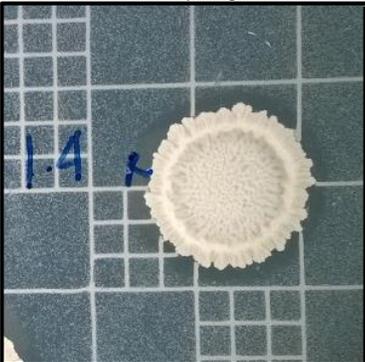
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
B. amyloliquefaciens BA5	1.4	No Inhibition	1.2
B. coagulans	3.1	1.8	1.9
B. licheniformis	2.4	No Inhibition	No Inhibition
B. megaterium	2.4	1.3	2.2
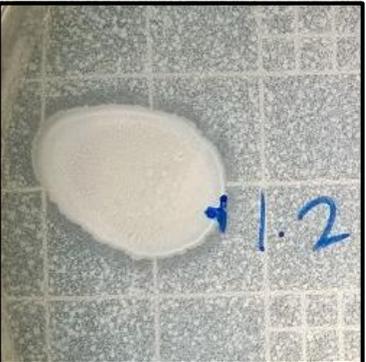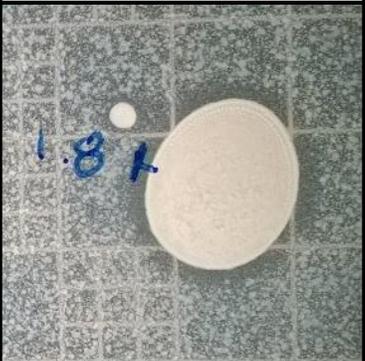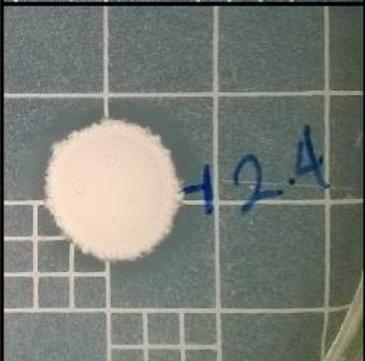
	L. monocytogenes	*Salmonella*	*E. coli* O157:H7
B. pumilus	0.9	No Inhibition	No Inhibition
B. thuringiensis BT2	1.8	0.8	1.8
S. plymuthica	1.5	No Inhibition	1.4
B. subtilis fermentate	1.4	0.8	1.1

Table D1. Optical densities of *Listeria monocytogenes*, *Salmonella*, and *Escherichia coli* O157:H7 grown in the presence of the CFS and NCFS of Lactic Acid Bacteria isolates after 48 h[1]

Treatment[2]	OD$_{600}$		
	Trial 1	Trial 2	Trial 3
Lb. plantarum			
CFS/LM	0.125	0.108	0.108
NCFS/LM	0.412	0.379	0.376
LM control	0.547	0.546	0.555
CFS/SAL	0.126	0.108	0.110
NCFS/SAL	1.193	1.117	0.905
SAL control	1.250	0.703	0.741
CFS/EC	0.130	0.108	0.107
NCFS/EC	1.241	1.148	0.927
EC control	1.380	0.815	0.846
P. acidilactici			
CFS/LM	0.134	0.110	0.110
NCFS/LM	0.516	0.548	0.629
LM control	0.547	0.563	0.536
CFS/SAL	0.124	0.110	0.109
NCFS/SAL	0.758	0.717	0.734
SAL control	1.250	0.706	0.697
CFS/EC	0.134	0.108	0.106
NCFS/EC	0.843	0.780	0.823
EC control	1.380	0.819	0.852
P. pentosaceus			
CFS/LM	0.121	0.115	0.298
NCFS/LM	0.522	1.152	1.179
LM control	0.547	0.791	0.846
CFS/SAL	0.124	0.118	0.273
NCFS/SAL	0.797	1.572	1.609
SAL control	1.250	1.338	1.345
CFS/EC	0.124	0.120	0.250
NCFS/EC	0.929	1.694	1.712
EC control	1.380	1.616	1.554

[1]Shaded rows indicate data sets not used in final statistical analysis.
[2]CFS: cell-free supernatant; NCFS: neutralized cell-free supernatant; LM: *Listeria monocytogenes*; SAL: *Salmonella*; EC: *Escherichia coli* O157:H7.c