QUANTIFICATION OF THE ANTIMICROBIAL SUBSTANCES PRODUCED BY LACTIC ACID BACTERIA USED AS AN INTERVENTION TO INHIBIT *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA IN VITRO* AND ON FRESH SPINACH (*SPINACIA OLERACEA*)

A Dissertation

by

THELMA FRANCISCA CALIX LARA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

December 2011

Major Subject: Food Science and Technology

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Approved by:

Chair of Committee,	Thomas Matthew Taylor
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ABSTRACT

Quantification of the Antimicrobial Substances Produced by Lactic Acid Bacteria Used as an Intervention to Inhibit *Escherichia coli* O157:H7 and *Salmonella in vitro* and on Fresh Spinach (*Spinacia oleracea*). (December 2011)

Thelma Francisca Calix Lara, B.S., Zamorano University;

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Chair of Advisory Committee: Dr. Thomas Matthew Taylor

The metabolic activity of bacterial microorganisms may influence the growth and metabolic activities of other microbes that are present in any specific niche. Lactic acid bacteria (LAB) are antagonistic to some microbial pathogens by the metabolic production of compounds with antimicrobial activity. Consequently, investigators have measured the effects of those antimicrobials to inhibit specific pathogens. However, the mode(s) of action of LAB against foodborne pathogens on products and/or in broth is not completely understood. Therefore, the objectives of this research were to (i) determine the LAB dose required for inhibition of *Escherichia coli* O157:H7 and *Salmonella enterica in vitro* and on spinach, and (ii) identify and quantify the major antimicrobial substances synthesized by LAB as a function of post-inoculation storage conditions. Assays were performed at 7 °C under aerobic conditions. The foodborne pathogens dose responses were assessed in a liquid microbiological medium (*in vitro*) and on spinach leaf surfaces. Different levels of foodborne pathogens and LAB cultures

were used. The addition of LAB cultures did not reduce *E. coli* O157:H7 or *Salmonella enterica* populations when performed *in vitro*. However, when LAB cultures were sprayed on the surfaces of spinach leaves at 8.0 $\log_{10}$ CFU/g, there were significant reductions on *E. coli* O157:H7 of 1.62 and 0.73 $\log_{10}$ CFU/g (after 3 days) and on *Salmonella enterica* of 1.85 and 0.71 $\log_{10}$ CFU/g (after 6 days) for treatments inoculated with an initial level of 2.0 and 4.0 $\log_{10}$ CFU/g, respectively.

After quantification of the antimicrobial compounds synthesized by LAB cultures, they were correlated against the population growth of targeted pathogens. The highest L-lactic acid (3.71±0.14 μmoles/ml, day 12) and hydrogen peroxide (3.72±3.34 μM, day 6) production were obtained from the *in vitro* sample inoculated with 8.0 $\log_{10}$ CFU/ml of LAB and 0.0 $\log_{10}$ CFU/ml of pathogens. The highest bacteriocin production (0.1±0.01 mg/ml) was obtained from the *in vitro* sample with 8.0 $\log_{10}$ CFU/ml of LAB and 2.0 $\log_{10}$ CFU/ml of pathogens. In conclusion, the LAB cultures were able to produce detectable amounts of antimicrobials that may be used as intervention and/or science-based practice against foodborne pathogens by producers and the industry.

To my husband, Jorge Cardona

To my parents, Sagrario and Winston

To my siblings, Winston and Lourdes

To those that have guided and inspired me

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I would like to thank my committee chair, Dr. Matthew Taylor, and my committee members, Dr. Alejandro Castillo, Dr. Ronda Miller, Dr. Steve Smith, and Dr. Joseph Sturino, for their guidance, support, assistance and time for my research.

Thanks also go to my friends and colleagues and the department faculty and staff for making my time at Texas A&M University a great experience. I also want to extend my gratitude to the Nutrition Physiology Corporation, which provided the resources, and to all the students who were willing to participate in the study.

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CHAPTER I

INTRODUCTION

The metabolic activity of bacterial species may influence the growth and activities of other microbes that are present in any specific niche (Schuenzel and Harrison 2002). Lactic acid bacteria (LAB) are antagonistic to some pathogens by the metabolic production of compounds with antimicrobial activity, such as organic acids, peroxides, bacteriocins, and others (Schillinger and others 1996). Consequently, several investigators have measured the abilities of those antimicrobials to inhibit specific pathogens, such as *Escherichia coli* O157:H7 and *Salmonella enterica*, both of which have recently been linked with produce-associated foodborne outbreaks (Ackers and others 1998; Aruscavage and others 2006; Erickson and others 2010).

The mode of action of the commercially available LAB antimicrobial LactiGuard™ against foodborne pathogens is not completely understood or known. Therefore, the primary purpose of this research was not only to determine the LactiGuard™ dose required for inhibition of two foodborne bacterial pathogens (*E. coli* O157:H7 and *Salmonella enterica*), but to also identify and quantify the major antimicrobial substances synthesized by LactiGuard™ and determine lengths of time required for onset and termination of antimicrobial production as a function of post-inoculation storage conditions.

This dissertation follows the style of Journal of Food Science.

The determination of dose response in microbiological medium and on the surface of spinach leaves, and the quantification of antimicrobials fermented by LactiGuard™, are crucial to the understanding of the antimicrobial mode of action and optimal conditions for foodborne pathogen inhibition. Furthermore, this research will enhance future research on produce and subsequent use of LAB as an intervention and/or science-based practice for controlling foodborne pathogens on produce surfaces.

Recently, studies have demonstrated the efficacy of active lactic acid bacteria from a commercial product named LactiGuard™ (Guardian Technologies, Inc.) to inhibit *Listeria monocytogenes* on ready-to-eat (RTE) meat products (Amézquita and Brashears 2002), *Escherichia coli* O157:H7 and *Salmonella enterica* spp. on ground beef (Smith and others 2005), and *Escherichia coli* O157:H7 on spinach (Gragg and others 2010; Gragg and Brasherars 2010).

Therefore, the objectives of this study were: (i) to identify the minimum levels of three strains of lactic acid bacteria (LAB) comprised on a LAB flash-freeze product LactiGuard™ required for the reduction and/or inhibition of *Escherichia coli* O157:H7 and *Salmonella enterica* in microbiological medium and on surfaces of spinach leaves, and (ii) to correlate the synthesis and quantification of antimicrobial substances produced by LactiGuard™ on surfaces of pathogen-inoculated spinach with observed inhibition of *E. coli* O157:H7 and *S. enterica* levels as a function of product storage conditions post-inoculation.

CHAPTER II

PRODUCE CONTAMINATION

2.1 Foodborne Disease and Safety of Produce

Food safety is a progressively important public health issue. The U.S. Centers for Disease Control and Prevention (CDC; Atlanta, GA) estimated that 9.4 million episodes of foodborne illness are caused each year in the United States by 31 major known pathogens, leading to 55,961 hospitalizations, and 1,351 deaths (Scallan and others 2011a). In addition, CDC states that about 38.4 million episodes of foodborne illness caused by unspecified agents occur each year in the United States (Scallan and others 2011b).

In recent years, fruits and vegetables have become significant vehicles in the transmission of human foodborne disease (Abadias and others 2008). From 1990 to 2005, fresh produce was associated with 713 outbreaks, resulting in 34,049 cases of illness (Solomon and Sharma 2009). Extensively consumed commodities such as apple cider, cantaloupe, raspberries, bagged lettuce and spinach, tomatoes, green onions, and sprouts have been involved in large outbreaks (Table 2-1) (Sapers and Doyle 2009). Human pathogens involved in reported outbreaks (2003 - 2006) associated with fruits and vegetables in US are listed in Table 2-2.

Table 2-1. Fresh produce implicated in outbreaks of foodborne disease in US.

Year	Produce Item	Outbreaks	Cases
2003	Green salads and lettuce	8	218
	Sprouts	4	62
	Melons	3	182
	Other vegetables (scallions, tomatoes, spinach)	3	962
	Other fruits (strawberries, mango)	2	30
	Lettuce	1	57
2004	Green salads and lettuce	17	630
	Tomatoes	4	671
	Other fruits (fruit salad)	3	184
	Melons	2	134
	Sprouts	2	37
	Other vegetables (cucumber salad, mixed vegetables)	2	329
2005	Green salads and lettuce	5	137
	Tomatoes	4	176
	Fruit salad	4	339
	Other vegetables (parsley, onion, carrots, basil)	4	633
	Other fruits (watermelon, strawberries)	2	58
	Lettuce	1	>18
2006	Green salads and lettuce	25	828
	Tomatoes	4	157
	Fruit salad	6	177
2008	Raw produce (jalapeño pepper, serrano peppers)	1	1442
	Cantaloupes	1	51
2009	Alfalfa sprouts	1	235
2010	Alfalfa sprouts	2	184
	Shredded romaine lettuce	1	26
2011	Alfalfa and spicy sprouts	1	25

From Sapers and Doyle (2009); FAO and WHO (2008); CDC (2011).

Table 2-2. Human pathogens involved in reported outbreaks associated with produce.

Year	Pathogen	Number of reported outbreaks	Cases
2003	*Escherichia coli* O157:H7	4	87
	Salmonella	10	719
	Shigella	2	62
	Cryptosporidium	1	144
	Hepatitis A	1	935
	Norovirus	4	110
	Campylobacter	2	22
2004	*Escherichia coli* O157:H7	3	308
	Salmonella	4	164
	Crystosporidium	1	212
	Norovirus	19	893
2005	*Escherichia coli* O157:H7	4	70
	Salmonella	7	367
	Cyclospora	1	592
	Hepatitis A	1	40
	Norovirus	6	431
2006	*Escherichia coli* O157:H7	7	380
	Salmonella	9	240
	Staphylococcus aureus	1	35
	Cyclospora	1	14
	Norovirus	25	770
2008	*Salmonella* Saintpaul	1	1442
	Salmonella Litchfield	1	51
2009	*Salmonella* Saintpaul	1	235
2010	*E. coli* O145	1	26
	Salmonella	2	184
2011	*Salmonella* Enteritidis	1	25

From Sapers and Doyle (2009); CDC (2011).

The economic impacts of produce-associated outbreaks are substantial because they include the medical costs and lost income of patients, the costs of product recalls, disposal of unmarketable products, cleanups, and retrofitting; and loss of production time (Sapers and Doyle 2009). In addition, outbreaks can affect an entire segment of the produce industry, and/or a production area by the significant reduction in sales, consumption, and consumer confidence in the fresh produce safety (Sapers and Doyle 2009).

In 2006, the Codex Committee on Food Hygiene (CCFH), through the Codex Alimentarius Commission (CAC), highlighted the need to address aspects related to the control of specific hazards in fresh fruits and vegetables (FAO and WHO 2008). Consequently, it was determined that leafy vegetables were the highest priority based on the ranking criteria of the frequency and severity of disease, size and scope of production, diversity and complexity of the production chain, potential for amplification of foodborne pathogens through the food chain, potential for control, and extent of international trade and economic impact (FAO and WHO 2008).

The unexpected increase in the prevalence of produce-associated outbreaks may be a result of the minimal processing practices used before packaging of convenient foods such as fresh-cut fruits and bagged salads (Abadias and others 2008; Solomon and Sharman 2009). Moreover, it may be due to greater consumption of fresh produce in response to the recommendations of health and nutrition professionals, which increased by 18% and 29% in the US from 1982 to 1997, and/or due to a change in surveillance

and/or reporting methodology by the CDC (Sapers and Doyle 2009; Solomon and Sharma 2009).

Fresh produce is grown in agricultural settings, on or close to the soil where contamination can occur (Solomon and Shaman 2009). The place where pathogens are normally found in nature is referred as the reservoir, which could be identified as a human and/or animal reservoir (Farrar and Guzewich 2009). Zoonotic bacterial pathogens such as *Salmonella* and *Escherichia coli* O157:H7 are easily transferred from other sources, such as feces (Solomon and Shaman 2009). The mechanisms by which fresh produce might become contaminated with such pathogens are shown in Figure 2-1. Therefore, contamination of fresh produce with bacterial pathogens may occur during pre-harvest, harvest, post-harvest, handling, and/or distribution (Ackers and others 1998; Johannessen and others 2004; Abadias and others 2008; Solomon and Shaman 2009).

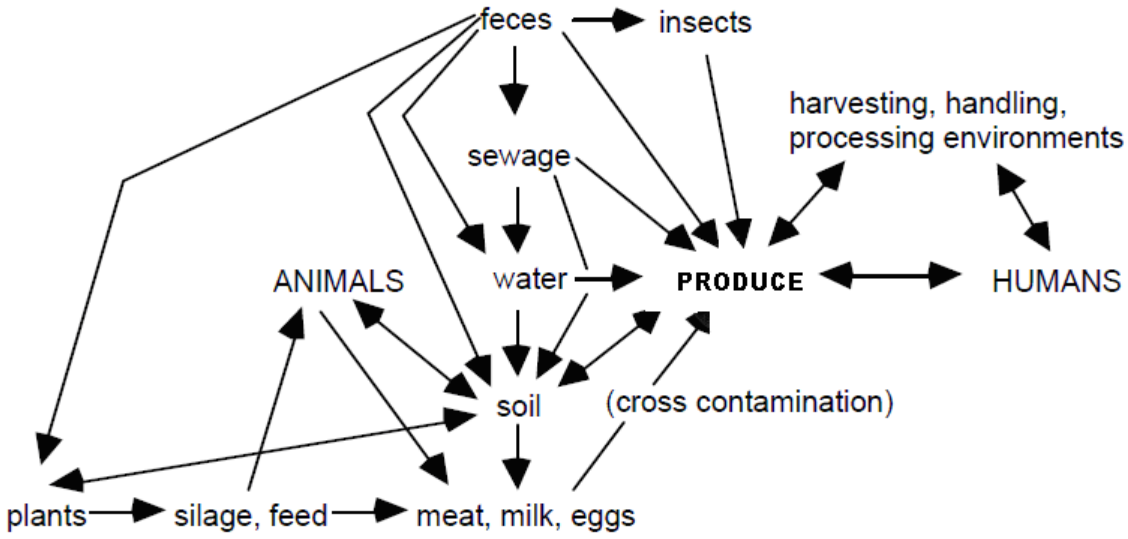

Figure 2-1. Mechanisms of fresh produce contamination (Beuchat and others 1998).

2.2 Potential Sources of Produce Contamination

2.2.1 Pre-harvest Sources of Contamination

Vegetables and fruits are mainly cultivated in open fields and protected cultivation that vary between and within countries (FAO and WHO 2008). However, regardless of the production environment and/or system, there is a wide range of accidental or intentional inputs that are potential sources of microbial foodborne hazards (FAO and WHO 2008). The use of untreated or fresh manure as fertilizer, soil and irrigation water contaminated with feces of domesticated animals, wildlife and/or humans are some examples of important sources of contamination during pre-harvest (Johannessen and others 2004; Gragg and others 2010; Sapers and Doyle 2009; Erickson and others 2010; Ongeng and others 2011).

Animal manure is used as a fertilizer on crop production land (Millner 2009). However, manure is a potential source of a wide variety of human pathogenic microorganisms, including bacteria (*Campylobacter coli*, *C. jejuni*, *Bacillus anthracis*, *Brucella abortus*, pathogenic and toxigenic strains of *E. coli*, *Leptospira* spp., *Listeria monocytogenes*, *Mycobacterium bovis*, *Mycobacterium avium paratuberculosis*, *Salmonella* spp., *Yersinia enterocolitica*), viruses (hepatitis A, norovirus, rotavirus, astrovirus), and parasitic protozoa (*Cryptosporidium parvum*) (Casteel and others 2008; Ortega and others 2008; Millner 2009; Morales-Rayas and others 2010; Laird and others 2011). According to Kudva and others (1998), *E. coli* O157:H7 can survive in sheep manure for up to 21 months, under fluctuating environmental conditions, with a bacterial concentration range of $<10^2$ to 10^6 CFU/g. In contrast, it is recommended that if raw

manure is used for organic produce, the harvest cannot occur before 90 to 120 days post-application (CFR 2010). Furthermore, no federal or state regulation determines a specific pathogen reduction or testing for animal manure prior to land application (Millner 2009).

Water is also an important vehicle for the transmission of foodborne pathogens (Gerba and Choi 2009; Berger and others 2010; Wood and others 2010; Fonseca and others 2011; Oliveira and others 2011). Several produce outbreaks have occurred from contamination in the field caused by irrigation (Gerba and Choi 2009; Hanning and others 2009; Behravesh and others 2011). The main reason of this is that raw water sources are subject to contamination by animal feces and sewage discharge (Gerba and Choi 2009). Therefore, it has been suggested a fecal coliform limit of 1,000 organisms per 100 ml of irrigation water as a bacteriological standard (Scott and others 2005). Also, the World Health Organization (WHO) developed guidelines for the use of treated wastewater for food crops in regions where water is a limited resource (WHO 2011). However, inadequate institutional capability and lack of resources have limited their correct application (Gerba and Choi 2009). Furthermore, the probability of the edible parts of a crop to become contaminated depends on several factors such as growing location (e.g. distance from the soil or water surface), frequency of irrigation, surface of the edible portion (e.g. smooth, webbed, rough), and type of irrigation method (furrow or flood, sprinkler, drip) (Gerba and Choi 2009).

2.2.2 Harvest and Post-Harvest Sources of Contamination

Farm management practices have a great impact in the prevalence of microbial foodborne hazard (Mukherjee and others 2004). Produce, in particular leafy vegetables,

may be harvested mechanically or by hand and unhygienic harvest devices may lead to contamination of crops (Matthews 2009; Johannessen and others 2004; Gragg and others 2010; Erickson and others 2010). Patel and others (2011) showed that *E. coli* O157:H7 can attach to spinach harvester blades under static and dynamic temperature conditions and that the nutrients from spinach extract promote cell growth on blade surfaces. It was also found that the populations of *E. coli* O157:H7 recovered from rusty blades ($p>0.05$, 0.54-0.66 $\log_{10}$ CFU/blade) were lower than the populations recovered from new blades (0.77-0.94 $\log_{10}$ CFU/blade) (Patel and other 2011). Spinach harvester blades are commonly suggested to be sanitized with hypochlorite solutions; however, the presence of organic compounds reduces the likelihood of free chlorine available to effectively kill any foodborne pathogen on harvester blades (Patel and others 2011).

The handling of produce during and immediately after harvest may result in a dramatic effect on its microbial safety (Ailes and others 2008; Matthews 2009). Leafy green are harvested into bins (Matthews 2009). If bins are placed directly onto the soil and stacked on top of the other for transport to the processing plant, it may result in contamination from the bottom of one bin to the content of the bin below (Matthews 2009). Following field harvest, it is recommended that during transportation to the post-harvest processing plant, the bins should be placed at low temperatures (4°C) to reduce the growth of potential foodborne microorganism (Matthews 2009).

Harvested produce, such as bagged spinach, may in some instances be classified as minimally processed (Gragg and others 2010). Commonly, post-harvest processing plants rely on washing steps, which may include the use of chlorinated agents, such as

hypochlorite (OCl^-) or chlorine dioxide (ClO_2), to decontaminate leafy vegetables (Beuchat and Ryu 1997; Lopez-Galvez and others 2010). Calcium hypochlorite ($CaClO_2$) is the most common form of dry bleach used in the industry, at concentrations of 120 ppm free chlorine (Khanna and Naidu 2000). The U.S. Food and Drug Administration (FDA) allows the use of sodium hypochlorite (NaOCl) at a maximum concentration of 0.2% to wash fruits and vegetables (FDA 2008). When applied in leafy greens for 1 to 10 min, chemical sanitizers generally provide a 1.0-2.0 $\log_{10}$ reduction in viable bacteria (Table 2-3).

Table 2-3. Chlorine-related sanitizers effect on fresh leafy vegetables.

Agent, Concentration and Exposure	Produce Item	Reduction ($\log_{10}$ CFU/g)	Reference
NaClO, 200 ppm, 1 min	Lettuce (pieces)	*E. coli* O157:H7: 0.86-0.88 *Salmonella*: 0.96-1.04	Koseki and others 2003
NaClO, 200 ppm, 10 min	Lettuce (leaves)	Aerobic bacteria: 2.5-3.0 Total coliforms: <2.0	Nascimento and others 2003
NaClO, 200 ppm, 10 min	Lettuce (pieces)	*E. coli* O157:H7: 1.2 *S. aureus*: 1.4 *Salmonella*: 1.2	Kondo and others 2006
NaClO, 100 ppm, 2 min	Lettuce (leaves)	*E. coli*: 2.6-2.9 *L. monocytogenes*: 1.5-1.7	Ölmez and Temur 2010
Chlorine, 10 ppm, 5 min	Lettuce (shredded)	*L. innocua*: 1.0-1.5	Francis and O'Beirne 2002

Although, a study that assayed for total aerobic bacteria and total coliforms demonstrated that the majority of produce categories showed higher microbial concentration when samples were obtained from post-packing as compared from immediately post-harvest stage (Ailes and others 2008). This effect may be caused by the contact with contaminated human hands, contaminated rinse water (without sanitizer or with inactivated sanitizer), equipment surface, waste products, or other contaminated produce (Aisles and others 2008). For example, Harris and others (2003) cited an outbreak of hepatitis A implicated with an infected food handler shredding lettuce by hand.

Table 2-4. Optimum cold chain conditions for leafy vegetables and fresh-cut salad.

Product	Temp[1] (°C)	RH[2] (%)	Suggested Shelf life	Reference
Bean sprouts	0	95-100	5-10 days	De Ell and others 2000
Lettuce (butterhead, crisphead, green leaf, iceberg, romaine)(chopped, shredded, whole leaf)	1-3	95-100	6-12 days	USDA 2009a
Spinach (whole leaves, cut leaves)	0-3	95-98	6-12 days	USDA 2009a

[1]Temp: Temperature, [2]RH: Relative humidity.

The level of surface hygiene in the produce processing plants can also compromise the safety of consumers (Beuchat and Ryu 1997). Packing equipment and the hands of packing workers are some examples of potential surface sources of human pathogen

contamination of fresh produce (Sapers and Doyle 2009). In addition, storage conditions also play an important role to reduce the growth ability of foodborne pathogens on leafy vegetables and fresh-cut salads; therefore, it is recommended to follow the cold chain suggestions listed on Table 2-4.

2.3 Microbial Attachment to Produce Surfaces

The plant leafy surface is not an ideal environment for enteric pathogen survival, since it is exposed to ultraviolet light, fluctuations in temperature and relative humidity (Matthews 2009). It also contains a very complex and large native community of microorganisms that may compete with foodborne pathogens for nutrients (Solomon and Sharma 2009). However, some bacterial foodborne pathogens such as *Listeria monocytogenes* have the ability to rapidly attach to both cut and intact cabbage tissues, as demonstrated by Ells and Hansen (2006). Production of extracellular fibrils and flagellins, and release of enzymes to facilitate bacterial attachment and infiltration have been reported to be used by *L. monocytogenes* (Ijabadeniyi and others 2011).

The normal numbers of aerobic bacteria may average 10^5 to 10^6 CFU/g of leaf tissue on leafy greens, with Gram-negative bacteria being the most predominant group of epiphytic microorganisms (Matthews 2009). The majority of plant surfaces are protected with a hydrophobic material called cuticle, which is mainly composed of fatty acids, waxes, and polysaccharides (Solomon and Sharma 2009). Epiphytic microorganisms commonly colonize the base of trichomes, around the stomata, and along veins in the leaves; however, foodborne pathogen bacteria are also capable of penetrating the epidermis of iceberg lettuce leaves through open stomata (Kroupitski and others 2009).

In addition, when the cuticle is cracked or damaged, the epidermal cells get exposed facilitating bacteria colonization and/or internalization (Solomon and Sharma 2009; Ijabadeniyi and others 2011). The presence of abrasions or cuts, and/or biofilms formation may also facilitate the attachment of bacterial pathogens (Seo and Frank 1999; Solomon and Sharma 2009). This has been confirmed by several investigations, such as Hora and others (2005) in spinach plants with *E. coli* O157:H7, and Golberg and others (2011) with *Salmonella* Typhimurium in leafy vegetables and herbs.

In some cases, the considerable incidence of internalized *Salmonella* may not be related with the stomatal closure as demonstrated Golberg and others (2011) among different plants and within the same crop, with the highest observed incidence in iceberg lettuce ($p<0.05$, 81±16%) and arugula (88±16%). The accessibility of carbon and nitrogen sources will also influence survival and growth of microorganisms (Matthews 2009). This has been illustrated by the higher persistence of pathogens on younger leaves since the exudate from young leaves is 2.9 and 1.5 times richer in total nitrogen and carbon than older leaves (Erickson and others 2010). Therefore, bacterial survival and/or attachment on plant surfaces is variable, depending on nutrient availability, competition with indigenous microflora, and relative humidity of the production environment and plant structure (Erickson and others 2010).

Attachment has also a significant impact on pathogen survival during and/or after washing and chlorine application due to the protective embedding arrangement formed in the plant tissue (Seo and Frank 1999; Iturriaga and Escartín 2010; Ijabadeniyi and others 2011). *Escherichia coli* cells were unable to be inhibited or reduced by sanitation

agents (ClO_2 at 3 mg/L or NaOCl at 100 mg/L) from inoculated and cross-contaminated lettuce leaves (Lopez-Galvez and others 2010). Furthermore, attachment of foodborne pathogens can occur as soon as four hours or less of incubation post contamination (Soloman and Sharma 2009).

2.4 Spinach (*Spinacia oleracea*)

2.4.1 Introduction

Spinach (*Spinacia oleracea*) is an annual plant that belongs to the *Chenopodiaceae* family (Grieve and Grieve 1971). It is cultivated for the consumption of its leaves that are good sources of vitamin A (672 UI/leaf), and ascorbic acid (3.0 mg/leaf) (Chick and Roscoe 1926). Spinach leaves also contain high amounts of nitrogenous substances, hydrocarbons, and iron sesquioxide (Fe_2O_3, 0.3 mg/leaf) (Grieve and Grieve 1971; Gebhardt and Thomas 2002). Calcium is present in high amounts in spinach leaves (10 mg/leaf); however, its bioavailability is very low due to the high oxalic acid content (0.97 g/ 100 g) that binds all the calcium present (5.1% of absorption efficiency) (Weaver and Heaney 2006; USDA 2009b).

2.4.2 Spinach Production and Trade

In 2008, the global spinach production reached an estimated 14,584,093 tonnes (FAO 2010). The world leader in spinach production was China, which contributed 86% of total global production, followed by the United States of America (2.4%), Japan (2.0%), and Turkey (1.5%) (Table 2-5) (FAO 2010). The major global exporter of spinach was the United States of America (Figure 2-2), representing 24% of the total world export (89,992 tonnes) (FAO 2010). The unit value of each tonne exported by

USA was about $2,182. In contrast, Trinidad and Tobago exported only one tonne with a unit value of about $36,000, which represents less than 1% of the total world export (FAO 2010).

Spinach has been increasingly consumed in the United States (Lucier and others 2004). The per capita consumption of fresh spinach in the U.S. totaled 2.35 pounds during 2000 to 2002, compared with 1.69 pounds during the 1990's and 1.57 pounds during 1980's (Lucier and others 2004). In 2009, U.S. growers produced 815 million pounds of spinach for all uses, representing 2.47% of the total world production (FAO 2010).

Table 2-5. Top ten global producing nations of spinach in 2008.

Ranking	Country	Production (MT[1])
1	China	12,512,005
2	United States of America	353,430
3	Japan	292,700
4	Turkey	225,746
5	Indonesia	152,130
6	France	123,500
7	Italy	99,800
8	Republic of Korea	93,441
9	Belgium	90,000
10	Pakistan	82,239

[1]MT: Metric Tonnes (Adapted from FAO 2010).

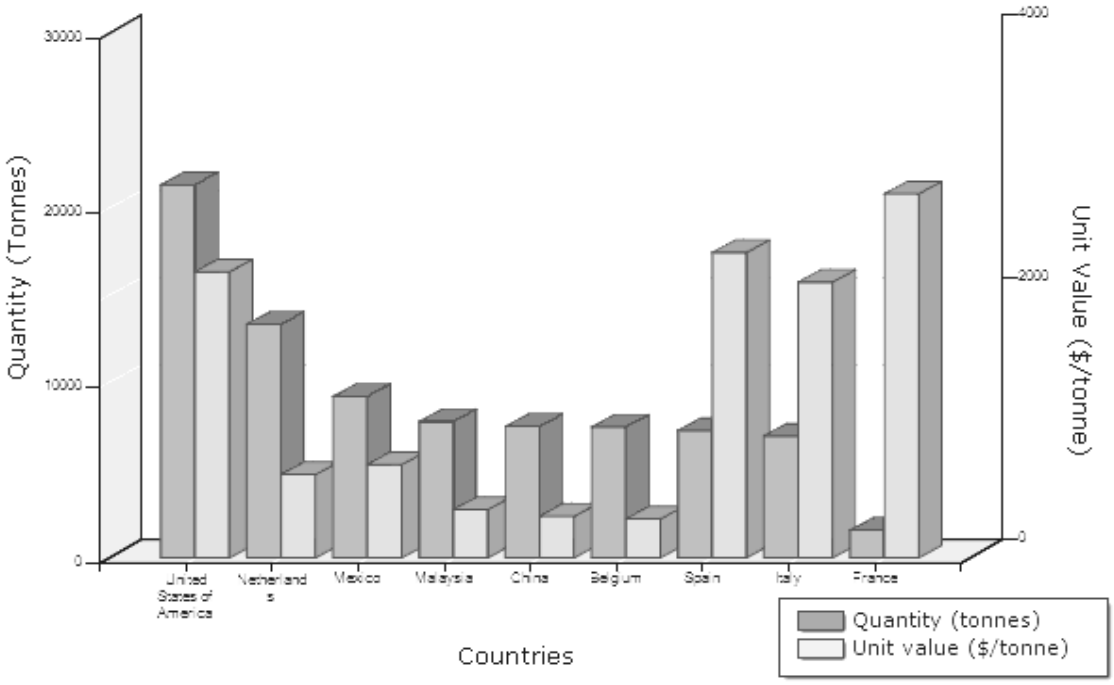

Figure 2-2. Top exports of spinach in 2008 (FAO 2010).

CHAPTER III

PRODUCE FOODBORNE *ESCHERICHIA COLI* O157:H7 AND *SALMONELLA ENTERICA*

3.1 *Escherichia coli*

3.1.1 Introduction

In 1885, Theodor Escherich first isolated and studied *Escherichia coli* (*E. coli*), which was originally named *Bacterium coli commune* (Willshaw and others 2000; Jay and others 2005). *E. coli* belongs to the family of *Enterobacteriaceae* (Jay and others 2005). *E. coli* is defined as a facultative anaerobe, oxidase-negative, Gram-negative rod (ranging in size 1-1.5 x 2-6 μm) (Doyle and others 1997; Madigan and others 2000; Willshaw and others 2000).

Gram-negative bacteria have a very complex cell wall (Figure 3-1), which is a multilayer structure composed of the lipopolysaccharide and outer membrane, the periplasm, and the cytoplasmic membrane (Madigan and others 2000). The outer membrane of Gram-negative bacteria makes them more resistant to lysozyme, hydrolytic enzymes, surfactants, bile salts, and hydrophobic antibiotics than Gram-positive bacteria (Kim and Gadd 2008). The outer membrane is mainly composed of lipopolysaccharides (LPS) that provide a semi-selective permeable barrier (Kim and Gadd 2008). The outer membrane components and their functions in *Escherichia col*i are listed in Table 3-1. The LPS structure consists of lipid A, core polysaccharide and repeating polysaccharide

(Kim and Gadd 2008). The repeating polysaccharide is involved in pathogenesis and is identified in the O-antigen profiling (Kim and Gadd 2008).

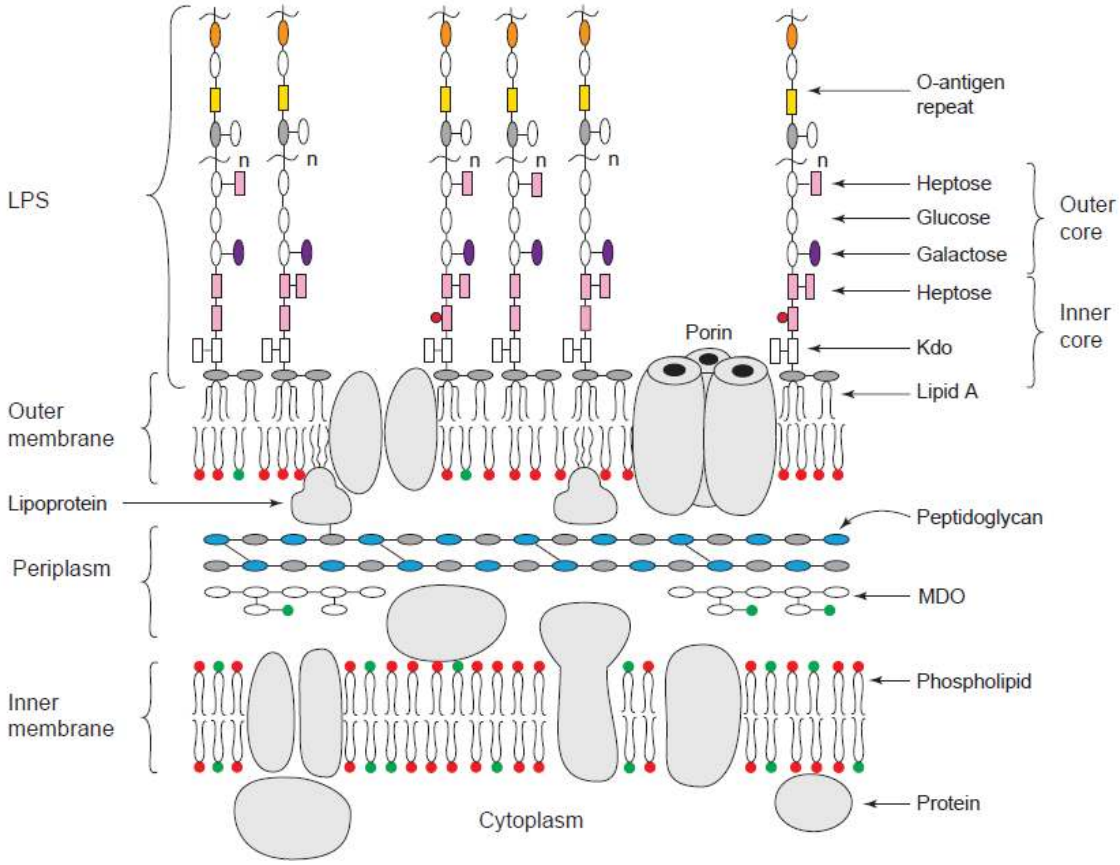

Figure 3-1. The Gram-negative cell wall structure (Wyckoff and others 1998).

Traditionally, *E. coli* isolates are identified by their IMViC pattern reactions: + + - - (*E. coli* Type I), and - + - - (*E. coli* Type II); where I = indole production, M = methyl red reaction, V = Voges-Proskauer reaction (production of acetoin), and C = citrate utilized as a sole source of carbon, respectively (Hitchins and others 1992; Willshaw and others 2000; Jay and others 2005). Also, *E. coli* may be differentiated on Sorbitol

MacConkey agar (red or pink colonies, indicating hydrolysis of bile with fermentation of sorbitol within 24 h), and on Levine's eosin-methylene blue (EMB) agar (dark center with greenish metallic sheen) (Hitchins and others 1992; Madigan and others 2000).

Table 3-1. *E. coli* outer membrane components and their functionality.

Component	Function
Phospholipid	Inner leaflet
Lipopolysaccharide	Outer leaflet, hydrophilic barrier. Stabilization of the surface structure by bonding with metal ions
Lipoprotein	Lipid section is embedded in the hydrophobic region of the outer membrane, and sugar section is bound to murein which stabilizes the outer membrane
Protein A	Uphold the outer membrane stability, receptor or amino acids and peptides
Porin	There are three different porins: OmpC, OmpF, and PhoE. They act as specific and non-specific channels for hydrophilic solutes
Receptor proteins	For sugars, vitamins, amino acids, etc
Other proteins	Enzymes, extracellular protein export

From Kim and Gadd (2008).

E. coli's growth temperature range is from 15 to 45 °C, with an optimal growth temperature of 37 °C (Willshaw and others 2000). Isolates are serologically

differentiated on the basis of three major surface antigens: the O (somatic), H (flagellar) and K (capsular) antigens (Doyle and others 1997; Jay and others 2005). *E. coli* is widely distributed as a commensal microorganism in the intestinal tracts of humans and warm-blooded animals (Doyle and others 1997; Willshaw and others 2000). However, some *E. coli* strains are pathogenic and capable of causing enteric disease (Doyle and others 1997; Willshaw and others 2000). Depending on its pathogenicity, *E. coli* is divided into specific pathotypes: enteropathogenic *E. coli* strains (EPEC), enterotoxigenic *E. coli* (ETEC), enteroinvasive *E. coli* (EIEC), diffuse-adhering *E. coli* (DAEC), enteroaggregative *E. coli* (EAEC), and enterohemorrhagic *E. coli* (EHEC) (Doyle and others 1997). Certain O serogroups are associated with the different pathogenic types of *E. coli* (Hitchins and others 1992). The major O serogroups associated with EPEC, ETEC, EIEC, and EHEC are listed in Table 3-2.

Table 3-2. Selected serotypes within groupings of pathogenic *Escherichia coli.*

Pathogenic type	Serogroups
EPEC	O55, O86, O11ab, O119, O125ac, O126, O127, O128ab, O142
ETEC	O6, O8, O15, O20, O25, O27, O63, O78, O85, O115, O128ac, O148, 0159, O167
EIEC	O112, O124, O136, O143, O144, O152, O164, O167
EHEC	O26, O103, O104, O111, O157

From Hitchins and others (1992), and Doyle and others (1997).

3.1.2 *Escherichia coli* Pathotypes

3.1.2.1 Enteropathogenic *Escherichia coli*

The enteropathogenic *E. coli* (EPEC) can cause severe watery diarrhea accompanied by vomiting and fever, and are mainly associated with infants and young children under the ages of 3-5 years (Knutton and others 1987; Clarke and others 2003; Jay and others 2005). These *E. coli* strains generally do not produce detectable quantities of enterotoxins (Doyle and others 1997; Jay and others 2005). They contain adherence factor plasmids, possess a chromosomal gene *eaeA* and deliver effector proteins, such as translocated intimin receptor (Tir), EspB, Map, EspF, EshH, and EspG, which induce the attaching and effacing (A/E) lesions on intestinal epithelial cells of the small bowel (Donnenberg and others 1989; Doyle and others 1997; Jay and others 2005; Shaw and others 2005). The A/E lesions are characterized by localized destruction of brush border microvilli, intimate bacterial attachment, and cytoskeletal accretion beneath intimately attached bacteria (Shaw and others 2005).

The adherence process starts at the first contact and it is hypothesized that plasmid-encoded bundle–forming pili aid the attachment (Jay and others 2005). Actin polymerization beneath bacteria results in bacteria sitting on raised pedestal-like structures (Clarke and others 2003; Shaw and others 2005). Consequently, the formation of A/E lesions results in a reduction in the absorptive capacity of the intestinal mucosa, leading to disruption of the electrolyte balance and diarrhea (Clarke and others 2003).

3.1.2.2 Enterotoxigenic *Escherichia coli*

Disease caused by infection with the enterotoxigenic *E. coli* (ETEC) is described as a watery diarrhea with abdominal cramps, fever, malaise, nausea with or without vomiting, chills, loss of appetite, headache, muscle aches, and bloating (Willshaw and others 2000; CDC 2005). Globally, 400 million ETEC-associated episodes of diarrhea occur annually, with an estimated 700,000 deaths (Mahdy and others 2010). These strains are known as the leading cause of travelers' diarrhea, and it has been estimated that 10^8-10^{10} CFU of an ETEC strain are necessary for diarrhea in adult humans (Willshaw and others 2000; Jay and others 2005). Sporadic ETEC outbreak infections have been related with consumption of contaminated fresh salad leaves (Shaw and others 2010). In 2010, a series of 11 outbreaks traced with lettuce occurred in Denmark, causing 260 cases with symptoms of gastroenteritis (Ethelberg 2010).

ETEC strains attach to and colonize the proximal small intestine by fimbrial colonization factor antigens (CFA I, II, III, and IV) (Doyle and others 1997; Jay and others 2005; Ochoa and others 2010). Bacterial pili or fimbriae have been proposed as the fibrils that mediate attachment to surface of other bacteria, to host cells of animals and plants, and to solid surfaces (Kim and Gadd 2008). Bacterial toxins damage the host cells by different modes of action; therefore, they are grouped into those that (i) help bacteria spread in tissues, (ii) lyse host cells, (iii) block protein synthesis, and (iv) act pharmacologically by elevating or depressing normal cell functions (Mahdy and others 2010). ETEC strains produce heat-labile (LT) or heat-stable (STa or ST-1, and STb or ST-II) enterotoxins that elicit fluid accumulation and diarrheal response (Doyle and

others 1997; Jay and others 2005; Mahdy and others 2010). The LT toxin (molecular weight of approximately 91 kDa, destroying time at 60 °C is about 30 minutes) is a protein with enzymatic activity similar to that of the cholera toxin, and is deposited into the periplasm of producing cells (Jay and others 2005). LT is also known as the major virulence determinant of ETEC strains (Mahdy and others 2010). ST toxins are destroyed at 100 °C in about 15 minutes, and some are methanol soluble (Jay and others 2005).

3.1.2.3 Enteroinvasive *Escherichia coli*

Enteroinvasive *E. coli* (EIEC) are biochemically atypical because they do not ferment lactose, are anaerogenic and lysine decarboxylase-negative (Willshaw and others 2000). This category of *E. coli* may produce an illness known as bacillary dysentery that occurs within 12 to 72 h following ingestion of contaminated food (FDA 2009). Bacillary dysentery is an acute ulcerative infection of the human large intestine (Hsu and others 2010). The strains generally do not produce enterotoxins; their invasive capacity is associated with the presence of 140 MDa plasmids that encode several outer membrane proteins involved in invasiveness (Doyle and others 1997; Jay and others 2005). EIEC principally invade and/or penetrate the colonic mucosa and multiply within the epithelial cells of the large intestine, resulting in an intense inflammatory response characterized by abscesses and ulceration, causing cell death (Gross and others 1983; Bando and others 2010). Illnesses caused by EIEC strains may also include non-bloody watery diarrhea, fever, malaise, toxemia, and abdominal cramps (FDA 2009; Hsu and

others 2010), and their infective dose is between 10^6 and 10^{10} organisms (Hsu and others 2010).

3.1.2.4 Diffuse-adhering *Escherichia coli*

According to Doyle and others (1997), the diffuse-adhering *E. coli* (DAEC) cause mild diarrhea without blood or fecal leucocytes. Diarrhea produced by DAEC is characterized by mucus-containing watery stools with some fever and vomiting (Willshaw and others 2000). The DAEC have been identified from covering the available cell surface uniformly following their characteristic diffuse-adherent pattern of adherence (Girón and others 1991; Doyle and others 1997; Scaletsky and others 2002; Servin 2005; Ochoa and others 2010).

DAEC do not produce heat-labile or heat-stable toxins (Doyle and others 1997). DAEC strains are grouped in two classes (Servin 2005). The first class of DAEC strains includes *E. coli* strains that comprise Afa/Dr adhesins which are associated with urinary tract infections, and the second class of DAEC strains includes *E. coli* strains that express an adhesin involved in diffuse adherence (Servin 2005). In developing countries, DAEC strains are highly associated with diarrhea, which is the major cause of infantile morbidity and mortality (Gomes and others 1988; Girón and others 1991; Gonzalez and others 1997; Scaletsky and others 2002).

3.1.2.5 Enteroaggregative *Escherichia coli*

Enteroaggregative *E. coli* (EAEC) have been related with persistent diarrhea that can last more than 14 days, especially in children in developing and developed countries and are also an important cause of traveler diarrhea (Willshaw and others 2000; Okeke

and Nataro 2001; Jay and others 2005). These EAEC strains are epidemiologically implicated in the onset of acute and chronic diarrhea, shortening of intestinal villi, edema, and mononuclear cell infiltration of the sub-mucosa (Girón and others 1991). EAEC strains do not show localized adherence and exhibit a stacked brick appearance type of adherence to HEp-2 cells, and aggregative expression due to a 60-MDa plasmid, which is needed for the production of fimbriae (Girón and others 1991; Yamamoto and others 1992; Okeke and Nataro 2001; Aslani and others 2010). Some EAEC strains are able to produce a heat-stable enterotoxin known as EAST1 (Jay and others 2005). EAEC is often seen in symptom-free people; however, EAEC is the probable cause of diarrhoea in AIDS patients (Okeke and Nataro 2001). Gastrointestinal inflammatory response resulting in appearance of gross mucus and blood cause by EAEC has been also reported (Steiner and others 1998).

3.1.2.6 Enterohemorrhagic *Escherichia coli*

Enterohemorrhagic *E. coli* (EHEC) strains possess a chromosomal gene *eaeA* and produce attachment effacement (A/E) lesions (Jay and others 2005). They produce a plasmid (60 MDa) that encodes fimbriae for attachment mediation to culture cells (Jay and others 2005). These strains of *E. coli* affect only the large intestine, producing cytotoxic factors that are described as verotoxins or Shiga-like toxins (SLTs) (Doyle and others 1997; Jay and others 2005). The two prototypes of EHEC shiga-like toxins are referred to as Stx1 and Stx2, and some EHEC Shiga-like toxins genes are encoded by temperate bacteriophages (Jay and others 2005). Serotype O157:H7 is the predominant cause of EHEC-associated disease in the United States (Scallan and others 2011a).

Escherichia coli O157:H7 is an enterohemorrhagic serotype that was first identified as foodborne pathogen in 1982 (Karmali and others 1983). *E. coli* O157:H7 is also a shiga toxin-producing E. coli (STEC) that has been recognized as a cause of severe human gastrointestinal disease (Wendel and others 2009).

3.2 *Escherichia coli* O157:H7

E. coli O157:H7 is an acid tolerant pathogen that has been studied in broth and food systems (Leyer and others 1995; Doyle and others 1997; Stopforth and others 2003). *E. coli* O157:H7, when inoculated at high levels, survived in mayonnaise (pH 3.6 to 3.9) for 5 to 7 weeks at 5 °C, and for 1 to 3 weeks at 20 °C (Zhao and Doyle 1994). It survives in acidic foods such as pressed fruit juices; researchers demonstrated previously the ability of the organism to survive in apple cider (pH 3.6 to 4.0) for 10 to 31 days at 8 °C, and 2 to 3 days at 25 °C (Doyle and others 1997; Jay and others 2005).

Thermal sensitivity studies have confirmed that *E. coli* O157:H7 does not have an unusual resistance to heat, with a $D_{60°C}$ of 45 sec (Doyle and others 1997). Thermal $D_{60°C}$ values of *E. coli* O157:H7 in different meat products are 45-47 sec for beef, 37-55 sec for pork sausage, 38-55 sec for chicken, and 55-58 sec for turkey (Jay and others 2005). When the fat content of the product increases, the $D_{60°C}$ value for *E. coli* O157:H7 increases (Jay and others 2005). In addition, *E. coli* O157:H7 thermal sensitivity increases when pH decreases in the presence of L-malic acid and benzoic acid (Doyle and others 1997; Jay and others 2005).

It has been demonstrated that *E. coli* O157:H7 can survive for extended periods of time in the environment, including cattle manure, soil, water and vegetables (Wang

and Doyle 1998; Johannessen and others 2004; Erickson and others 2010). In addition, according to Wang and Doyle (1998), *E. coli* O157:H7 survival in water is better at 8 °C than at 15 °C and 25 °C.

E. coli O157:H7 strain incidence and prevalence in meat, milk, poultry, and seafood products is very variable (Jay and others 2005). In fact, outbreaks of *Escherichia coli* O157:H7 are generally associated with ground beef and dairy products. However, from 1991 to 2002, 21% of *E. coli* O157:H7 outbreaks were linked to water, fruits and vegetables, including cantaloupe, lettuce, alfalfa, and apple cider products (Ackers and others 1998; Aruscavage and others 2006; Gragg and others 2010). In addition, according to Amber Waves (2007), *E. coli* O157:H7 illnesses linked to leafy green produce reached record highs in 2006 (Figure 3-2).

In September 2006, a multistate outbreak of *E. coli* O157:H7 infection related with the consumption of packaged spinach occurred in Wisconsin, involving 205 laboratory-confirmed cases in the United States. This outbreak resulted in 103 hospitalizations, 31 cases of hemolytic uremic syndrome (HUS), and 3 deaths (Wendel and others 2009). The California Department of Public Health (CDPH 2007) reported that the presence of wild pigs in and around spinach fields and the proximity of irrigation wells exposed to feces from cattle and wildlife were potential environmental risk factors for *E. coli* O157:H7 contamination on spinach.

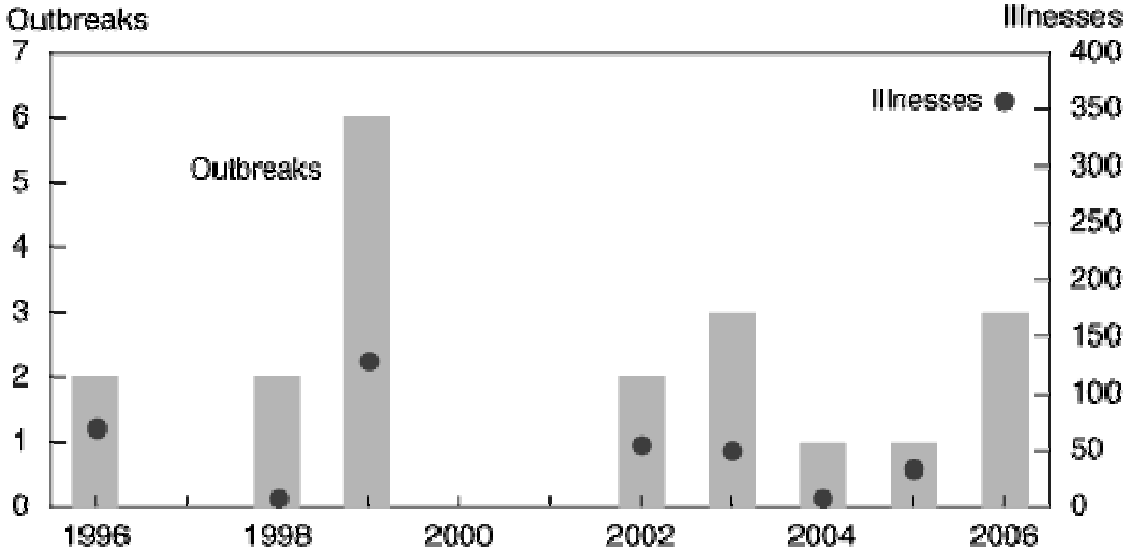

Figure 3-2. *E. coli* O157:H7 illnesses linked to leafy greens reached (Amber Waves 2007).

3.3 *Salmonella enterica*

3.3.1 Introduction

Salmonella enterica subsp. *enterica* are facultative anaerobic, Gram-negative, and non-sporulating rods belonging to the family *Enterobacteriaceae* (D'Aoust 1997). Based on DNA hybridization and electrophoretic characterization, *Salmonella* serovars are divided into two species: *S. enterica* and *S. bongori* (D'Aoust 2000). In addition, *S. enterica* specie type consists of six subspecies (*enterica, salamae, arizonae, diarizonae, houtenaem* and *indica*) (D'Aoust 2000). Members of this genus are motile by peritrichous flagella, except serovars Pullorum and Gallinarum (D'Aoust 2000). Epidemiologically, *Salmonella* can be placed intro three groups: human infectious only, host-adapted serovars, and un-adapted serovars (Jay and others 2005). Those that infect humans only include *S.* Typhi, *S.* Paratyphi A and C (Jay and others 2005). Smith and

others (2005) refer to *Salmonella* as the deadliest foodborne pathogen that cause more than 1,000 deaths each year. Enteric (typhoid) fever, uncomplicated enterocolitis, and systemic infections are some of the clinical conditions due the human *Salmonella* foodborne infections (D'Aoust 1997).

Salmonella grow optimally at 35 to 37 °C, are oxidase negative, catalase positive, use citrate as the sole carbon source, and do not hydrolyze urea (D'Aoust 1997; Jay and others 2005). When tested on triple sugar iron (TSI), *Salmonella* typically produces gas and acid from glucose, but do not utilize lactose and sucrose at 35 °C for 24 ± 2 h (D'Aoust 1997; Jay and others 2005). The typical appearance for a *Salmonella* isolate on TSI consist of a positive yellow butt from glucose fermentation and a positive blackenig from hydrogen sulfite (H_2S) formation. For *Salmonella* serotyping, species and serovars are placed in groups designated A, B, C, and so on, in relation to similarities in content of one or more O antigen (Jay and others 2005). For additional identification, the flagellar (H) antigens are determined as either specific phase (phase 1), or group phase (phase 2) type (Jay and others 2005; D'Aoust 1997). Furthermore, to characterize antibiotic resistance of *Salmonella spp.*, such as *S.* Typhimurium definitive type 104 (DT104), the resistance to antibiotics such as the penicillins (e.g. amipicillin), chloramphenicol, streptomycin, sulfa drugs, and tetracyclines is tested (Jay and others 2005).

According to Aruscavage and others (2006), *Salmonella* is the most common bacterial pathogen associated with fruits and vegetables. During 2005 and 2006, four large multistate outbreaks of *Salmonella* infections linked to raw tomatoes at restaurants

occurred in the United States, which resulted in 459 culture-confirmed cases of salmonellosis in 21 states (CDC 2011). In addition, in 2008, a very large multistate outbreak associated with raw produce, including jalapeno, serrano peppers, and tomatoes, was caused by *Salmonella* Saintpaul, reporting 1442 persons infected with Salmonella Saintpaul in 43 states, the District of Columbia, and Canada (CDC 2008; Pan and Schaffner 2010). Currently, the range of *Salmonella* prevalence on U.S. domestic produce is estimated at 0.0 to 6.3% (Erickson and others 2010). Table 3-3 provides more examples of human outbreaks of salmonellosis due to produce contamination.

Table 3-3. Salmonellosis outbreaks in the United States due to fresh produce.

Produce	*Salmonella* serotype	Year	Cases
Alfalfa	Enteriditis	2011	21
Alfalfa	Saintpaul	2009	235
Alfalfa	I 4	2010	140
Cantaloupe	Litchfield	2008	51
Cantaloupe	Panama	2011	20
Tomatoes	Typhimurium	2006	183
Serrano peppers	Saintpaul	2008	1442

From CDC 2011.

CHAPTER IV

LACTIC ACID BACTERIA

4.1 Introduction

The lactic acid bacteria (LAB) are a group of Gram-positive, non-spore forming, aerotolerant anaerobic catalase-negative bacteria that mainly produce lactic acid as a fermentation product (Axelsson 1998; Madigan and others 2000). This group is comprised of the following genera: *Aerococcus*, *Alloicoccus, Carnobaacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus*, *Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus*, *Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella* (Axelsson 1998).

The industrial uses of LAB (e.g. production of cheeses, cocoa, coffee, beer, wine, fermented vegetables, etc.) depend on the fermentation product(s) formed from carbohydrates, which provide specific characteristics to a food product (Mayra-Makinen and Bigret 1993; Buckenhuskes 1997; Thompson and others 1997; Campbell 1997; Fleet 1997). The fermentation products formed by glycolysis (oxidative conversion of glucose to pyruvic acid) are shown in Figure 4-1 (Madigan and others 2000; Pelicano and others 2006). The homofermentative group produces only lactate from the reduction of pyruvic acid (Axelsson 1998; Tortora and others 1982). The heterofermentative subgroup may also produce ethanol, acetate and CO_2, in addition to lactate (Axelsson 1998).

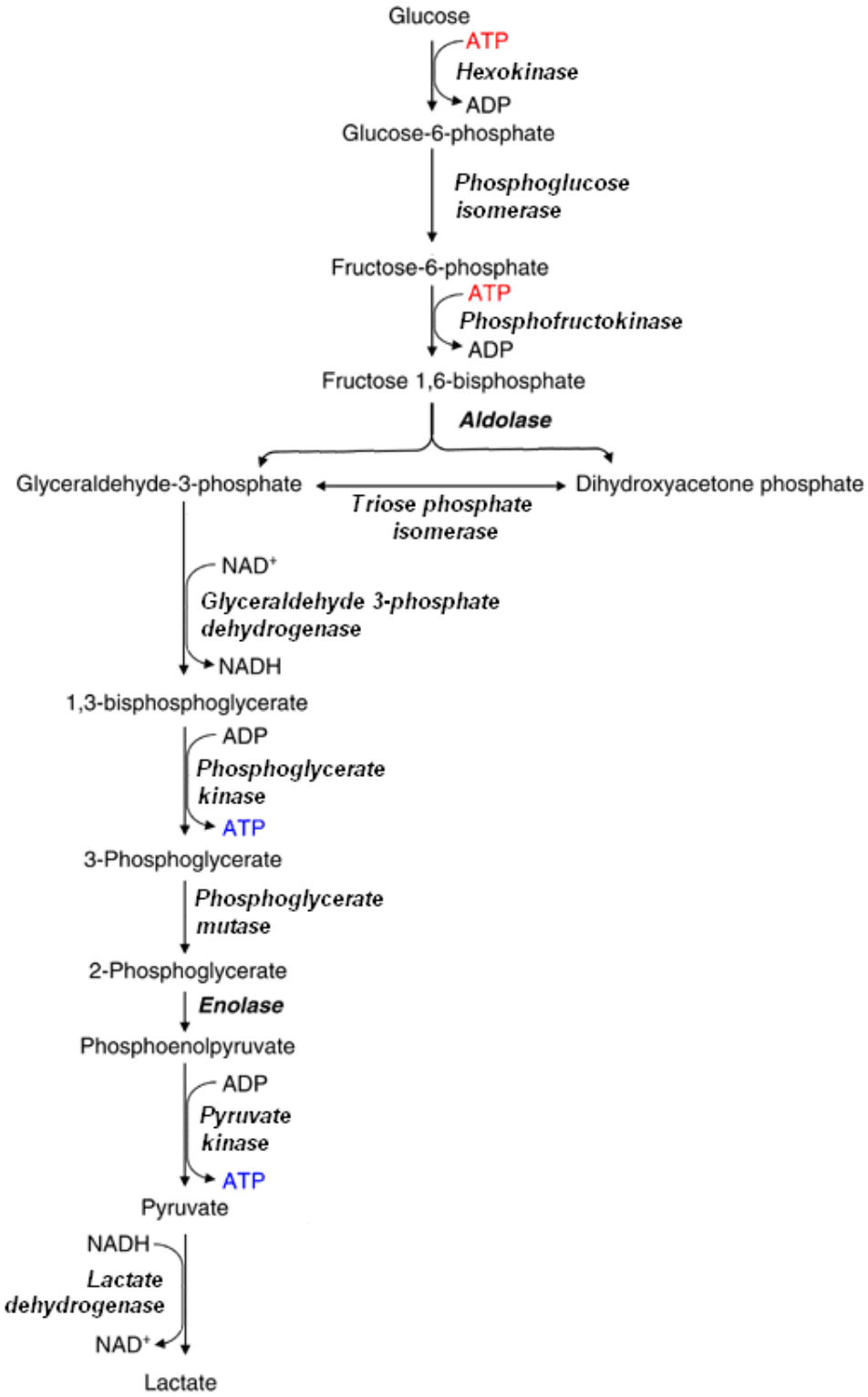

Figure 4-1. The glycolytic pathway. Adapted from Pelicano and others (2006).

Since Metchnikoff discussed mechanisms of action of lactobacilli in inhibiting undesirable intestinal microflora, the antimicrobial activity of lactic acid bacteria has been studied (Barefoot and Klaenhammer 1983). Consequently, LAB have been more recently used to control *E. coli* O157:H7, *Salmonella*, and *Listeria monocytogenes* in ground meat (Smith and others 2005), ready-to-eat (RTE) meats (Amézquita and Brashears 2002), and fresh or minimally processed vegetables, including fresh spinach (Gragg and Brashears 2010, Gragg and others 2010). The LAB strains used for previous studies are *Lactobacillus animalis* (LA51), *Lactobacillus amylovorus* (M35), and *Pediococcus acidilactici* (D3), which are commercialized as a LAB flash-freeze product (LactiGuard™, Nutrition Physiology Corp) (Gragg and Brashears 2010; Gragg and others 2010). The metabolic activity of LAB produces inhibitory compounds that may be able to reduce pathogens in food products (Gragg and Brashears 2010; Carvalho and others 2009). These antimicrobial compounds are divided in two groups: non-peptide inhibitors, and peptide/protein inhibitors (Davidson and Hoover 1993).

4.2 Non-peptide Antimicrobial Substances Produced by LAB

4.2.1 Introduction

Acetic acid, lactic acid, hydrogen peroxide, and diacetyl are some non-peptide inhibitors produced by members of the lactic acid bacteria (Davidson and Hoover 1993). Some LAB, such as *Leuconostoc citrovorum*, are inhibitory for *Salmonella* Gallinarum, *Pseudomonas fragi*, *P. putrefaciens*, *P. fluorescens*, *Staphylococcus aureus*, *Aerobacter aerogenes*, *Alcaligenes viscolactis*, and *E. coli* because of their ability to produce these and other non-peptide antimicrobials (Keenan 1968; Sorrells and Speck 1970). Likewise,

diacetyl has been shown to exhibit inhibitory ability against *L. monocytogenes* (O'Bryan and others 2009), *Mycobacterium tuberculosis*, *Corynebacterium diphtheriae*, *Enterobacter aerogenes*, *E. coli*, *K. pneumoniae*, *Mycobacterium phlei*, *Neisseria gonorrhoeae*, *Pseudomonas* spp., *Salmonella* spp., *S. aureus*, and *Streptococcus* spp. (Jay and thers 2005). This is supported by the hypothesis that when the bacterial cell is exposed to low pH conditions (pH < 6.0), the cell tries to maintain a pH consistent with viability through three progressively mechanisms, which are the homeostatic response, the acid tolerance response, and the synthesis of acid shock proteins, until the environment becomes suitable; otherwise, lysis of the cell occurs (Hartman 1997).

4.2.2 Lactic Acid

Lactic acid ($CH_3CHOHCOOH$), also known as 2-hydroxypropionic acid, is a milk-associated acid first isolated by Carl Wilhelm Scheele, a Swedish chemist, in 1780 (Bogaert and Naidu 2000; Yadav and others 2011). About a decade ago, the market for lactic acid and its derivatives amounts to 100,000 metric tons (Bogaert and Naidu 2000). Biologically, lactic acid is formed by reduction of pyruvic acid during anaerobic conditions or in case of increased energy demands (Bogaert and Naidu 2000). *Pediococcus acidilactici* produces lactic acid from dextrose and has been used in chicken salad to prevent the formation of botulinum toxin (Davidson and Hoover 1993). Lactic acid is also produced by several other bacterial species (i.e. *Aerococcus*, *Alloicoccus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus*, *Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Streptococcus, Tetragenococcus, Vagococcus,* and *Weissella*) (Axelsson 1998).

Table 4-1. Lactic acid production as a function of substrate.

Substrate	Microorganism	Lactic acid (g/l)	pH
Glucose	*L. casei* NRRL B441^{-}	82	–
	L. delbrueckii IFO 3534	81	5.0
	L. rhamnosus ATCC 10863	65	5.0
	L. lactis ATCC19435	5.4	5.0
Sucrose	*L. rhamnosus* ATCC 10863	77	6.0
Maltose	*L. lactis* ATCC19435	5.1	5.0
Lactose	*L. delbrueckii* (sp. bulgaricus) NRRL B-548	25	4.5
	L. lactis (var diacetylactis) CNRZ 2125	7	5.0
Whey	*L. delbrueckii* (sp. bulgaricus) ATCC 55163	35	5.4
	L. helveticus NCDO 1844	31	5.6
Sorghum	*L. delbrueckii* (sp. bulgaricus) ATCC11842	–	5.5
	L. plantarum ATCC 14917	–	5.5
Maize + Barley H.	*L. delbrueckii* (mixture of several strains)	59	5.0
Wheat H.	*L. lactis* ATCC19435	3.3	6.0
Soy molasses	*L. salivarius* ATCC11742	5.5	5.6
Cellulose	*L. delbrueckii* (sp. bulgaricus) NRRL B-548	27	4.2

H: Hydrolysate. Adapted from Yadav and others (2011)

Industrial lactic acid productions use species of *Lactobacillus* such as *L. delbrueckii*, *L. amylophilus*, *L. bulgaricus*, and *L. leichmanii* (Jamshidian and others

2010). Trontel and others (2010) modeled the effects of three different substrates (glucose, sucrose and starch) and temperature on the growth and lactic acid production by *Lactobacillus amylovorus*, and it was demonstrated that the maximum values for substrate consumption rate, growth rate, and productivity of lactic acid occurred at 45 °C. Table 4-1 shows the lactic acid production as a function of different substrates (Yadav and others 2011).

4.2.3 Hydrogen Peroxide

Some LAB are aerotolerant obligative anaerobes that possess superoxide dismutase to detoxify superoxide ($O_2^{\cdot-}$) to produce hydrogen peroxide (H_2O_2) (Kim and Gadd 2008). Hydrogen peroxide, formed by superoxide dismutase ($2O_2^- + 2H \rightarrow H_2O_2 + O_2$) and/or by the uncatalyzed reaction of hydrogen radicals, is scavenged by catalase which calalyzes the dismutation of hydrogen peroxide into water and oxygen ($H_2O_2 + H_2O_2 \rightarrow 2\ H_2O + O_2$) (Stryer 1995). Peroxidase is another enzyme that removes hydrogen peroxide ($H_2O_2 + RH_2 \rightarrow 2H_2) + R$) (Kim and Gadd 2008; Marty-Teysset and others 2000).

Delbes-Paus and others (2009) showed the capability of *Lactococcus garviae* to inhibit *S. aureus* by the production of hydrogen peroxide in raw milk. In addition, Batdorj and others (2007) demonstrated that *L. delbrueckii* subsp. *lactis* are able to inhibit Gram-positive (e.g. *S. aureus* and *Listeria innocua*) and Gram-negative bacteria (e.g. *E. coli*) through H_2O_2 production. However, the presence of catalase or other peroxidases in these foodborne pathogens can increase tolerance to lower concentrations (McDonnell and Russell 1999). Hydrogen peroxide acts as an oxidant, producing free

radicals of hydroxyl ($^{+}$OH), damaging cell components, such as lipids, proteins, and DNA (McDonnell and Russell 1999).

4.3 Polypeptide Antimicrobial Substances Produced by LAB

4.3.1 Introduction

Bacteriocins are antimicrobial peptides or proteins produced by members of the LAB able to inhibit various foodborne pathogens and spoilage bacteria (Zouhir and others 2010; Jack and others 1995), and are usually related to the producer strain (Ayad and others 2002; Todorov 2010). In general, bacteriocins are cationic (i.e. contain excess of lysyl and arginyl residues) amphipathic molecules composed of 12-45 amino acid residues (Moll and others 1999). They can vary in their molecular weight, biochemical properties, activity spectra and mechanism of action (Davidson and Hoover 1993).

Bacteriocins produced by LAB are grouped into four classes (Figure 4-2): class I (lantibiotic peptides), class II (small non-modified peptides with molecular mass <10kDa), class III (large heat labile proteins with molecular mass >10kDa), and class IV (complex cyclic peptides whose activity requires the association of carbohydrate or lipid moities (Cotter and others 2006; Nissen-Meyer and others 2009; Carvalho and others 2010).

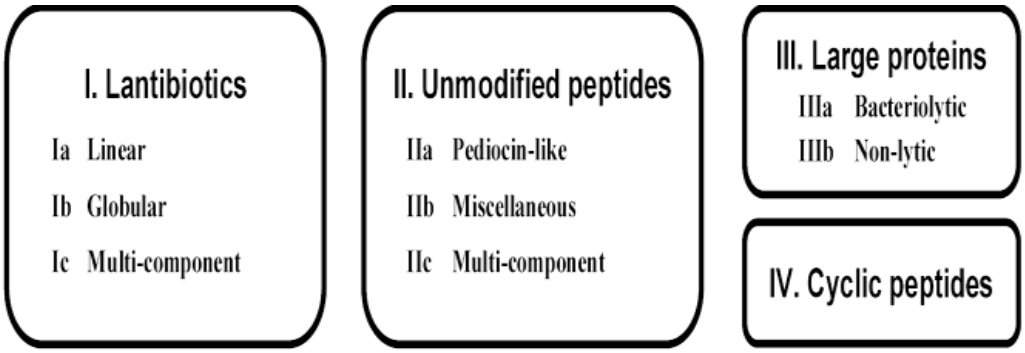

Figure 4-2. Bacteriocin classification scheme (Heng and Tagg 2006).

Class I and II bacteriocins are the best characterized (Callewaert and others 1999). Class I lantibiotic bacteriocins undergo post-translational modifications before they are exported from the cell that result in the formation of thioether amino acids or lanthionine (Moll and others 1999; Callewaert and others 1999; Foulquié Moreno and others 2008; Héchard and Sahl 2002). The lantibiotic designation originates from the presence of lanthionine ring-containing peptide antibiotics which is a nonproteinogenic aminoacid (Héchard and Sahl 2002; Levengood and others 2009). Nisin is classified as a class IA lantibiotic (Miller and others 2010; Héchard and Sahl 2002; Asaduzzaman and Sonomoto 2009). It is a small heat-stable polypeptide produced by some strains of *Lactococcus lactis* subsp. *lactis* (Mastromatteo and others 2010; Miller and others 2010). Some natural nisin variants, such as A, Z, F, U and Q, have been characterized (Piper and others 2011; Delves-Broughton and others 1996; Yoneyama and others 2008). Nisin Z was described to have a substitution of His^{27} for Asn^{27} (Figure 4-3), and Nisin Q exhibited 82% homology with nisin A cluster (Yoneyama and others 2008). It is a ribosomally synthesized peptide that contains 34 amino acids; post-translationally

modified residues include β-methyllanthionine, dehydroalanine, and dehydrobutyrine (Miller and others 2010; Gross and Morell 1971).

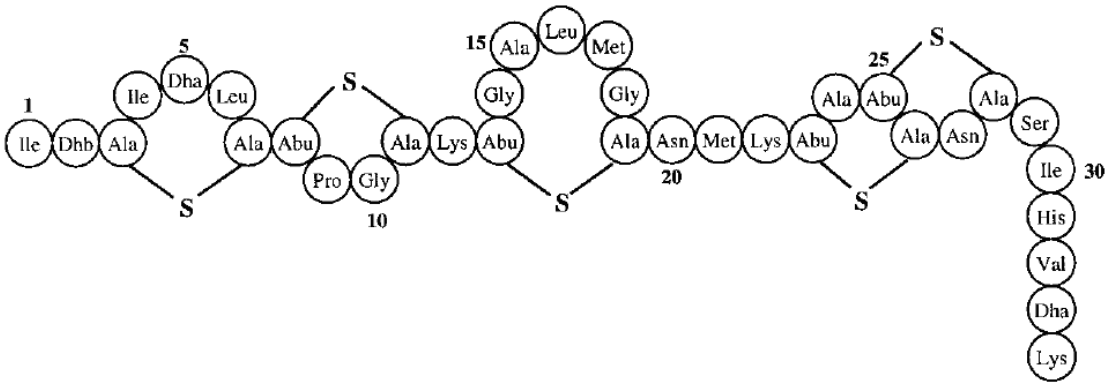

Figure 4-3. Structure of Nisin Z (Gross and Morell 1971).

Class II bacteriocins are small, heat stable, unmodified, cationic and hydrophobic peptides (Moll and others 1999; Callewaert and others 1999; Héchard and Sahl 2002). They have a proteolytic cleavage of the N-terminal extension at a Gly-Gly processing site (Callewaert and others 1999). Pediocins (produced by *Pediococcus* strains) are class IIa bacteriocins, which are small, heat-stable, non-modified peptides with strong antilisterial activity (Eom and others 2010; Davidson and Hoover 1993; Mandal and others 2010), and are characterized by a YGNGVXC motif in their N terminus (Callewaert and others 1999). Class IIb bacteriocin activity depends on two-peptide distinct peptides (Héchards and Salh 2002). Lactacin F, lactococcin G, plantaricin EF and JK are some examples of bacteriocins included in this group (Héchards and Salh 2002). *Lactobacillus acidophilus* strains are able to produce multiple bacteriocin-type

compounds: lactocidin, acidolin, acidophilin, lactacin B, and lactacin F, which are also class IIa bacteriocins (Davidson and Hoover 1993). Class IIc bacteriocins includes miscellaneous peptides, which are diffent from Class IIa and IIb (Callewaert and others 1999; Hechard and Sahl 2002).

Diplococcin is produced by some strains of *L. lactis* ssp. *cremoris* (Davidson and Hoover 1993; Ayad and others 2002), and *Streptococcus cremoris* (Davey and Richardson 1981). Diplococcin is a class III bacteriocin, which is considered a normal constituent of the bacterial cell and its named is due to the diplococcal arrangement that the producing bacteria exhibit (Oxford 1944).

4.3.2 Mode of Action

The LAB-synthesized bacteriocins inactivate sensitive bacteria by inserting themselves into the cytoplasmic membrane, resulting in pore formation, membrane permeabilization, and leakage of essential molecules (Moll and others 1999; Foulquié Moreno and others 2008). Particularly, the mode of action of nisin is one of pore formation initiated by the electrostatic attraction of the cationic nisin to the negatively charged phospholipids of the Gram-positive bacteria cell membrane, which is forced and bent by perpendicular orientation, allowing the pore to open (Abee and others 1994; O'Bryan and others 2009). The affected bacteria die as a result of energy depletion and stagnation of intracellular biosynthetic processes (Ruhr and Sahl 1985).

The mode of action of class II bacteriocins is thought to result from a bundle of α-helical peptides, which may enhance membrane permeability by the formation of a barrel stave and the carpet mechanisms (Moll and others 1999). The barrel stave-like

pore is formed when the hydrophilic faces of a bundle of amphipathic α-helical peptides form the inner wall of the water-filled pore (Moll and others 1999). The carpet mechanism takes place when the outer hydrophobic side of single peptide molecules is oriented parallel to the membrane surface and interfere with the membrane bilayer organization, the membrane will temporarily collapse because of local and transient permeability due to strong phospholipid mobilizing activity (Moll and others 1999).

4.3.3 Isolation, Characterization, and Quantification

Bacteriocin isolation, characterization, and quantification has been achieved by different methods. Chromatographic isolation protocols by ion-exchage on carboxymethyl cellulose have been proposed for the analytical purification of lactic acid bacteria bacteriocins (Hickey and others 2003). Chromatography is usually applied after a first concentration step by salt and/or ammonium sulfate precipitation (Bhunia and others 1987; Yang and others 1992; Hickey and others 2003; Foulquié Moreno and others 2008; Korobov and others 2009; Abdel-Mohsein and others 2011; Xie and others 2011), or acid extraction (Chumchalova and others 2004; Taylor and others 2007; Carvalho and others 2010). The high concentration of salts reduces the protein solubility, creating the effect called salting out (Lubert 1995); in contrast, the efficiency of extraction with acids or organic solvents relies on the hydrophobic character of the bacteriocin molecule (Chumchalova and others 2004).

The electrospray mass spectrometric and SDS-PAGE (sodium dodecyl sulfate polyacrylamide gel electrophoresis) methods are used to determine molecular mass of a bacteriocin (Callewaert and others 1999). Electrospray mass spectrometry consists of an

atmospheric pressure mass spectrometer equipped with an electrospray ionization source (micromass) (Callewaert and others 1999). Electrophoresis is the migration of charged particles through viscous medium (gel or buffer) in an electric field (Stryer 1995). The anionic detergent SDS solubilizes most proteins, denatures proteins to provide uniform shape, and makes insignificant the natural charge controlled by pH of proteins, allowing a direct relationship of the mobility of each protein with the logarithm of the molecular weight (Stryer 1995). Following, the protein bands are then stained with Coomassie brilliant blue or silver dye to be compared against the standard proteins (Bhunia and others 1987; Contreras and others 1997).

The N-terminal amino acid sequencing is also a method of bacteriocin characterization and can be performed by an automated Edman degradation sequencer (Edman 1950; Callewaert and others 1999). Following isolation of the peptide, 2 ml of an anhydrous solution of hydrogen chloride in nitromethane is added to a tube containing the dehydrated peptide (Edman 1950). The tube is immersed in a water bath at 40 °C and then stirred to form a fine suspension (Edman 1950). Finally, the insoluble material is filtered off and the filtrate transferred to a different test tube to be evaporated with nitrogen (Edman 1950). Then, 2 ml of 0.25N barium hydroxide are added and the tube is sealed and incubated for about 48 h (Edman 1950). This procedure sequentially removes one residue at a time form the amino end of a peptide (Lubert 1995).

To measure antimicrobial activity of bacteriocins and inhibitory spectrum, the smallest amount of antimicrobial agent or the minimum inhibitory concentration (MIC) needed to inhibit the growth of a target organism is determined by the tube dilution

technique (Madigan and others 2000) or the recently modified method know as the fractional inhibitory concentration which is calculated using the checkerboard method with 96-well microtitre plates (Gutierrez and others 2008). Another commonly used procedure to measure bacteriocin activity is the agar spot test or agar diffusion method (Yang and others 1992; Chumchalova and others 2004; Batdorj and others 2007; Foulquié Moreno and others 2008). This technique consists of a Petri plate overlayed with an indicator microorganism (such as *L. delbruekii* subsp *lactis*) inoculated agar (Chumchalova and others 2004). Then, known amounts of antimicrobial agent are added to wells previously created on the solidified inoculated agar (Batdorj and others 2007). After incubation, a zone of inhibition or halo is created around the well and the diameter is measured, which depends upon the unit of measurement targeted (direct concentration, bioactivity), the solubility of the agent, the diffusion coefficient, and the overall effectiveness of the agent against the indicator organism (Tramer and Fowler 1964).

Following SDS-PAGE, enzyme activity may be measured by gel zymograms (Thammasirirak and others 2006). Polyacrylamide gels containing separated proteases are incubated with specific naphthyl ester substrates and diazotized dye (Lantz and Ciborowski 1994). The proteases are allowed to diffuse from polyacrylamide gels into an underlying agarose indicator gel containing a protein substrate (Lantz and Ciborowski 1994). When lysis (proteolytic degradation of the substrate) in the indicator gel is produced by active bands, it will be visualized as clear zones against an opaque background on dark-field illumination (fibrin-agar gels) or as clear zones against a dark blue background after Coomassie Brilliant Blue staining (Lantz and Ciborowski 1994).

CHAPTER V

MATERIALS AND METHODS

5.1 Foodborne Bacterial Cultures

5.1.1 Pathogen Preparation and Maintenance

Five strains of rifampicin-resistant *Escherichia* coli O157:H7 were used in this study as marker pathogens. Rifampicin-resistant strains were obtained by the procedure reported by Kaspar and Tamplin (1993). The strains are designated as R1, R8, R18, R34, and R41. Strains were obtained from the Center for Food Safety culture collection in the Department of Animal Science at Texas A&M University (College Station, TX) and were isolated from cattle fecal swabs. Cultures were preserved on Tryptic Soy Agar (TSA; Becton, Dickinson and Co., Sparks, MD) slants at 4 °C. Before experimentation, cultures were transferred to 10 ml of Tryptic Soy Broth (TSB; Becton, Dickinson and Co.), and incubated aerobically for 24 hr at 35 °C twice consecutively. Enterotube™ II (Becton, Dickinson and Co.) testing was accomplished according to manufacturer procedures to biochemically confirm isolates as *E. coli*. The RIM® *E. coli* O157:H7 latex agglutination test (Remel, Lenexa, KS) was performed to identify and confirm isolates as *E. coli* serogroup O157:H7 according to manufacturer's instructions.

Rifampicin-resistant strains of *Salmonella enterica* serovars Agona BAA-707 (ATCC, Manassas, VA; alfalfa sprout isolate), Anatum BAA-1592 (ATCC, isolated from the Pennsylvania tomatoes outbreak in 2004), Montevideo BAA-710 (tomato isolate), Michigan (cantaloupe isolate), and Saintpaul (tomato, pepper outbreak isolate

from 2008; CDC 2011), obtained from the Department of Animal Science Center for Food Safety culture collection at Texas A&M University, were used for this study. To obtain rifampicin-resistant strains, the procedure described by Kaspar and Tamplin (1993) was completed. Cultures were maintained on TSA slants at 4 °C. Isolates were transferred to TSB, and incubated at 35 °C for 24 hr of working cultures were performed twice consecutively to gain working cultures. The Enterotube™ II (Becton, Dickinson and Co.) test was also accomplished according to manufacturer procedures to biochemically confirm the *Salmonella* specie. Serovars were serologically identified and confirmed by the U.S. Department of Agriculture – Animal and Plant Health Inspection Service (USDA–APHIS) National Veterinary Diagnostic Laboratory (Ames, IA).

5.1.2 Foodborne Bacterial Cocktail Preparation Procedure

Following duplicate sub-culturing of bacterial pathogens, two ml of each culture (approximately 9.0 $\log_{10}$ CFU/ml) were dispensed with a sterile pipette into a sterile conical tube (50 ml vol., Thermo-Fisher Scientific, Waltham, MA), and centrifuged at 25 °C for 15 min at 1623 x g in a Jouan B4i centrifuge (Thermo Electron Corp.). After gently pouring off the resulting supernatant, the pellet was suspended with 20 ml of 0.1% (w/v) peptone water (Becton, Dickinson and Co.) and washed twice by centrifugation at the same conditions previously mentioned. After the third centrifugation, the pellet was suspended in 20 ml of 0.1% peptone water. Finally, the re-suspended bacterial cocktail was diluted with 0.1% peptone water to a desired number of bacterial cells in diluent prior to experimentation.

5.2 Lactic Acid Bacteria Cultures

The lactic acid bacteria (LAB) viable cultures were obtained from the flash-frozen commercial LAB product LactiGuard™ (Guardian Food Technologies, Overland Park, KS), and were shipped overnight in 5 g pouches (Lot # 101019; Batch # 2994525). As they were received, pouches were stored and maintained at -85 °C until required for use according to manufacturer instructions. Each pouch was opened for single use only. The concentration of the LAB flash-freeze cultures received was approximately 11.0 $\log_{10}$ CFU/g, confirmed by serial dilution and enumeration on de Man, Rogosa, and Sharpe lactobacilli agar (MRS; Becton, Dickinson and Co.) plates, incubated aerobically for 48 hr at 35 °C. Working LAB cultures were weighed aseptically and serially diluted in 0.1% peptone water (Becton, Dickinson and Co.) to achieve a desired number of cells for inoculation and experimentation.

5.3 Preliminary Experiments

5.3.1 Growth of Pathogens and Lactic Acid Bacteria in Liquid Microbiological Media

To validate the ability of pathogens to achieve predictable concentrations prior inoculation of produce surfaces, growth curves of rifampicin-resistant strains of *E. coli* O157:H7, rifampicin-resistant strains of *Salmonella enterica* serovars, and of the LAB cultures comprising the LactiGuard™ product were accomplished in three different liquid microbiological media. In addition, this experiment was performed to identify and validate a medium that would simultaneously satisfy the nutritional requirements of both enteric pathogens and the LAB cultures, working to avoid any possible false positive

bacterial pathogenic inhibition/reduction results. The three media tested were MRS, TSB, and TSB supplemented with 1 g/L Tween 80 (Sigma-Aldrich Co., St Louis, MO; TTSB). Working bacterial cultures were revived and prepared as described above. All cultures were serially diluted in 0.1% (w/v) peptone water, aseptically inoculated (1.0 ml) into bottles containing 99.0 ml of the broth medium to achieve 2.0 $\log_{10}$ CFU/ml, and homogenized by shaking for 1 min. Inoculated bottles were incubated aerobically without shaking at 25 °C. Enumeration of bacterial organisms was performed at 0, 1, 3, 6, 12, 18, 24, and 48 hr post-inoculation. Pathogenic cultures were plated on TSA and lactic acid cultures are plated on MRS.

5.3.2 Validation of Bacterial Recovery from Inoculated Spinach

To experimentally validate the recovery of the pathogen bacterial cocktail by the spot inoculation method, it was necessary to determine the most consistent drying time (0, 30, 60 min, y 24 hr) at a known level of bacterial inoculum and at 25 °C. Commercial bagged spinach obtained from a local supermarket was stored at 4 °C and used within 2 days. Spinach samples of approximately 2 g were aseptically weighed and placed on Petri dishes (100x15 mm) under a bio-safety cabinet. About 10 spots of 10 µl each per 2 g of spinach were inoculated. Then, each sample was aseptically loaded into sterile filtered-stomacher bags (VWR, Arlington Heights, IL) and suspended with 18 ml of 0.1% peptone water. Samples were blended and homogenized (230 rpm) for 1.0 min. Homogenized samples were serially diluted in 0.1% peptone water. Enumeration of *Salmonella enterica* and *E. coli* O157:H7 was performed by selective/differential plating on Petri dishes (100 x 15 mm) and loading inoculated plates with tempered (45 °C)

sterile (15 min, 121 °C) lactose-sulfite-phenol red-rifampicin (LSPR) agar, which contained rifampicin at a concentration of 100 mg/L (Castillo and others 1998). The rifampicin-resistant *Escherichia coli* O157:H7 produced yellow colonies (indicative of lactose fermentation but no sulfite reduction) while colonies with a black center and pink halo were produced by rifampicin-resistant *Salmonella enterica* (indicative of no lactose fermentation and sulfite reduction) (Castillo and others 1998). LSPR plates were incubated aerobically at 35 °C for 24 hr. The assay was replicated identically three times. After the drying time was established, it was necessary to determine the required bacterial inoculum concentration to achieve the target inoculated level on the spinach with inoculums at three concentrations (7.0, 6.0, and 5.0 $\log_{10}$ CFU/ml). Sampling, inoculation, dilution and incubation procedure was performed as mentioned before.

To validate the recovery of the lactic acid bacteria from LactiGuard™ by the spray inoculation method, it was necessary to verify the inoculum concentration required to achieve the targeted inoculation level on the spinach. Commercial bagged spinach was also obtained from a local supermarket, stored at 4 °C and used within 2 days. Spinach samples of approximately 25 g were aseptically weighed and placed on sterilized frames under the biological cabinet. Approximately 10 sprays of 0.1 ml each per 25 g of spinach were inoculated. The inoculum concentrations were 8.0 and 10.0 $\log_{10}$ CFU/ml. Following x min for organisms to adhere to spinach surfaces, samples were aseptically loaded into sterile filtered-stomacher bags (VWR), suspended with 225 ml of 0.1% peptone water, blended and homogenized (230 rpm) for 1.0 min. Homogenized samples

were serially diluted in 0.1% peptone water and plated on MRS, which were incubated aerobically at 35 °C for 48 hr. The assay was replicated identically three times.

5.4 Study 1: Dose Response

5.4.1 *In vitro* Dose Response Assay Measuring Pathogen Inhibition by Lactic Acid Bacteria

The *in vitro* dose response assay consisted of 12 treatments comprised of four inoculation levels of LAB cultures comprised on the (LactiGuard™) (0.0, 5.0, 6.0 and 8.0 $\log_{10}$ CFU/ml), and three levels of cocktailed foodborne pathogens (*Salmonella enterica*, and *E. coli* O157:H7) (0.0, 2.0 and 4.0 $\log_{10}$ CFU/ml). Table 5-1 shows the inoculation levels of combined cultures for each of the 12 treatments of the dose response *in vitro* assay. This assay was performed at 7 °C, under simulated retail aerobic conditions. Temperature was read two times per day by a thermometer placed inside the cold incubator.

Survival of pathogens was quantified by enumeration after 0, 1, 3, 6, 9 and 12 days post-inoculation. Before enumeration of cultures on each testing day, sample tubes were properly homogenized by vortexing for about 1 min. Serial dilutions were completed using 9.9 ml of 0.1% peptone water tubes. Enumeration of *Salmonella enterica* and *E. coli* O157:H7 was performed by selective/differential plating LSPR agar. LAB cultures were enumerated by plating on De Man, Rogosa and Sharpe (MRS) agar. LSPR plates were incubated aerobically at 35 °C for 24 hr. Plates of MRS were aerobically incubated for 48 hr at 35 °C. Due to pathogen cultures ability to growth on MRS plates, enumeration from MRS plates was blanked against population obtained from the

corresponding LSPR plates for each sample. The assay was triplicated identically, and all samples were duplicated for each of the post-inoculation testing days.

Table 5-1. Levels of foodborne cocktailed pathogens and LactiGuard™ to be tested by *in vitro* dose response assays.

Treatment number	Targeted Inoculum Level (log_{10} CFU/ml)	
	Lactic acid bacteria	Pathogen Cocktail
1	0.0	0.0
2	0.0	2.0
3	0.0	4.0
4	5.0	0.0
5	5.0	2.0
6	5.0	4.0
7	6.0	0.0
8	6.0	2.0
9	6.0	4.0
10	8.0	0.0
11	8.0	2.0
12	8.0	4.0

5.4.2 Dose Response on Spinach

About 6 kg of spinach were shipped overnight by Dole Food Company, Inc (Soledad, California) in an isolated container box labeled as perishable product. Upon arrival, spinach was stored at 4 °C and used within 3 days post-receipt. Samples of spinach were aseptically weighed and separated in portions of 25 g, and inoculated according to the treatment level specifications on Table 5-2. Treatments of dose response on spinach were selected depending on results obtained from the in vitro dose response, in which it was expected to have an impact on those with the highest concentration of lab (LactiGuard™) against the three levels of foodborne pathogens. The foodborne pathogen cocktail was inoculated by spot-inoculation, with an approximate of 112 spots of 20 µl each per 25 grams of spinach. Inoculated cocktailed pathogens were allowed to adhere to spinach surfaces for 60 min at 25 °C in a biological cabinet prior to treatment with LAB cultures. LactiGuard™ inoculation was achieved by spray-inoculation to simulate a spray-washing intervention step employed by some post-harvest processing plants, previously described as an efficient application method of antimicrobials (e.g. chlorine solutions) (Beuchat and others 1998). LAB cultures were also allowed to adhere to spinach surfaces for 60 min at 25 °C in a biological-cabinet prior to beginning of experimentation.

Samples of inoculated and treated spinach were aseptically loaded into sterile filtered-stomacher bags (VWR) and stored aerobically at 7 °C for 0, 1, 3, 6, 9, and 12 days. On the experimental day, samples were removed from refrigeration and suspended with 225 ml of 0.1% peptone water. Following dilution, samples were blended and

homogenized (230 rpm) for 1.0 min. Homogenized samples were serially diluted in 0.1% peptone water and bacterial organisms plated on LSPR and MRS. *Salmonella enterica* and *E. coli* O157:H7 survival enumeration was completed by plating with LSPR agar; LAB cultures from LactiGuard™ were enumerated by pour-plating on MRS agar. Pathogen-inoculated LSPR plates were incubated aerobically at 35 °C for 24 hr. Plates of MRS were aerobically incubated for 48 hr at 35 °C.

Table 5-2. Levels of foodborne cocktailed pathogens and LactiGuard™ to be tested by spinach surface dose response assays.

Treatment number	Targeted Inoculum Level (log_{10} CFU/g)	
	Lactic acid bacteria (LactiGuard™)	Pathogen Cocktail
1	0.0	0.0
2	0.0	2.0
3	0.0	4.0
10	8.0	0.0
11	8.0	2.0
12	8.0	4.0

Bacterial enumeration from MRS plates was corrected or blanked against counts from the uninoculated and untreated spinach control plated on MRS plates, and against bacterial enumeration obtained from corresponding LSPR plates for each sample. The assay was repeated identically three times, and all samples were duplicated within each

replicate for each of the post-inoculation testing days. Appropriate controls (samples of untreated-intact spinach) were prepared and handled in identical manner.

5.5 Study 2: Antimicrobial Synthesis *in vitro* and on Spinach Surface by LAB Cultures

The fermentation of antimicrobials by lactic acid bacteria cultures present in the LactiGuard™ product in liquid medium and on surface of spinach leaves was analyzed. Following the dose response assay, microbiologically assayed samples were centrifuged at 2272 x g for 20 min at 25 °C to remove cells, and the cell-free supernatant aliquot was collected and held at 0 °C until further use for following assays (Bhunia and others 1987; De Vuyst and others 1996; Chumchalova and others 2004; Abdel-Mohsein and others 2011; Xie and others 2011). Samples were stored at 4 °C prior to antimicrobial assay.

5.5.1 Detection and Quantification of Fermented L-Lactic Acid

Synthesis of L-lactic acid was confirmed spectrophotometrically using previously reported methods (Bergmeyer and Bernt 1974; Gutmann and Wahlefeld 1974). These methods rely on the formation of nicotinamide adenine dinucleotide (NADH) by the increase in extinction of L-lactate and NAD^+ with the addition of the enzyme lactate dehydrogenase (LDH). Reagent solutions of potassium carbonate (5.0 M, Sigma-Aldrich Co.), perchloric acid (1.0 N, Sigma-Aldrich Co.), hydrazine/glycine buffer (0.4 M hydrazine, Fisher Scientific; 0.5 M glycine, Sigma-Aldrich Co.), β-NAD (40 mM, Fisher Scientific), and LDH suspension (5 mg protein/ml, Sigma-Aldrich Co.) were prepared in advance and kept in closed vessels at 4 °C until required for use. The NAD solution is stable for 4 weeks, the hydrazine/glycine solution is stable for 3 months, and all other

solutions for 1 year (Gutmann and Wahlefeld 1974). All reagents were of highest purity and were bought fresh.

The first procedures of the assays were to treat and stabilize the cell-free samples by deproteinization and neutralization. For deproteinization, 0.5 ml of cell-free supernatant was mixed with 1 ml of the perchloric acid solution. The mixture was then centrifuged at 3000 x g for 10 min at 25 °C. The supernate was collected and neutralized. The neutralization step consisted of the addition of 50 µl of potassium carbonate solution; samples were then incubated for 10 min at 25 °C.

Following neutralization and incubation, 14.0 µl of neutralized protein-free supernatant sample was collected and transferred with a pipette into wells of a clear and flat bottom 96-well microplate (Becton Dickinson Falcon, Sparks, MD), and mixed with 175 µl of buffer solution (hydrazine/glycine solution), and 14 µl of NAD^+ solution (Fisher Scientific). For the blank preparation, 14 µl of sample was replaced by perchloric acid solution (1.0 N, Sigma Aldrich) and was processed in identical fashion as treatment samples. The first absorbance reading (E_1) was taken at 25 °C using an Infinite 200 Tecan spectrophotometer (Model M200, Tecan Group Ltd., Durham, NC) at 340 nm. Then, 1.4 µl of LDH suspension (Sigma-Aldrich Co.) was added and mixture was allowed to stand for 60 min at 25 °C. A second absorbance reading (E_2) was measured at the same conditions previously mentioned. The extinction difference of the sample was calculated by following equation:

$$\Delta E \times 2.35 \times DF \qquad (1)$$

in which ΔE is the extinction difference ($\Delta E = E_S - E_B$), and DF is the dilution factor. The extinction difference of the sample (E_S) was determined by the subtraction $E_2 - E_1$. The blank extinction absorbance reading is represented by E_B. The assay was repeated identically three times. Figure 5-1 and Figure 5-2 illustrate the sequence map of treatments on the 96-wells microplate for the *in vitro* dose response-derived cell-free samples and the spinach leaf-derived cell-free samples, respectively. The lactate concentration (µmol/ml) of the sample was therefore calculated by determining the DF with the following equation:

$$DF = \left[\frac{V}{v}\right]_{deproteinization} \times \left[\frac{V}{v}\right]_{neutralization} \qquad (2)$$

in which V is the volume of the assay, and v is the volume of sample used in assay. The calculated DF was 3.06.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Day 0 TRT 1	Day 0 TRT 2	Day 0 TRT 3	Day 0 TRT 4	Day 0 TRT 5	Day 0 TRT 6	Day 0 TRT 7	Day 0 TRT 8	Day 0 TRT 9	Day 0 TRT 10	Day 0 TRT 11	Day 0 TRT 12
C	Day 1 TRT 1	Day 1 TRT 2	Day 1 TRT 3	Day 1 TRT 4	Day 1 TRT 5	Day 1 TRT 6	Day 1 TRT 7	Day 1 TRT 8	Day 1 TRT 9	Day 1 TRT 10	Day 1 TRT 11	Day 1 TRT 12
D	Day 3 TRT 1	Day 3 TRT 2	Day 3 TRT 3	Day 3 TRT 4	Day 3 TRT 5	Day 3 TRT 6	Day 3 TRT 7	Day 3 TRT 8	Day 3 TRT 9	Day 3 TRT 10	Day 3 TRT 11	Day 3 TRT 12
E	Day 6 TRT 1	Day 6 TRT 2	Day 6 TRT 3	Day 6 TRT 4	Day 6 TRT 5	Day 6 TRT 6	Day 6 TRT 7	Day 6 TRT 8	Day 6 TRT 9	Day 6 TRT 10	Day 6 TRT 11	Day 6 TRT 12
F	Day 9 TRT 1	Day 9 TRT 2	Day 9 TRT 3	Day 9 TRT 4	Day 9 TRT 5	Day 9 TRT 6	Day 9 TRT 7	Day 9 TRT 8	Day 9 TRT 9	Day 9 TRT 10	Day 9 TRT 11	Day 9 TRT 12
G	Day 12 TRT 1	Day 12 TRT 2	Day 12 TRT 3	Day 12 TRT 4	Day 12 TRT 5	Day 12 TRT 6	Day 12 TRT 7	Day 12 TRT 8	Day 12 TRT 9	Day 12 TRT 10	Day 12 TRT 11	Day 12 TRT 12

Figure 5-1. Layout for L-lactic acid quantification for *in vitro* dose response-derived cell-free samples on a 96-wells microplate.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank						
B	Day 0 TRT 1	Day 0 TRT 2	Day 0 TRT 3	Day 0 TRT 10	Day 0 TRT 11	Day 0 TRT 12						
C	Day 1 TRT 1	Day 1 TRT 2	Day 1 TRT 3	Day 1 TRT 10	Day 1 TRT 11	Day 1 TRT 12						
D	Day 3 TRT 1	Day 3 TRT 2	Day 3 TRT 3	Day 3 TRT 10	Day 3 TRT 11	Day 3 TRT 12						
E	Day 6 TRT 1	Day 6 TRT 2	Day 6 TRT 3	Day 6 TRT 10	Day 6 TRT 11	Day 6 TRT 12						
F	Day 9 TRT 1	Day 9 TRT 2	Day 9 TRT 3	Day 9 TRT 10	Day 9 TRT 11	Day 9 TRT 12						
G	Day 12 TRT 1	Day 12 TRT 2	Day 12 TRT 3	Day 12 TRT 10	Day 12 TRT 11	Day 12 TRT 12						
H	Sp-CT Day 0	Sp-CT Day 1	Sp-CT Day 3	Sp-CT Day 6	Sp-CT Day 9	Sp-CT Day 12						

Figure 5-2. Layout for L-lactic acid quantification for spinach leaf-derived cell-free samples on a 96-wells microplate. Sp-CT: spinach control aliquot (un-inoculated and untreated).

5.5.2 Spectrophotometric Determination of Hydrogen Peroxide Production

Hydrogen peroxide (H_2O_2) was tested and quantified by the Leuco-Crystal Violet method, a spectral method with maximum absorbance at 590 nm at a pH range of 3.6 – 4.2 (Mottola and others 1970; Cohn and others 2005). This method is sensitive for detection of micromolar concentrations of H_2O_2 (Mottola and others 1970; Cohn and others 2005). When H_2O_2 and horseradish peroxidase (HRP) are present, oxidation of leuco-crystal violet (LCV, $C_{25}H_{31}N_3$) occurs, leading to the formation of stable crystal violet ions (Cohn and others 2005). All reagents used for this assay were of highest purity and obtained from Sigma-Aldrich Co. Solutions of HRP (10 mg in 10 ml of distilled water), LCV (1.31 mM, 50 mg in 100 ml of 0.5% hydrochloric acid), buffer (pH

4.21, 2 M sodium acetate and 2 M acetic acid), and hydrogen peroxide (0.1 M, from 30% hydrogen peroxide solution) were prepared and stored at 4 °C in closed bottles. Before analysis, reagents were brought to 25 °C.

Cell-free samples were first homogenized using via vortexing. The procedure consisted of the transfer of 171.2 µl of homogenized sample with a pipette to wells of a 96-well (300 µl capacity) microplate (Becton, Dickinson and Co.). The sequence map for *in vitro* dose response-derived samples is shown in Figure 5-3, and for spinach leaf-derived cell-free samples in Figure 5-4. Following well inoculation, 20 µl of buffer at pH 4.2, and 6.3 µl (41 µM) of LCV solution were added and mixed. Then, when 2.5 µl (2.5 µg) of HRP solution were added to the mixture (final well volume of 200 µl), a color changed occurred from transparent to blue. The samples were incubated in the dark covered with aluminum foil at 25 °C for 30 min, as suggested by Cohn and others (2005) to ensure stability of the absorbance. After incubation, absorbance values were measured at 596 nm of wavelength using an Infinite 200 spectrophotometer (Model M200, Tecan Group Ltd.). A hydrogen peroxide standard curve was performed for each replicate at the concentrations of 0.0, 0.1, 1, 2, 5, 10, 20, 25, 50, and 100 µM. Absorbance values were measured at the same conditions described for the samples.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank	Blank
B	Day 0 TRT 1	Day 0 TRT 2	Day 0 TRT 3	Day 0 TRT 4	Day 0 TRT 5	Day 0 TRT 6	Day 0 TRT 7	Day 0 TRT 8	Day 0 TRT 9	Day 0 TRT 10	Day 0 TRT 11	Day 0 TRT 12
C	Day 1 TRT 1	Day 1 TRT 2	Day 1 TRT 3	Day 1 TRT 4	Day 1 TRT 5	Day 1 TRT 6	Day 1 TRT 7	Day 1 TRT 8	Day 1 TRT 9	Day 1 TRT 10	Day 1 TRT 11	Day 1 TRT 12
D	Day 3 TRT 1	Day 3 TRT 2	Day 3 TRT 3	Day 3 TRT 4	Day 3 TRT 5	Day 3 TRT 6	Day 3 TRT 7	Day 3 TRT 8	Day 3 TRT 9	Day 3 TRT 10	Day 3 TRT 11	Day 3 TRT 12
E	Day 6 TRT 1	Day 6 TRT 2	Day 6 TRT 3	Day 6 TRT 4	Day 6 TRT 5	Day 6 TRT 6	Day 6 TRT 7	Day 6 TRT 8	Day 6 TRT 9	Day 6 TRT 10	Day 6 TRT 11	Day 6 TRT 12
F	Day 9 TRT 1	Day 9 TRT 2	Day 9 TRT 3	Day 9 TRT 4	Day 9 TRT 5	Day 9 TRT 6	Day 9 TRT 7	Day 9 TRT 8	Day 9 TRT 9	Day 9 TRT 10	Day 9 TRT 11	Day 9 TRT 12
G	Day 12 TRT 1	Day 12 TRT 2	Day 12 TRT 3	Day 12 TRT 4	Day 12 TRT 5	Day 12 TRT 6	Day 12 TRT 7	Day 12 TRT 8	Day 12 TRT 9	Day 12 TRT 10	Day 12 TRT 11	Day 12 TRT 12
H	H_2O_2 0 µM	H_2O_2 0.1 µM	H_2O_2 1 µM	H_2O_2 2 µM	H_2O_2 5 µM	H_2O_2 10 µM	H_2O_2 20 µM	H_2O_2 25 µM	H_2O_2 50 µM	H_2O_2 100 µM		

Figure 5-3. Layout for the leuco crystal violet (LCV) assay for *in vitro* cell-free samples. TRT: treatment.

To reduce the occurrence of false positives (Cohn and others 2005), a second 96-well microplate containing catalase (10000 to 40000 U/mg bovine liver enzyme, Sigma-Aldrich Co.) was completed for all samples and replicates. After the mixture of the cell-free aliquot sample and the buffer solution in the respective well, 12 µl of catalase were added. Following catalase addition, the LCV and HRP solutions were added and incubation was pursued at the same conditions described before. To prepare blanks, 171.2 µl of distilled water were added in place of the sample volume. The whole assay was repeated identically three times.

	1	2	3	4	5	6	7	8	9	10	11	12
A	Day 0 TRT 1	Day 0 TRT 2	Day 0 TRT 3	Day 0 TRT 10	Day 0 TRT 11	Day 0 TRT 12	Sp-CT Day 0					
B	Day 1 TRT 1	Day 1 TRT 2	Day 1 TRT 3	Day 1 TRT 10	Day 1 TRT 11	Day 1 TRT 12	Sp-CT Day 1					
C	Day 3 TRT 1	Day 3 TRT 2	Day 3 TRT 3	Day 3 TRT 10	Day 3 TRT 11	Day 3 TRT 12	Sp-CT Day 3					
D	Day 6 TRT 1	Day 6 TRT 2	Day 6 TRT 3	Day 6 TRT 10	Day 6 TRT 11	Day 6 TRT 12	Sp-CT Day 6					
E	Day 9 TRT 1	Day 9 TRT 2	Day 9 TRT 3	Day 9 TRT 10	Day 9 TRT 11	Day 9 TRT 12	Sp-CT Day 9					
F	Day 12 TRT 1	Day 12 TRT 2	Day 12 TRT 3	Day 12 TRT 10	Day 12 TRT 11	Day 12 TRT 12	Sp-CT Day 12					
G	H_2O_2 0 µM	H_2O_2 0.1 µM	H_2O_2 1 µM	H_2O_2 2 µM	H_2O_2 5 µM	H_2O_2 10 µM	H_2O_2 20 µM	H_2O_2 25 µM	H_2O_2 50 µM	H_2O_2 100 µM		
H												

Figure 5-4. Layout for the leuco crystal violet (LCV) assay for spinach cell-free samples. TRT: treatment, Sp-CT: spinach control.

5.5.3 Determination of Antimicrobial Polypeptide Synthesis via Agar Diffusion Assay

Antimicrobial polypeptide activity was detected and characterized via agar diffusion assay (Yang and others 1992; Hickey and others 2003; Chumchalova and others 2004; Batdorj and others 2007; Carvalho and others 2010). This assay was performed using *Listeria monocytogenes* Scott A as the indicator microorganism, given its sensitivity to bacteriocins produced by LAB cultures (Abee and others 1994; Harris and others 1989; Schillinger and Lucke 1989). The indicator microorganism was obtained from the Department of Animal Science Center for Food Safety culture collection at Texas A&M University (College Station, TX), and maintained on TSA

slants at 4 °C. Sub-culturing in TSB, followed by static incubation at 35 °C for 24 hr of the indicator culture was performed twice consecutively. *Listeria* isolate was confirmed to the genus level with API® *Listeria* (API® Gram positive identification), incubated for 24 hr at 35 °C. The microbiological medium used to complete bacteriocin assays contained 10 g of peptone, 3 g of sodium chloride (NaCl), 1.5 g of yeast extract, 1 g of glucose, 7.5 g of granulated agar, 10 g of sodium phosphate dibasic (Na_2HPO_4, buffer), and 10 ml of Tween 20 per liter of distilled water, according to previously reported studies (Tramer and Fowler 1964; Wolf and Gibbons 1996). After autoclaving (15 min, at 121 °C) and tempering to about 45 °C, the indicator culture was diluted and seeded into the microbiological medium to a final concentration of 7.0 $\log_{10}$ CFU/ml. Petri dishes (100x15 mm) were loaded with 30 ml of medium seeded with indicator *L. monocytogenes* Scott A to according to previously methods (Taylor and others 2007). Loaded plates were incubated at 4 °C for about 2 hr to allow agar to solidify prior to boring of wells and loading of bacteriocin-containing cell-free supernates.

Each treatment was assayed in a single loaded Petri plate. Six wells (approximately 6 mm diameter) were bored in a cooled agar plate using a sterile (121 °C, 15 min.) glass Pasteur pipette. Each well contained 87.5 µl of cell-free aliquot sample treated with catalase (3 µl, 10,000-40,000 units/mg protein, Sigma-Aldrich Co.), proteinase K (4 µl Prot K, ≥30 units/mg protein, Sigma-Aldrich Co.), and/or sodium hydroxide (4 µl, 0.1 N NaOH, Sigma-Aldrich Co.) according to procedure suggested by Concha-Meyer and others (2011) (Figure 5-5). To reach a final volume of 100 µl sample, it was necessary to compensate the volume with sterile distilled water. The first well (A)

consisted of the untreated cell free aliquot sample. The second well (B) had treated cell-free sample with Prot-K and catalase. The cell-free sample treated with Prot K and NaOH was placed in the third well (C). Catalase, NaOH, and pediocin treated cell-free sample was placed in the forth well (D). The fifth well (E) included a cell-free aliquot with catalase and pediocin, and the last well (F) consisted of the cell-free aliquot sample treated with catalase, NaOH, and Prot-K. Samples were treated with this Prot K, catalase and NaOH to confirm the proteinaceous nature of the inhibitor, avoid presence of H_2O_2, and to neutralize the sample, respectively.

Pediocin (0.1% of concentration, from *Pediococcus acidilactici*, Sigma-Aldrich) and Nisin (0.1% of concentration, from *Lactococcus lactis*, Sigma-Aldrich) were used as a bacteriocin standard reference. Pediocin and Nisin were diluted in sodium acetate (1.36%, pH 5.0) to different known concentrations (0.1, 0.05, 0.01, 0.005, 0.0001 mg/ml) dispensed and assayed in wells in identical manner as treatment samples. Standard references were obtained by placing different concentrations of bacteriocin standard solutions in wells cut in cooled agar plates. Following preparation and sample loading, all plates were aerobically incubated for 2 h at 4 °C and then at 35 °C for 24 h to allow diffusion of bacteriocin and bacteriocin concentration-specific inhibition of the indicator microbe (Naghmouchi and others 2007; Li and others 2011). After incubation, zones of clearing (halos) were measured with a digital-readout caliper in two planes (horizontal and vertical). Readings were averaged and reported as the sample-specific zone of inhibition.

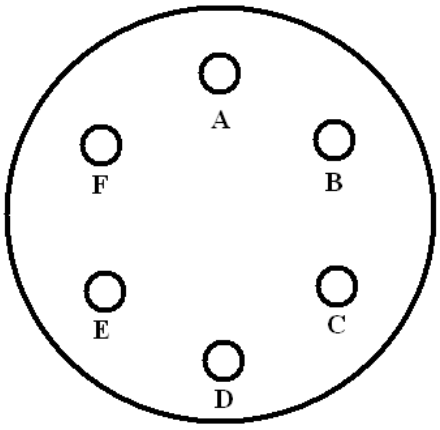

Figure 5-5. Orientation of the cell-free aliquot samples for bacteriocin agar diffusion assays.

5.6 Experimental Design and Data Analysis

5.6.1 Dose Response Assay

All experiments were completed in triplicate with duplicate identical samples prepared for each replicate. Microbiological data (plate counts) were converted to logarithmic values (base 10) before statistical analysis. Statistical analysis consisted of completing a one-way analysis of variance (ANOVA) by the general linear model procedure (GLM), and a means separation procedure was completed by Tukey's Honestly Significant Differences (HSD) test ($p<0.05$) to evaluate the differences between observed inhibition of pathogen growth as a function of LactiGuard^{TM} dosage *in vitro* and on spinach surfaces. Lactic acid bacteria counts on MRS agar plates were corrected against corresponding counts on LSPR agar plates, since pathogens were also able to grow on MRS agar, by subtracting the UFC/ml or g on LSPR agar plates from

the UFC/ml or g on MRS agar plates. Analyses were conducted using the software program SPSS v16.0 (SPSS Inc., Chicago, IL).

5.6.2 Antimicrobial Synthesis Assays

The antimicrobial synthesis by lactic acid bacteria from LactiGuard^{TM} as a function of incubation duration and inoculum applied in broth medium (TTSB) and on spinach surfaces was analyzed up to 12 days. The statistical procedures performed consisted of performing a one-way ANOVA by the GLM procedure, with separation of means via Least Significant Differences (LSD) ($p< 0.05$). All experiments were replicated 3 times. Analyses were conducted using SPSS v16.0 (SPSS Inc.). One standard curve per replication was necessary to complete for the hydrogen peroxide and bacteriocin production assays.

A correlation evaluation between the antimicrobials synthesized by lactic acid bacteria and the pathogenic dose response was completed within treatments and storage days. The statistical procedure performed consisted of running a two-tailed Pearson's correlation to determine any statistical relationship between the two studies. Analyses were conducted using SPSS v16.0 (SPSS Inc.).

CHAPTER VI

RESULTS AND DISCUSSION

6.1 Preliminary Experiments

6.1.1 Growth Curve Medium Validation

Three different media (MRS, TSB, and TTSB) were microbiologically analyzed to identify a medium suitable for growth of the two pathogens and lactic acid bacteria (LAB) cultures when co-inoculated into a sample tube. Figures 6-1, 6-2, and 6-3 illustrate the growth of *E. coli* O157:H7, *Salmonella enterica*, and LAB cultures in the three media, respectively. Plating counts were converted to logarithmic units (base 10).

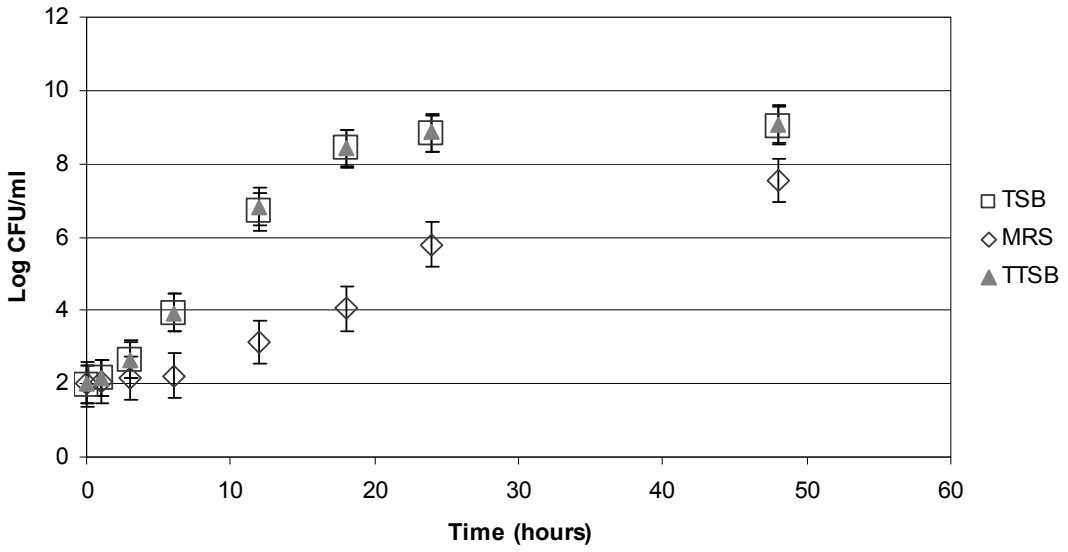

Figure 6-1. Growth of *Escherichia coli* O157:H7 on MRS, TSB, and TTSB liquid media at 25 °C for up to 48 h.

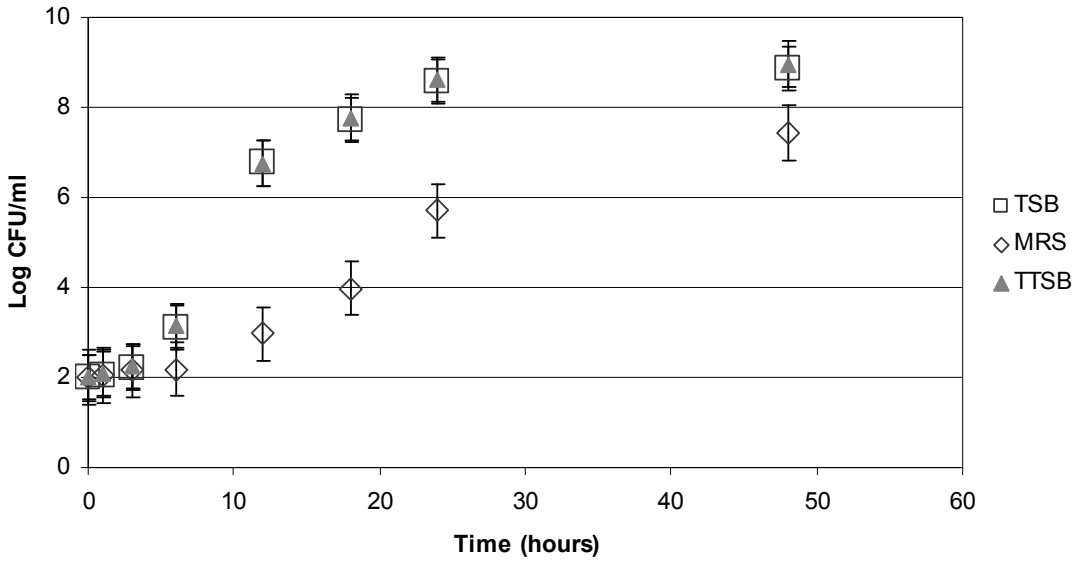

Figure 6-2. *Salmonella enterica* growth on MRS, TSB, and TTSB liquid media at 25 °C for up to 48 h.

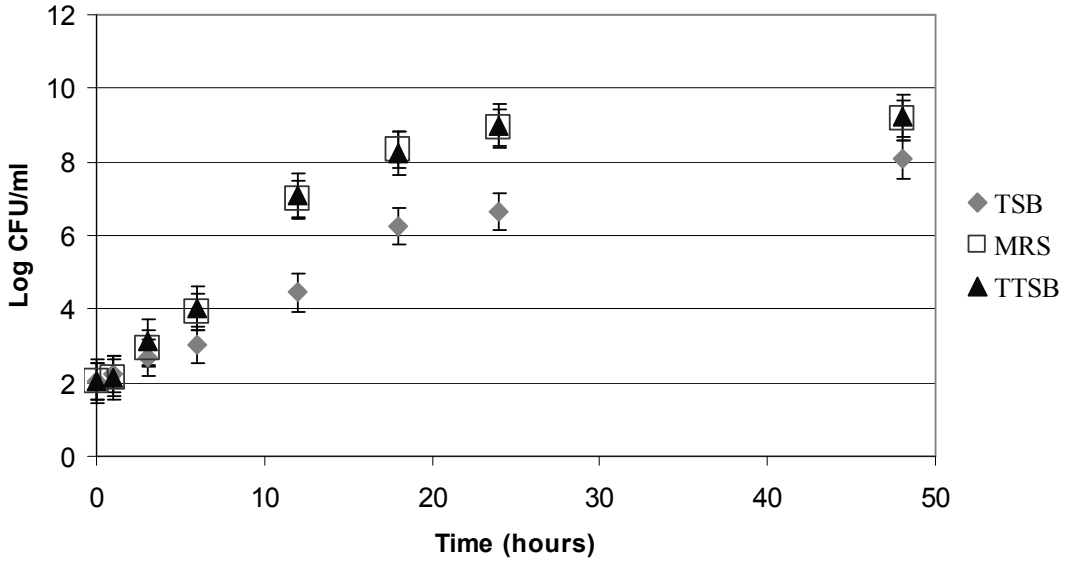

Figure 6-3. Growth of lactic acid bacteria cultures from LactiGuard™ growth on MRS, TSB, and TTSB liquid media at 25 °C for up to 48 h.

The microbiological analysis performed to validate a medium suitable to growth pathogenic and lactic acid bacteria (LAB) cultures demonstrated that Tryptic Soy Broth (TSB) supplemented with Tween 80 (TTSB) supported the nutritional requirements to achieve a growth curve that was not significantly different from a growth curved completed with traditional suggested medium for enteric pathogens (TSB) and LAB cultures (MRS), simultaneosly. Figures 6-1, 6-2, and 6-3 show the growth at 25 °C of the five strains of *E. coli* O157:H7, five serovars of *Salmonella enterica*, and the lactic acid bacteria cultures, respectively.

The growth curve results at 25 °C for the five strains of *E. coli* O157: H7, five *Salmonella enterica* serovars, and the LAB cultures from LactiGuard™ in the three different media are shown in Tables 6-1, 6-2, and 6-3, respectively. Tables 6-1 shows that the growth of the five strains of *E. coli* O157:H7 on Tryptic Soy Broth (TSB) and on TTSB was not significantly different for up to 48 h; whereas, on MRS liquid medium there was a signififcant difference in the growth of about 1.0 $\log_{10}$ CFU/ml and 1.5 $\log_{10}$ CFU/ml after 6 and 48 h, respectively for all strains. The same behaviour was observed in Table 6-2, which illustrates the growth of *Salmonella enterica*. Therefore, it is stated that MRS medium did not support a normal growth of the enteric pathogenic cultures at 25 °C. In contrast, Table 6-3 demonstrated that MRS liquid medium significantly supported the growth of LAB cultures as TTSB medium. In this occasion, the lactic acid bacteria (LAB) from LactiGuard™ showed a slower rate of generation on TSB medium and a faster rate of generation on MRS and TTSB after the 6 h point.

Table 6-1. Growth of five strains of *E. coli* O157:H7 (Rif +) in three media incubated at 25 °C.

M[2]	Time (h)	*Escherichia coli* O157:H7 (log_{10} CFU/ml)[1]				
		R1	R8	R18	R34	R41
TSB	0	2.02±0.11^{a}	2.02±0.10^{a}	1.83±0.10^{a}	2.01±0.04^{a}	1.99±0.08^{a}
	1	2.29±0.09^{a}	2.25±0.20^{a}	2.15±0.12^{a}	2.11±0.22^{a}	2.03±0.21^{ab}
	3	2.90±0.16^{ac}	2.73±0.38^{ac}	2.69±0.38^{ac}	2.54±0.51^{ac}	2.45±0.39^{ab}
	6	3.98±0.40^{b}	3.95±0.37^{b}	4.03±0.57^{b}	3.87±0.51^{bd}	3.87±0.61^{bc}
	12	6.84±0.93^{e}	6.52±0.35^{d}	6.74±0.42^{d}	6.57±0.43^{e}	6.76±0.62^{e}
	18	8.28±0.07^{fg}	7.99±0.23^{fg}	8.63±0.25^{fg}	8.66±0.13^{g}	8.54±0.11^{fg}
	24	8.97±0.05^{g}	8.60±0.03^{g}	8.68±0.09^{g}	8.92±0.06^{g}	8.90±0.06^{g}
	48	9.14±0.24^{g}	9.05±0.38^{g}	8.98±0.19^{g}	9.00±0.24^{g}	8.98±0.48^{g}
MRS	0	2.01±0.12^{a}	2.03±0.09^{a}	2.09±0.06^{a}	1.92±0.08^{a}	1.87±0.05^{a}
	1	2.08±0.05^{a}	2.03±0.01^{a}	2.15±0.05^{a}	2.00±0.04^{a}	2.02±0.16^{a}
	3	2.24±0.16^{a}	2.22±0.31^{a}	2.27±0.21^{a}	2.09±0.18^{a}	2.01±0.14^{a}
	6	2.26±0.14^{a}	2.21±0.18^{a}	2.30±0.12^{a}	2.09±0.19^{a}	2.27±0.18^{ab}
	12	2.98±0.11^{bc}	3.13±0.09^{bc}	3.29±0.03^{bc}	3.14±0.08^{bc}	3.09±0.09^{b}
	18	3.64±0.50^{b}	3.82±0.03^{b}	4.39±0.12^{b}	4.19±0.10^{d}	4.17±0.03^{c}
	24	5.63±0.40^{d}	5.54±0.01^{d}	5.99±0.40^{d}	5.93±0.48^{e}	5.89±0.58^{d}
	48	7.43±0.62^{ef}	7.41±0.75^{ef}	7.67±0.54^{ef}	7.60±0.36^{f}	7.60±0.46^{ef}
TTSB	0	2.02±0.11^{a}	1.98±0.11^{a}	1.99± 0.13^{a}	1.98±0.10^{a}	1.99±0.07^{a}
	1	2.25±0.08^{a}	2.24±0.15^{a}	2.17± 0.12^{a}	2.04±0.29^{a}	2.03±0.19^{a}
	3	2.81±0.29^{a}	2.75±0.23^{a}	2.68± 0.39^{a}	2.52±0.55^{a}	2.45±0.46^{a}
	6	4.02±0.38^{b}	3.96±0.47^{b}	3.96± 0.59^{b}	3.88±0.47^{b}	3.87±0.54^{b}
	12	7.09±0.97^{c}	6.67±0.33^{c}	6.81± 0.35^{c}	6.80±0.62^{c}	6.76±0.60^{c}
	18	8.27±0.08^{d}	8.14±0.20^{d}	8.57± 0.18^{d}	8.48±0.13^{d}	8.54±0.12^{d}
	24	8.82±0.08^{d}	8.89±0.05^{de}	8.77± 0.02^{d}	8.84±0.06^{d}	8.90±0.08^{d}
	48	9.17±0.11^{d}	9.15±0.39^{e}	9.07± 0.29^{d}	9.05±0.23^{d}	8.98±0.28^{d}

[1]Values represent means of triplicate replications with duplicate identical samples processed per replicate (n=6) ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between *Escherichia coli* O157:H7 strains within columns.
[2]M: medium; TSB: tryptic soy broth; MRS: de Man, Rogosa, and Sharpe broth; TTSB: tryptic soy broth supplemented with 1 g/L Tween 80.

Table 6-2. Growth of five servovars of *Salmonella enterica* (Rif +) in differing media at incubated 25 °C.

M[2]	Time (h)	*Salmonella enterica* serovars (log_{10} CFU/ml)[1]				
		Agona	Anatum	Michigan	Montevideo	Saintpaul
TSB	0	2.13±0.08^{a}	2.05±0.06^{a}	1.84±0.30^{a}	2.09±0.11^{a}	1.92±0.08^{a}
	1	2.16±0.05^{a}	2.05±0.26^{a}	1.74±0.08^{a}	2.31±0.11^{a}	2.02±0.03^{ab}
	3	2.34±0.06^{a}	2.31±0.05^{b}	1.85±0.08^{a}	2.30±0.08^{a}	2.17±0.07^{b}
	6	3.52±0.38^{b}	3.10±0.07^{c}	2.77±0.05^{b}	3.45±0.18^{b}	2.72±0.14^{c}
	12	6.68±0.09^{c}	6.74±0.10^{d}	6.69±0.11^{c}	7.04±0.33^{c}	6.65±0.07^{d}
	18	7.83±0.08^{d}	7.80± 0.14^{e}	7.81±0.05^{d}	7.61±0.08^{d}	7.55±0.08^{e}
	24	8.89±0.03^{e}	8.71±0.05^{f}	8.56±0.26^{e}	8.79±0.06^{e}	7.94±0.08^{f}
	48	9.25±0.19^{f}	8.89±0.19^{f}	8.53±0.08^{e}	9.09±0.05^{f}	8.57±0.08^{g}
MRS	0	2.10±0.09^{a}	2.08±0.06^{a}	1.74±0.16^{a}	2.12±0.06^{a}	1.98±0.06^{a}
	1	2.12±0.10^{a}	2.07±0.13^{a}	1.75±0.12^{a}	2.17±0.03^{a}	2.09±0.07^{a}
	3	2.29±0.11^{a}	2.13±0.15^{a}	1.84±0.14^{a}	2.36±0.19^{a}	2.15±0.19^{ab}
	6	2.29±0.20^{a}	2.28±0.08^{b}	1.97±0.17^{a}	2.22±0.15^{a}	2.12±0.10^{ab}
	12	2.99±0.07^{b}	3.03±0.09^{c}	2.78±0.04^{b}	3.07±0.04^{b}	2.96±0.14^{c}
	18	3.64±0.35^{b}	3.88±0.22^{g}	4.09±0.17^{e}	4.11±0.16^{g}	4.15±0.26^{h}
	24	5.89±0.30^{c}	5.63±0.15^{h}	5.74±0.39^{f}	5.44±0.56^{h}	5.83±0.46^{i}
	48	7.41±0.45^{d}	7.53±0.62^{e}	7.35±0.55^{d}	7.43±0.75^{cd}	7.44±0.52^{ef}
TTSB	0	2.11±0.13^{a}	2.04±0.06^{a}	1.71±0.20^{a}	2.08±0.09^{a}	1.97±0.05^{a}
	1	2.24±0.07^{a}	2.05±0.29^{a}	1.70±0.07^{a}	2.34±0.10^{a}	2.14±0.05^{ab}
	3	2.36±0.11^{a}	2.41±0.01^{b}	1.81±0.02^{a}	2.30±0.13^{a}	2.30±0.07^{b}
	6	3.54±0.40^{b}	3.14±0.15^{c}	2.54±0.14^{b}	3.52±0.03^{b}	2.94±0.15^{c}
	12	6.74±0.10^{c}	6.69±0.16^{d}	6.72±0.14^{c}	7.04±0.30^{c}	6.57±0.04^{d}
	18	7.83±0.05^{d}	7.83±0.08^{e}	7.82±0.06^{d}	7.74±0.16^{d}	7.62±0.06^{e}
	24	8.85±0.04^{e}	8.75±0.08^{f}	8.74±0.08^{e}	8.78±0.08^{e}	7.93±0.10^{f}
	48	9.29±0.21^{f}	9.02±0.18^{f}	8.71±0.07^{e}	9.09±0.09^{f}	8.64±0.20^{g}

[1]Values represent means of triplicate replications with duplicate identical samples processed per replicate (n=6) ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between *Salmonella* serovars within columns. [2]M: medium; TSB: tryptic soy broth; MRS: de Man, Rogosa, and Sharpe broth; TTSB: tryptic soy broth supplemented with 1 g/L Tween 80.

Table 6-3. Growth of lactic acid bacteria from LactiGuard™ in differing media incubated at 25 °C.

Medium[2]	Time (h)	Lactic acid bacteria cultures (log_{10} CFU/ml)[1]
TSB	0	2.02 ± 0.14^{a}
	1	2.24 ± 0.09^{a}
	3	2.67 ± 0.19^{a}
	6	3.04 ± 0.21^{bc}
	12	4.44 ± 0.40^{d}
	18	6.23 ± 0.28^{e}
	24	6.66 ± 0.28^{e}
	48	8.06 ± 0.25^{f}
MRS	0	2.03 ± 0.32^{a}
	1	2.15 ± 0.14^{ab}
	3	2.94 ± 0.09^{ab}
	6	3.92 ± 0.68^{cd}
	12	6.97 ± 0.40^{e}
	18	8.31 ± 0.20^{fg}
	24	8.91 ± 0.07^{fg}
	48	9.16 ± 0.31^{g}
TTSB	0	2.03 ± 0.30^{a}
	1	2.15 ± 0.11^{ab}
	3	3.10 ± 0.04^{bc}
	6	4.03 ± 0.63^{cd}
	12	7.08 ± 0.60^{e}
	18	8.24 ± 0.08^{fg}
	24	8.98 ± 0.02^{fg}
	48	9.20 ± 0.19^{g}

[1]Values represent means of triplicate replications with duplicate identical samples processed per replicate (n=6) $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between LactiGuard™ sample means within columns. [2]TSB: tryptic soy broth; MRS: de Man, Rogosa, and Sharpe broth; TTSB: tryptic soy broth supplemented with 1 g/L Tween 80.

6.1.2 Bacterial Recovery Validation from the Spinach Surface

Table 6-4 shows the results from the validation procedure to recover the enteric pathogenic cocktail inoculated by the spot inoculation method on the surface of spinach leaves at different drying times. Table 6-5 shows the validation of the recovery of different levels of inoculum cocktail of pathogens inoculated by the spot inoculation method. The validation of the application and recovery of the LAB cultures is shown in Table 6-6.

Table 6-4. Recovery validation at four drying times of the pathogen bacterial cocktail (7.61±0.35 $\log_{10}$ CFU/ml) of *Escherichia coli* O157:H7 and *Salmonella enterica* by the spot inoculation method at at 25 °C.

Drying times	*Escherichia coli* O157:H7 ($\log_{10}$ CFU/g)[1]	*Salmonella enterica* ($\log_{10}$ CFU/g)[1]
0 min	5.28 ± 0.05^{a}	5.17 ± 0.06^{a}
30 min	5.23 ± 0.14^{a}	5.10 ± 0.14^{a}
60 min	5.32 ± 0.04^{a}	5.32 ± 0.12^{a}
24 hrs	1.56 ± 0.24^{b}	1.44 ± 0.43^{b}

[1]Values represent means of triplicate replications ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between sample means within columns.

The spot inoculation procedure for the pathogen bacterial cocktail was validated to determine the appropriate drying time for attachment and to determine the required inoculum concentration to achieve the inoculation level on the spinach leaves. Table 6-4 illustrates the *Escherichia coli* O157:H7 and *Salmonella enterica* (log10 CFU/g) population recovered from spinach leaves after 0, 30, 60 min, and 24 hr at 25 °C. It was

determined that after 0, 30, and 60 min there was no significant difference in the recovery levels of *E. coli* O157:H7 and *S. enterica*. The reduction in recovery after 24 hr of drying may be due to the continuos airflow of the bacteriological cabinet which desiccated to some extend the spinach leaves and resulted in a reduction in viability of pathogen cells which are desiccation intolerant (Scott 2000). Therefore, it was stablished that the drying time for attachment of pathogen bacterial cultures was 60 min because it allowed enough time to handle several samples at the same time. In Table 6-5, it was determined that between the recovered pathogen culture and the inoculum cocktail level, there was an overall difference of approximately 2.5 $\log_{10}$ CFU which helped to predict the inoculum concentration to achieve the targeted cocktail pathogen inoculation.

Table 6-5. Recovery validation at different inoculum cocktail concentrations of *Escherichia coli* O157:H7 and *Salmonella enterica* by the spot inoculation method at at 25 °C for 1 h drying time at different inoculation levels.

Inoculum cocktail concentrations ($\log_{10}$ CFU/mL)[1]	*Escherichia coli* O157:H7 ($\log_{10}$ CFU/g)[1]	*Salmonella enterica* ($\log_{10}$ CFU/g)[1]
7.31 ± 0.41	4.83 ± 0.05[a]	4.82 ± 0.04[a]
6.34 ± 0.23	3.82 ± 0.30[b]	3.80 ± 0.24[b]
5.39 ± 0.28	2.93 ± 0.39[c]	2.93 ± 0.22[c]

[1]Values represent means of triplicate replications ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between sample means within columns.

The recovery validation of lactic acid bacteria from LactiGuard™ cultures (Table 6-6) by spray inoculation method demonstrated that there was not a significant

difference in the the drying time between 0 and 60 min, and that there was an overall difference between the inoculated concentration and the recovered LAB culture of about 1.5 $\log_{10}$ CFU.

Table 6-6. Recovery validation of lactic acid bacteria from LactiGuard™ cultures by the spray inoculation method at at 25 °C for 1 h drying time.

Inoculum concentrations ($\log_{10}$ CFU/ml)[1]	Drying time (min)	Lactic acid bacteria cultures ($\log_{10}$ CFU/g)[1]
10.02 ± 0.22	0	8.92 ± 0.06[a]
	60	8.90 ± 0.10[a]
8.25 ± 0.15	0	6.80 ± 0.08[b]
	60	6.66 ± 0.07[b]

[1]Values represent means of triplicate replications ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripts indicate significant differences between sample means within columns.

6.2 Study A: Dose Response

6.2.1 Dose Response *in vitro* Assays

The *in vitro* dose responses of the foodborne pathogens *E. coli* O157:H7 ($\log_{10}$ CFU/ml) and *Salmonella enterica* ($\log_{10}$ CFU/ml) in TTSB liquid medium at 7 °C for days 0, 1, 3, 6, 9, and 12 are illustrated on Table 6.7 and Table 6.8, respectively. The *in vitro* dose response of lactic acid bacteria (LAB) from LactiGuard™ cultures on TTSB for 0, 1, 3, 6, 9 and 12 days at 7 °C is demonstrated on Table 6-9. When applicable, according to the treatment, counts of LAB were corrected or blanked against population of pathogens obtained from LSPR plates.

Table 6-7, Table 6-8 and Table 6-9 demonstrated that the initial population for all treatments achieved the targeted levels. All treatments were compared within columns

and rows. By comparing each individual treatment within rows in Table 6-7 and Table 6-8, it was demonstrated that *E. coli* O157:H7 and *Salmonella enterica*, respectively, were able to growth during the storage period at 7 °C because there were significant grow differences between days. When comparing treatments 2, 5, 8, and 11 within columns, it was determined that there was no significant difference ($p>0.05$) within columns during the storage period at 7 °C for the 2.0 $\log_{10}$ CFU/ml pathogenic level after the LAB dosage levels, demonstrating that the LAB intervention did not result in a inhibition and/or reduction of *E. coli* O157:H7 and *Salmonella enterica*, even at the highest initial population level of LAB (8.0 $\log_{10}$ CFU/ml). In addition, when treatments 3, 6, 9, and 12 were compared against each other within columns, there was not a significant difference of the inoculated pathogens level during the storage period for up to 12 days at 7 °C.

Table 6-9 showed and verified the *in vitro* levels of LAB from LactiGuard™ for all treatments. By comparing treatments 4, 5, and 6 within columns, it was observed that as the pathogenic levels increased, the growth of LAB increased from day 3 to day 12. The same behavior was observed when treatments 7, 8, and 9 were compared within columns. Therefore, it can be determined that an aggressive behavior by the LAB occurs when the presence of *E. coli* O157:H7 and *Salmonella enterica* increased.

Table 6-7. *E. coli* O157:H7 i*n vitro* dose response in TTSB medium at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on LSPR agar plates.

Treatment	Bacterial concentration levels (log_{10} CFU/ml)[1]		*Escherichia coli* O157:H7 (log_{10} CFU/ml)[2]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	1.88 ± 0.14^{b}	2.63 ± 0.18^{d}	3.97 ± 0.18^{ch}	6.09 ± 0.16^{ij}	7.11 ± 0.26^{klmn}	7.53 ± 0.17^{no}
3	0.0	4.0	3.83 ± 0.13^{c}	4.72 ± 0.24^{eg}	5.87 ± 0.29^{ij}	7.35 ± 0.25^{lmno}	7.37 ± 0.30^{mno}	7.23 ± 0.10^{klmno}
4	5.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	5.0	2.0	2.10 ± 0.27^{b}	2.73 ± 0.13^{d}	4.04 ± 0.20^{ch}	5.76 ± 0.29^{j}	6.95 ± 0.18^{klm}	7.30 ± 0.14^{lmno}
6	5.0	4.0	3.77 ± 0.10^{c}	4.72 ± 0.12^{eg}	6.16 ± 0.15^{ij}	7.06 ± 0.45^{klmn}	7.68 ± 0.21^{o}	7.27 ± 0.36^{lmno}
7	6.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
8	6.0	2.0	1.94 ± 0.23^{b}	2.78 ± 0.08^{d}	4.18 ± 0.19^{cf}	6.16 ± 0.15^{ij}	7.30 ± 0.35^{lmno}	7.53 ± 0.16^{no}
9	6.0	4.0	3.88 ± 0.13^{c}	4.76 ± 0.12^{eg}	6.02 ± 0.32^{ij}	7.30 ± 0.29^{lmno}	7.01 ± 0.53^{klm}	7.25 ± 0.31^{klmno}
10	8.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
11	8.0	2.0	1.84 ± 0.15^{b}	3.02 ± 0.45^{d}	4.40 ± 0.26^{fgh}	5.94 ± 0.55^{ij}	6.86 ± 0.12^{kl}	6.74 ± 0.37^{k}
12	8.0	4.0	3.79 ± 0.07^{c}	4.61 ± 0.15^{ef}	6.33 ± 0.20^{i}	7.36 ± 0.25^{lmno}	7.25 ± 0.16^{klmno}	7.16 ± 0.26^{klmn}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-8. *In vitro* dose response of *Salmonella enterica* in TTSB medium at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on LSPR agar plates.

Treatment	Bacterial concentration levels (log_{10} CFU/ml)[1]		*Salmonella enterica* (log_{10} CFU/ml)[2]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	1.90 ± 0.21^{b}	2.54 ± 0.25^{c}	3.98 ± 0.21^{de}	5.77 ± 0.30^{gh}	6.62 ± 0.41^{kl}	7.24 ± 0.31^{m}
3	0.0	4.0	3.72 ± 0.08^{d}	4.57 ± 0.29^{f}	5.98 ± 0.24^{ghij}	7.26 ± 0.20^{m}	7.14 ± 0.34^{lm}	7.34 ± 0.31^{m}
4	5.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
5	5.0	2.0	1.83 ± 0.11^{b}	2.64 ± 0.22^{c}	3.98 ± 0.23^{de}	5.54 ± 0.29^{g}	6.51 ± 0.19^{jk}	7.05 ± 0.34^{lm}
6	5.0	4.0	3.62 ± 0.27^{d}	4.70 ± 0.21^{f}	5.96 ± 0.16^{ghi}	6.59 ± 0.51^{jkl}	7.41 ± 0.26^{m}	7.29 ± 0.38^{m}
7	6.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
8	6.0	2.0	1.85 ± 0.06^{b}	2.67 ± 0.22^{c}	3.80 ± 0.12^{d}	5.83 ± 0.14^{gh}	6.68 ± 0.19^{kl}	7.23 ± 0.31^{m}
9	6.0	4.0	3.75 ± 0.13^{d}	4.63 ± 0.20^{f}	5.85 ± 0.21^{gh}	7.10 ± 0.20^{lm}	6.71 ± 0.70^{kl}	7.32 ± 0.35^{m}
10	8.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
11	8.0	2.0	1.81 ± 0.05^{b}	2.62 ± 0.16^{c}	4.01 ± 0.25^{de}	5.73 ± 0.37^{gh}	6.47 ± 0.15^{ijk}	6.90 ± 0.41^{klm}
12	8.0	4.0	3.76 ± 0.13^{d}	4.42 ± 0.20^{ef}	6.08 ± 0.13^{hij}	7.16 ± 0.16^{lm}	7.07 ± 0.33^{lm}	7.20 ± 0.29^{m}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-9. *In vitro* response of lactic acid bacteria from LactiGuard™ cultures on TTSB at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on MRS agar plates.

Treatment	Bacterial concentration levels (log_{10} CFU/ml)[1]		Lactic acid bacteria (log_{10} CFU/ml)[2]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	0.0	4.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
4	5.0	0.0	4.48 ± 0.83^{b}	5.02 ± 0.25^{bc}	5.03 ± 0.10^{bc}	5.21 ± 0.21^{cd}	5.01 ± 0.08^{bc}	5.11 ± 0.13^{bc}
5	5.0	2.0	5.10 ± 0.13^{bc}	4.93 ± 0.15^{bc}	5.04 ± 0.70^{bc}	6.55 ± 0.33^{e}	7.74 ± 0.28^{f}	8.34 ± 0.15^{fghi}
6	5.0	4.0	5.00 ± 0.19^{bc}	4.99 ± 0.20^{bc}	6.51 ± 0.11^{e}	8.09 ± 0.27^{fghi}	7.91 ± 0.72^{fghi}	8.52 ± 0.29^{hi}
7	6.0	0.0	6.03 ± 0.26^{e}	6.08 ± 0.15^{e}	5.99 ± 0.07^{e}	5.88 ± 0.11^{de}	5.84 ± 0.07^{de}	5.96 ± 0.19^{e}
8	6.0	2.0	5.93 ± 0.18^{e}	6.07 ± 0.23^{e}	6.11 ± 0.12^{e}	6.37 ± 0.37^{e}	7.86 ± 0.15^{fgh}	8.46 ± 0.16^{ghi}
9	6.0	4.0	6.03 ± 0.19^{e}	6.00 ± 0.24^{e}	6.43 ± 0.48^{e}	7.72 ± 0.46^{f}	8.00 ± 0.67^{fghi}	8.58 ± 0.16^{i}
10	8.0	0.0	8.03 ± 0.22^{fghi}	8.06 ± 0.36^{fghi}	7.70 ± 0.69^{f}	7.97 ± 0.13^{fghi}	8.00 ± 0.18^{fghi}	8.24 ± 0.09^{fghi}
11	8.0	2.0	8.05 ± 0.14^{fghi}	7.82 ± 0.36^{fg}	7.92 ± 0.33^{fghi}	7.91 ± 0.30^{fghi}	8.04 ± 0.08^{fghi}	8.06 ± 0.07^{fghi}
12	8.0	4.0	8.03 ± 0.26^{fghi}	8.02 ± 0.23^{fghi}	8.02 ± 0.15^{fghi}	8.27 ± 0.40^{fghi}	8.37 ± 0.10^{fghi}	8.22 ± 0.41^{fghi}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

6.2.2 Dose Response on Spinach

The dose response on the spinach leaves surface of *E. coli* O157:H7 (log_{10} CFU/g) and *Salmonella enterica* (log_{10} CFU/g) at 7 °C for days 0, 1, 3, 6, 9, and 12 are shown in Table 6-10 and Table 6-11, respectively, in which the untreated and uninoculated spinach control samples counts are also illustrated. In Table 6-12, the LAB cultures counts on MRS plates were corrected against population obtained from the untreated and uninoculated spinach control samples from MRS plates and against population of pathogens obtained from LSPR plates when applicable.

All initial population levels satisfied the targeted levels for *E. coli* O157:H7, *Salmonella enterica* and LAB cultures (Tables 6-10, 6-11, and 6-12, respectively) for all treatments. It was also observed that *E. coli* O157:H7 and *Salmonella enterica* cultures were able to grow significantly during the storage period (from day 0 to day 12) on the spinach surface at 7 °C, with an increase on the population of approximately 3.0 – 3.5 log_{10} CFU/ml for treatment 2, and 1.0 - 2.0 log_{10} CFU/ml for treatment 3. After 3 days, the dose response of *E. coli* O157:H7 on spinach surface showed that there were significant differences ($p<0.05$) between treatments 2 (3.88±0.22 log_{10} CFU/ml) and 11 (2.26±0.37 log_{10} CFU/ml), and treatments 3 (5.63±0.26 log_{10} CFU/ml) and 12 (4.90±0.46 log_{10} CFU/ml), which indicated inhibition of about 1.62 and 0.73 log CFU/ml, respectively.

Furthermore, in day 6 there was evidence of inhibition (about 2.1 $\log_{10}$ CFU/ml) between treatments 2 (5.02±0.37 $\log_{10}$ CFU/ml) and 11 (2.92±0.30 $\log_{10}$ CFU/ml) when compared within columns. The dose response of *Salmonella enterica* on spinach surface demonstrated significant inhibition differences on day 6 between treatments 2 (4.11±0.42 $\log_{10}$ CFU/ml) and 11 (2.26±0.39 $\log_{10}$ CFU/ml), and treatments 3 (5.11±0.02 $\log_{10}$ CFU/ml) and 12 (4.40±0.48 $\log_{10}$ CFU/ml) of approximately 1.85 and 0.71 $\log_{10}$ CFU/ml.

The initial inoculated levels (day 0) of LAB cultures from LactiGuard™ were relatively constant after 12 days. However, treatment 10 was significantly different after 6, 9 and 12 day. Treatments 11 and 12 showed the same behavior after 9 and 12 days. In contrast, the normal microflora population from the intact non-inoculated spinach increased significantly after 12 days (about 3.27 $\log_{10}$ CFU/ml). Therefore, the significant reduction on LAB cultures may be an effect of surface attachment competition against the proliferating normal microflora.

Table 6-10. Dose response of *E. coli* O157:H7 on spinach surface at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on LSPR agar plates.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/ml)[2]		*Escherichia coli* O157:H7 (log_{10} CFU/g of spinach)[3]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	2.60 ± 0.11^{b}	2.94 ± 0.28^{b}	3.88 ± 0.22^{c}	5.02 ± 0.37^{defgh}	5.17 ± 0.47^{efgh}	6.23 ± 0.32^{kl}
3	0.0	4.0	4.48 ± 0.17^{cd}	4.98 ± 0.02^{defgh}	5.63 ± 0.26^{hijk}	5.87 ± 0.08^{ijkl}	5.90 ± 0.12^{ijkl}	6.42 ± 0.29^{l}
10	8.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
11	8.0	2.0	2.45 ± 0.16^{b}	2.39 ± 0.28^{b}	2.26 ± 0.37^{b}	2.92 ± 0.30^{b}	4.68 ± 0.68^{def}	5.92 ± 0.28^{jkl}
12	8.0	4.0	4.39 ± 0.13^{cd}	4.53 ± 0.10^{cde}	4.90 ± 0.46^{defg}	5.25 ± 0.42^{fghi}	5.46 ± 0.37^{ghij}	6.51 ± 0.23^{l}
SC	N/A	N/A	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-11. *Salmonella enterica* dose response on spinach surface at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on LSPR agar plates.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/ml)[2]		*Salmonella enterica* (log_{10} CFU/g of spinach)[3]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	2.56 ± 0.09^{b}	2.60 ± 0.50^{b}	3.41 ± 0.22^{b}	4.11 ± 0.42^{cde}	4.09 ± 0.68^{cde}	5.38 ± 0.33^{h}
3	0.0	4.0	4.45 ± 0.18^{ef}	4.63 ± 0.08^{efg}	5.17 ± 0.25^{gh}	5.11 ± 0.02^{fgh}	5.22 ± 0.35^{gh}	5.64 ± 0.31^{h}
10	8.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
11	8.0	2.0	2.40 ± 0.17^{b}	2.25 ± 0.20^{b}	1.96 ± 0.31^{b}	2.26 ± 0.39^{b}	3.72 ± 0.42^{cd}	4.65 ± 0.32^{efg}
12	8.0	4.0	4.35 ± 0.15^{de}	4.39 ± 0.13^{de}	4.61 ± 0.44^{efg}	4.40 ± 0.48^{de}	4.58 ± 0.31^{efg}	5.49 ± 0.25^{h}
SC	N/A	N/A	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-12. Dose Response of lactic acid bacteria from LactiGuard™ cultures on spinach surface at 7 °C for 0, 1, 3, 6, 9 and 12 days plated on MRS agar plates.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/mL)[2]		Lactic acid bacteria from LactiGuard™ (log_{10} CFU/g of spinach)[3]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	0.0	4.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
10	8.0	0.0	8.19 ± 0.14^{i}	8.11 ± 0.13^{fghi}	8.08 ± 0.14^{fghi}	7.53 ± 0.59^{defgh}	7.20 ± 0.13^{d}	7.51 ± 0.04^{defg}
11	8.0	2.0	8.16 ± 0.12^{hi}	7.80 ± 0.40^{defghi}	8.00 ± 0.15^{efghi}	7.61 ± 0.09^{defghi}	7.48 ± 0.10^{def}	7.43 ± 0.14^{de}
12	8.0	4.0	8.20 ± 0.19^{i}	8.00 ± 0.26^{efghi}	8.17 ± 0.21^{hi}	7.69 ± 0.03^{defghi}	7.17 ± 0.16^{d}	7.43 ± 0.06^{de}
SC	N/A	N/A	2.52 ± 0.40^{b}	2.79 ± 0.11^{b}	3.29 ± 0.40^{b}	3.95 ± 0.50^{b}	4.04 ± 0.82^{b}	5.79 ± 0.15^{c}

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

6.3 Study B: Antimicrobial Synthesis *in vitro* and on Spinach Surface by LAB Cultures

The antimicrobial synthesis by LAB cultures *in vitro* and on spinach surface is illustrated in three sections as follow: L-lactate quantification, hydrogen peroxide quantification, and agar diffusion assay to determine the antimicrobial activity.

6.3.1 Lactate Quantification

The L-lactate synthesis was measured spectrophotometrically using a method reported by Bergmeyer and Bernt (1974), and Gutmann and Wahlefeld (1974). Table 6-13 shows the results in μmoles/ml for the *in vitro* assay and Table 6-14 showed the measurement obtained from the spinach surface in μmoles/g. For the *in vitro* assay, the initial content of L-lactic acid (day 0) on treatments 10 (0.43±0.10 μmoles/ml), 11 (0.55±0.17 μmoles/ml) and 12 (0.50±0.29 μmoles/ml) compared against the initial L-lactic acid content of treatments 1 (0.00±0.00 μmoles/ml) and 4 (0.01±0.01 μmoles/ml) demonstrated that the commercial LactiGuard^{TM} product may contain small amounts of L-lactic acid by the observation that as the inoculated level of LAB increased, the amount of L-lactatic acid detected also increased. In addition, Table 6-14 shows the same trend on day 0 between treatment 1 (0.04±0.07 μmoles/ml) against treatments 10 (0.64±0.29 μmoles/ml), 11 (1.03±0.10 μmoles/ml), and 12 (0.87±0.33 μmoles/ml). Furthermore, the *in vitro* treatments 10, 11 and 12 showed that the fermentation of L-lactic acid was influenced by the storage period at 7 °C. After 12 days *in vitro*, it was observed an increased of about 3.28, 2.90, and 2.86 μmoles/ml on treatments 10, 11, and 12, respectively.

Table 6-13. Quantification of *in vitro* synthesized L-lactic acid by LAB cultures at 7 °C.

Treatment	Bacterial concentration levels (log_{10} CFU/ml)[1]		L-Lactic acid (μmoles/ml)[2]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
2	0.0	2.0	0.03±0.03^{a}	0.01±0.02^{a}	0.00±0.00^{a}	0.03±0.06^{a}	0.00±0.00^{a}	0.13±0.17^{ab}
3	0.0	4.0	0.04±0.03^{a}	0.00±0.00^{a}	0.02±0.03^{a}	0.00±0.00^{a}	0.03±0.05^{a}	0.00±0.00^{a}
4	5.0	0.0	0.01±0.01^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.02±0.02^{a}	0.02±0.03^{a}	0.00±0.00^{a}
5	5.0	2.0	0.10±0.10^{ab}	0.02±0.03^{a}	0.02±0.04^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.05±0.08^{a}
6	5.0	4.0	0.06±0.10^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.06±0.05^{a}
7	6.0	0.0	0.02±0.04^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.01±0.02^{a}	0.00±0.00^{a}	0.04±0.07^{a}
8	6.0	2.0	0.04±0.05^{a}	0.00±0.00^{a}	0.05±0.08^{a}	0.03±0.05^{a}	0.06±0.06^{a}	0.10±0.08^{ab}
9	6.0	4.0	0.04±0.06^{a}	0.00±0.00^{a}	0.17±0.30^{abc}	0.05±0.04^{a}	0.08±0.08^{a}	0.05±0.06^{a}
10	8.0	0.0	0.43±0.10^{bcd}	1.50±0.66^{e}	2.64±0.50^{f}	3.12±0.26^{hi}	3.91±0.37^{j}	3.71±0.14^{ij}
11	8.0	2.0	0.55±0.17^{d}	1.21±0.90^{e}	2.52±0.15^{f}	3.39±0.42^{i}	3.60±0.26^{ij}	3.45±0.02^{i}
12	8.0	4.0	0.50±0.29^{cd}	1.38±0.65^{e}	2.73±0.12^{fg}	3.03±0.28^{gh}	3.09±0.67^{hi}	3.36±0.01^{hi}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication ± one standard deviation from the mean. Means were separated by LSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-14. Quantification of L-lactic acid produced on spinach surface by LAB cultures at 7 °C.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/mL)[2]		L-Lactic acid (μmoles/g of spinach)[3]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.04 ± 0.07^{ab}	0.06 ± 0.07^{ab}	0.08 ± 0.07^{ab}	0.09 ± 0.04^{ab}	0.09 ± 0.08^{ab}	0.09 ± 0.11^{ab}
2	0.0	2.0	0.02 ± 0.03^{ab}	0.10 ± 0.08^{ab}	0.07 ± 0.07^{ab}	0.08 ± 0.05^{ab}	0.11 ± 0.09^{ab}	0.39 ± 0.60^{c}
3	0.0	4.0	0.05 ± 0.06^{ab}	0.06 ± 0.05^{ab}	0.09 ± 0.09^{ab}	0.10 ± 0.05^{ab}	0.13 ± 0.07^{ab}	0.09 ± 0.08^{ab}
10	8.0	0.0	0.64 ± 0.29^{de}	0.56 ± 0.13^{cd}	0.50 ± 0.11^{cd}	0.57 ± 0.02^{cd}	0.39 ± 0.10^{c}	0.44 ± 0.03^{cd}
11	8.0	2.0	1.03 ± 0.10^{f}	0.40 ± 0.05^{c}	0.24 ± 0.26^{bc}	0.53 ± 0.01^{cd}	0.38 ± 0.11^{c}	0.42 ± 0.02^{cd}
12	8.0	4.0	0.87 ± 0.33^{ef}	0.53 ± 0.09^{cd}	0.38 ± 0.23^{c}	0.56 ± 0.12^{cd}	0.49 ± 0.14^{cd}	0.47 ± 0.03^{cd}
SC	N/A	N/A	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

6.3.2 Hydrogen Peroxide Quantification

Tables 6-15 and 6-16 illustrate the spectrophotometric hydrogen peroxide quantification from the *in vitro* (μmoles/ml) and on spinach surface (μmoles/g) assays, respectively. The μmoles of hydrogen peroxide were obtained from the corresponding absorbance and concentration of the standard curves performed.

The *in vitro* and on spinach surface quantification of hydrogen peroxide (H_2O_2) are listed in Table 6-15 and Table 6-16, respectively. Overall, both tables showed no effect at 7 °C for most of the treatments. However, the H_2O_2 content of treatment 10 *in vitro* decreased about 4.34 μmoles/ml after 12 days (Table 6-15), and treatment 12 on spinach surface decreased about 2.42 μmoles/ml from day 1 to day 12 (Table 6-16). Therefore, this observation may be attributed to a reduction in the ability of the LAB cultures to produce H_2O_2 during storage at 7 °C, or to instability of H_2O_2 under storage conditions (overall medium pH was about 6.5). Stability of H_2O_2 is highly influenced by the pH (more stability at pH below 3.0) (Nicoll and Smith 1955). Table 6-12 also illustrates that particularly the initial content (day 0) of H_2O_2 quantified *in vitro* for treatment 10 (4.36±7.02 μmoles/ml) was significantly higher than treatment 1 (0.00±0.00 μmoles/ml) and 4 (0.00±0.00 μmoles/ml). This significant difference may indicate that as the inoculated level of LAB cultures from LactiGuard™ increases, the amount of H_2O_2 also increases.

Table 6-15. Quantification of *in vitro* synthesized hydrogen peroxide by LAB cultures at 7 °C.

Treatment	Bacterial concentration levels (log_{10} CFU/m)[1]		Hydrogen peroxide (μmoles/ml)[2] Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
2	0.0	2.0	0.64±0.11^{ab}	0.00±0.00^{a}	0.26±0.45^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
3	0.0	4.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
4	5.0	0.0	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.35±0.36^{ab}	0.00±0.00^{a}	1.24±2.15^{abc}
5	5.0	2.0	0.04±0.06^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{aabc}	0.00±0.00^{a}
6	5.0	4.0	0.00±0.00^{a}	0.54±0.94^{ab}	2.06±3.56^{abc}	2.70±4.65^{abc}	0.00±0.00^{a}	2.80±4.84^{abc}
7	6.0	0.0	1.10±0.95^{abc}	0.80±0.64^{ab}	0.30±0.53^{ab}	0.52±0.87^{ab}	2.12±3.35^{abc}	0.87±1.01^{ab}
8	6.0	2.0	0.94±1.64^{abc}	0.30±0.52^{ab}	0.21±0.27^{ab}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}
9	6.0	4.0	0.74±1.29^{ab}	0.92±1.60^{abc}	0.00±0.00^{a}	0.00±0.00^{a}	0.00±0.00^{a}	1.34±2.31^{abc}
10	8.0	0.0	4.36±7.02^{c}	2.49±3.85^{abc}	1.67±2.90^{abc}	3.72±3.34^{bc}	1.63±2.82^{abc}	0.02±0.04^{a}
11	8.0	2.0	3.54±5.79^{bc}	1.44±2.50^{abc}	1.88±3.25^{abc}	1.48±2.58^{abc}	1.22±2.12^{abc}	1.16±2.01^{abc}
12	8.0	4.0	3.31±5.71^{abc}	1.63±2.83^{abc}	2.68±4.65^{abc}	2.19±3.21^{abc}	2.30±3.99^{abc}	1.48±2.56^{abc}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication ± one standard deviation from the mean. Means were separated by LSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-16. Quantification of hydrogen peroxide synthesized on spinach surface by LAB cultures at 7 °C.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/ml)[2]		Hydrogen peroxide (μmoles/g of spinach)[3]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]
2	0.0	2.0	0.00±0.00[a]	0.00±0.00[a]	0.06±0.10[a]	0.15±0.26[a]	0.00±0.00[a]	0.09±0.15[a]
3	0.0	4.0	0.00±0.00[a]	0.65±1.03[a]	0.00±0.00[a]	0.13±0.22[a]	0.00±0.00[a]	0.00±0.00[a]
10	8.0	0.0	0.00±0.00[a]	0.97±0.36[a]	0.19±0.18[a]	0.24±0.42[a]	0.66±0.70[a]	0.40±0.18[a]
11	8.0	2.0	0.00±0.00[a]	1.00±0.21[a]	0.00±0.00[a]	0.41±0.71[a]	0.00±0.00[a]	0.13±0.23[a]
12	8.0	4.0	0.00±0.00[a]	2.54±1.78[b]	0.00±0.00[a]	1.78±1.89[b]	0.32±0.56[a]	0.12±0.22[a]
SC	N/A	N/A	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]	0.00±0.00[a]

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication ± one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

6.3.3 Agar Diffusion Assay

To determine the antimicrobial activity of the synthesized peptidous compounds by the lactic acid bacteria (LAB) cultures comprised on LactiGuard^{TM}, it was necessary to performe the agar diffusion assay for the *in vitro* and on spinach surface shown in Tables 6-17 and 6-18, respectively. Table 6-17 illustrates the *in vitro* results and there was detected an initial indication of antimicrobial activity (day 0) on treatments 10 (4.85±0.51 mm/100 µl of sample aliquot), 11 (4.24±0.24 mm/100 µl of sample aliquot), and 12 (4.89±0.99 mm/100 µl of sample aliquot) which increased during the storage period in about 7.19, 7.85, 4.42 mm/100 µl of sample aliquot, respectively. Therefore, the previous statement demonstrated that during the *in vitro* assay at 7 °C there was evidence of bacteriocin formation by the LAB cultures present on LactiGuard at treatment levels of 8.0 $\log_{10}$ CFU/ml that was able to inhibit *Listeria monocytogenes* Scott A (Schillinger and Lucke 1989). In the other hand, Table 6-18 shows that there was not significant difference influenced by the storage time, and temperature condition on the spinach surface assay. However, it was observed indication of same influence by the LAB inoculation level during the entire storage period.

Table 6-17. Antimicrobial activity from the *in vitro* assay.

Treatment	Bacterial concentration levels (log_{10} CFU/ml)[1]		Bacteriocin concentration (mg/ml of sample aliquot)[2]					
			Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
2	0.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
3	0.0	4.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{ab}
4	5.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.01^{b}
5	5.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{ab}	$0.00\pm0.0.01^{ab}$
6	5.0	4.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}
7	6.0	0.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{ab}	0.01 ± 0.01^{ab}
8	6.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.01^{ab}	$0.01\pm0.0.01^{c}$
9	6.0	4.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.01^{c}	0.02 ± 0.00^{c}
10	8.0	0.0	0.04 ± 0.02^{def}	0.05 ± 0.01^{fgh}	0.05 ± 0.01^{h}	0.04 ± 0.01^{gh}	0.06 ± 0.02^{i}	0.10 ± 0.01^{l}
11	8.0	2.0	0.04 ± 0.01^{d}	0.04 ± 0.01^{efg}	0.04 ± 0.01^{def}	0.04 ± 0.01^{de}	0.10 ± 0.01^{k}	0.10 ± 0.01^{l}
12	8.0	4.0	0.04 ± 0.01^{def}	0.05 ± 0.01^{h}	0.04 ± 0.01^{efg}	0.05 ± 0.01^{gh}	0.10 ± 0.00^{l}	0.08 ± 0.01^{j}

[1]LAB: lactic acid bacteria; PT: cocktailed pathogens. [2]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by LSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

Table 6-18. Antimicrobial activity from the spinach surface assay.

Treatment[1]	Bacterial concentration levels (log_{10} CFU/ml)[2]		Bacteriocin concentration (mg/ml of spinach aliquot)[3] Day					
	LAB	PT	0	1	3	6	9	12
1	0.0	0.0	0.01 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.01^{a}	0.01 ± 0.00^{a}	0.01 ± 0.01^{a}
2	0.0	2.0	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.01^{a}	0.00 ± 0.01^{a}
3	0.0	4.0	0.01 ± 0.01^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.01 ± 0.02^{ab}	0.00 ± 0.00^{a}	0.01 ± 0.01^{a}
10	8.0	0.0	0.05 ± 0.01^{defgh}	0.06 ± 0.01^{fghi}	0.05 ± 0.01^{de}	0.05 ± 0.01^{def}	0.05 ± 0.01^{defg}	0.06 ± 0.01^{hi}
11	8.0	2.0	0.06 ± 0.01^{efghi}	0.05 ± 0.01^{defgh}	0.04 ± 0.01^{c}	0.06 ± 0.02^{efghi}	0.06 ± 0.01^{fghi}	0.06 ± 0.01^{hi}
12	8.0	4.0	0.07 ± 0.01^{hi}	0.06 ± 0.01^{efghi}	0.02 ± 0.00^{b}	0.04 ± 0.02^{cd}	0.06 ± 0.01^{ghi}	$0.07\pm0.0.1^{hi}$
SC	N/A	N/A	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}	0.00 ± 0.00^{a}

[1]SC: Spinach control (untreated and uninoculated). [2]LAB: lactic acid bacteria; PT: cocktailed pathogens. N/A: not applicable. [3]Values represent means of triplicate replication $\pm$ one standard deviation from the mean. Means were separated by Tukey's HSD ($p<0.05$). Different superscripted letters denote significant difference between treatments within columns and rows.

6.4 Correlation Analysis between the Dose Response and Antimicrobial Synthesis Results

To determine the relationship of the antimicrobial production by LAB cultures and the dose response assay, it was necessary to performe a correlation analysis by the treatment and by the days of storage as effects at 7 °C. The correlation between the pathogenic dose response and the antimicrobial synthesis by the LAB cultures from LactiGuard (*in vitro* and on spinach) was illustrated on Tables 6-19, 6-20, 6-21, 6-22, 6-23 and 6-24. For the *in vitro* assay, the corresponding correlation values were not determined for treatments 1, 2, and 3 because there was not antimicrobial production by LAB cultures at a inoculated level of 0.0 $\log_{10}$ CFU/ml, and for treatments 1, 4, 7, and 10 because the inoculated level of pathogenic cocktail was 0.0 $\log_{10}$ CFU/ml. For the spinach surface assay, correlation for treatments 1, 2, 3, and 10 was not calculated because the bacterial culture (LAB culture or pathogenic cocktail culture) inoculated level was 0.0 $\log_{10}$ CFU/ml.

Table 6-19 shows the correlation for each treatment between dose response of the pathogic cultures and the L-lactic acid production. The correlation for each *in vitro* treatment between dose response of pathogenic cultures and L-lactic acid production demonstrated that there was a significant correlation of 0.54 for treatment 12, indicating that the increase of the foodborne pathogen population was related by 54% (correlation significance = 0.02) with the increase in production of L-lactic acid by the LAB cultures.

Table 6-19. Correlation for each treatment between dose response of pathogenic cultures and L-lactic acid production during storage at 7 °C.

Treatment	Bacterial Concentration Levels (log_{10} CFU/ml)		*In vitro*	On spinach surface
	LAB[1]	PT[2]		
1	0.0	0.0		
2	0.0	2.0		0.46 (0.06[3])
3	0.0	4.0		0.34 (0.16[3])
4	5.0	0.0		N/A[5]
5	5.0	2.0	0.26 (0.30[3])	N/A
6	5.0	4.0	0.08 (0.77[3])	N/A
7	6.0	0.0		N/A
8	6.0	2.0	0.21 (0.41[3])	N/A
9	6.0	4.0	0.39 (0.11[3])	N/A
10	8.0	0.0		
11	8.0	2.0	-0.15 (0.54[3])	-0.19 (0.45[3])
12	8.0	4.0	0.54 (0.02[4])	-0.39 (0.11[3])

[1]LAB: lactic acid bacteria. [2]PT: cocktailed pathogens. [3]Correlation significance (2-tailed). [4]Correlation is significant ($p<0.05$). [5]N/A: not applicable.

Table 6-20. Correlation between dose response of pathogenic cultures and L-lactic acid production for all treatments during storage at 7 °C.

Day	*In vitro*	On spinach surface
0	0.04 (0.98[1])	0.10 (0.69[1])
1	-0.03 (0.87[1])	-0.09 (0.72[1])
3	0.35 (0.04[2])	-0.19 (0.44[1])
6	-0.03 (0.88[1])	-0.16 (0.53[1])
9	0.23 (0.18[1])	0.04 (0.87[1])
12	0.01 (0.95[1])	0.15 (0.54[1])

[1]Correlation significance (2-tailed). [2]Correlation is significant ($p<0.05$).

Table 6-21. Correlation for each treatment between dose response of pathogenic cultures and hydrogen peroxide production during storage at 7 °C.

Treatment	Bacterial Concentration Levels (log_{10} CFU/ml)		*In vitro*	On spinach surface
	LAB[1]	PT[2]		
1	0.0	0.0		
2	0.0	2.0		0.27 (0.28[3])
3	0.0	4.0		-0.21 (0.39[3])
4	5.0	0.0		N/A[5]
5	5.0	2.0	-0.29 (0.24[3])	N/A
6	5.0	4.0	-0.15 (0.56[3])	N/A
7	6.0	0.0		N/A
8	6.0	2.0	0.33 (0.18[3])	N/A
9	6.0	4.0	0.12 (0.63[3])	N/A
10	8.0	0.0		
11	8.0	2.0	0.92 (0.01[4])	-0.24 (0.34[3])
12	8.0	4.0	0.90 (0.01[4])	-0.21 (0.41[3])

[1]LAB: lactic acid bacteria. [2]PT: cocktailed pathogens. [3]Correlation significance (2-tailed). [4]Correlation is significant ($p<0.05$). [5]N/A: not applicable.

Table 6-22. Correlation between dose response of pathogenic cultures and hydrogen peroxide production for all treatments during storage at 7 °C

Day	*In vitro*	On spinach surface
0	0.06 (0.71[1])	-
1	-0.09 (0.96[1])	0.35 (0.15[1])
3	0.05 (0.76[1])	-0.34 (0.16[1])
6	0.01 (0.97[1])	0.28 (0.26[1])
9	-0.04 (0.81[1])	-0.46 (0.06[1])
12	-0.04 (0.81[1])	-0.30 (0.23[1])

[1]Correlation significance (2-tailed). [2]Correlation is significant ($p<0.05$).

Table 6-23. Correlation for each treatment between dose response of pathogenic cultures and bacteriocin production during storage at 7 °C.

Treatment	Bacterial Concentration Levels (log_{10} CFU/mL)		*In vitro*	On spinach surface
	LAB[1]	PT[2]		
1	0.0	0.0		
2	0.0	2.0		
3	0.0	4.0		
4	5.0	0.0		N/A[5]
5	5.0	2.0	0.55 (0.02[3])	N/A
6	5.0	4.0		N/A
7	6.0	0.0		N/A
8	6.0	2.0	0.65 (0.01[3])	N/A
9	6.0	4.0	0.54 (0.02[3])	N/A
10	8.0	0.0		
11	8.0	2.0	0.74 (0.01[3])	0.87 (0.01[3])
12	8.0	4.0	0.54 (0.02[3])	0.82 (0.01[3])

[1]LAB: lactic acid bacteria. [2]PT: cocktailed pathogens. [3]Correlation is significant ($p<0.05$). N/A5: not applicable.

Table 6-24. Correlation between dose response of pathogenic cultures and bacteriocin production for all treatments during storage at 7 °C.

Day	*In vitro*	On spinach surface
0	0.04 (0.98[1])	-
1	0.04 (0.82)	-
3	-0.01 (0.96)	-
6	-0.02 (0.92)	0.36 (0.14[1])
9	0.14 (0.42)	0.46 (0.06[1])
12	-0.04 (0.82)	0.48 (0.05[2])

[1]Correlation significance (2-tailed). [2]Correlation is significant ($p<0.05$).

The correlation for all treatments during storage (0, 1, 3, 6, 9, and 12 days) between dose response of pathogenic cultures and L-lactic acid production is illustrated on Table 6-20. Values not calculated were due to undetected production of antimicrobial concentration.When the dose response for all treatments of pathogenic cultures was correlated by storage days with the L-lactic acid production, it was detected that the population of *E. coli* O157:H7 and *Salmonella enterica* increased was correlated in a 35% (correlation significance = 0.04) with the increase in fermentation of L-lactic acid *in vitro*. For the spinach surface leaves assay, there was not observed a significant correlation ($p<0.05$) of the dose response with the L-lactic acid production when analyzed by each treatment and by storage day.

Table 6-21 showed hydrogen peroxide correlation with dose response of pathogens for each treatment and it was identified that treatments (*in vitro*) 11 and 12 were significantly positively correlated in a 0.92 and 0.90, respectively. The previous observation indicated that the increase in the production of hydrogen peroxide by LAB at initial 8.0 $\log_{10}$ CFU/ml level is highly and positively correlated with the population increase of the two foodborne pathogens when inoculated in TTSB. In contrast, there was not a significant correlation detected when all treatments were correlated by storage day between dose response and hydrogen peroxide production, in vitro and on spinach surface (Table 6-22).

The antimicrobial activity by bacteriocin production for each treatment was correlated against the dose response of *E. coli O157:H7* and *Salmonella enterica*, in vitro and on spinach leaves surface (Table 6-23). The correlation between dose response

of pathogenic cultures and the antimicrobial activity by bacteriocin production for all treatment during storage is illustrated on Table 6-24.

It was observed that the *in vitro* treatments 5 (0.55), 8 (0.65), 9 (0.54), 11 (0.74), and 12(0.54), and on spinach treatments 11 (0.87) and 12 (0.82) significantly correlated the production of bacteriocin by LAB and the population of the pathogenic cultures in a positive trend. In addition, the correlation of the production bacteriocin and the pathogen population was significant after 12 days (0.48) on the spinach surface inoculation assay. Therefore, it can be stated that the production of bacteriocin increases as a response of the increase in population of *E. coli* O157:H7 and *Salmonella enterica*, within treatments and storage days.

CHAPTER VII

SUMMARY AND CONCLUSIONS

7.1 Summary

In recent years, fruits and vegetables have become significant vehicles of human foodborne disease, and have been associated with 713 outbreaks, from 1990 to 2005. Consequently, they have been considered as highest priority to control specific hazards associated with foodborne pathogens. Lactic acid bacteria (LAB) are antagonistic to some pathogens by the production of antimicrobials. Therefore, they have been proposed as an active intervention option to inhibit and/or reduce foodborne pathogens, such as *Escherichia coli* O157:H7 and *Salmonella enterica* in a microbiological medium and on the surface of spinach leaves.

The determination of dose response in microbiological medium (*in vitro*) and on the surface of spinach leaves, and the quantification of antimicrobials produced by LAB are essential for the understanding of the antimicrobial mode of action for pathogen inhibition. However, it was observed that there was not a significant difference of the inoculated pathogens level *in vitro* during the storage period for up to 12 days at 7 °C. The dose response of *Salmonella enterica* on spinach surface demonstrated significant inhibition differences on day 6 between treatment 2 (4.11±0.42 $\log_{10}$ CFU/ml) and 11 (2.26±0.39 $\log_{10}$ CFU/ml), and treatments 3 (5.11±0.02 $\log_{10}$ CFU/ml) and 12 (4.40±0.48 $\log_{10}$ CFU/ml) of approximately 1.85 and 0.71 $\log_{10}$ CFU/ml. In addition, after 3 days, the dose response of *E. coli* O157:H7 on spinach surface showed that there

were significant differences between treatments 2 (3.88±0.22 $\log_{10}$ CFU/ml) and 11 (2.26±0.37 $\log_{10}$ CFU/ml), and treatments 3 (5.63±0.26 $\log_{10}$ CFU/ml) and 12 (4.90±0.46 $\log_{10}$ CFU/ml), which indicated inhibition of about 1.62 and 0.73 $\log_{10}$ CFU/ml, respectively. Furthermore, in day 6 there was evidence of inhibition (about 2.1 $\log_{10}$ CFU/ml) between treatments 2 (5.02±0.37 $\log_{10}$ CFU/ml) and 11 (2.92±0.30 $\log_{10}$ CFU/ml) when compared within columns.

When the dose response of the specific foodborne pathogens was correlated with the fermentation of antimicrobials by LAB cultures from LactiGuard™, it was determined that the *in vitro* increase in the population of foodborne pathogens with an initial population of 4.0 $\log_{10}$ CFU/ml treated with 8.0 $\log_{10}$ CFU/ml of LAB cultures (treatment 12) was significantly correlated in a 54% with the increase in production of L-lactic acid by the LAB cultures. In addition, the increase in the production of hydrogen peroxide by LAB at initial 8.0 $\log_{10}$ CFU/ml level was highly correlated (treatment 11 = 0.92 and treatment 12 = 0.90) with the population increase of the two foodborne pathogens when inoculated in TTSB (*in vitro*). In contrast, there was not a significant correlation detected when all treatments were correlated by day between dose response and hydrogen peroxide production, in vitro and on spinach surface. For the bacteriocin fermentation, it was observed that the *in vitro* treatments 5 (0.55), 8 (0.65), 9 (0.54), 11 (0.74), and 12 (0.54), and on spinach treatments 11 (0.87) and 12 (0.82) were significantly correlated with the population of the pathogenic cultures in a direct way.

7.2 Conclusions

It is possible to conclude that the metabolic activity of the lactic acid bacteria (LAB) cultures from the commercial LactiGuard™ product (*Lactobacillus animalis,* LA51; *Lactobacillus amylovorus,* M35; and *Pediococcus acidilactici,* D3) was not able to reduce and/or inhibit two foodborne bacterial pathogens (*Escherichia coli* O157:H7 and *Salmonella enterica*) in a microbiological medium (TTSB). However, it was observed a potential inhibitory LAB activity when sprayed on the surface of spinach leaves after 3 and 6 days.

In addition, it was demonstrated that the LAB cultures have the capability to produce detectable antimicrobial substances, such as L-lactic acid, hydrogen peroxide, and bacteriocins in the microbiological medium TTSB and on spinach leaves surface, which may have a significant potential to inhibit and/or reduce Gram-positive pathogens, such as *Listeria monocytogenes* Scott A in produce and/or ready-to-eat meat products.

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VITA

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