EFFECT OF NATURAL ANTIMICROBIALS AGAINST *Salmonella, Escherichia coli* O157:H7 AND *Listeria monocytogenes*

A Thesis

by

MARY PIA CUERVO PLIEGO

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

MASTER OF SCIENCE

May 2007

Major Subject: Food Science and Technology

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ABSTRACT

Effect of Natural Antimicrobials Against *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes.* (May 2007)

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Salmonella, Escherichia coli O157:H7 and *Listeria monocytogenes* are pathogens that have caught the attention of federal agencies and researchers due to their great economic impact when illnesses occur. To reduce the presence of these pathogens, different approaches have been used. However, since the global consumer's demand for natural ingredients is steadily increasing, the investigation of the effectiveness of potential natural antimicrobials is necessary. In this study, the *in vitro* antimicrobial activity of *Hibiscus sabdariffa* L extracts against *Salmonella, E. coli* O157:H7 and *L. monocytogenes* was investigated. Furthermore, *H. sabdariffa* L and ε-polylysine were evaluated to reduce populations of *Salmonella* and *E. coli* O157:H7 in ground beef.

The minimum inhibitory concentration (MIC) of *H. sabdariffa* L extracts against *Salmonella* and *E. coli* O157:H7 was 6,489 μg/mL and for *L. monocytogenes*, 5,309 μg/mL. The minimum bactericidal concentration (MBC) of *H. sabdariffa* L extracts against *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* was 19,467, 58,400 and 29,200 μg/mL, respectively. The exposure to 58,400 μg/mL of *H. sabdariffa* extract at 25 ^{o}C for 12 h resulted in reductions of more than 6.0 log CFU/mL for any of the 3

pathogens tested. Ground beef inoculated with *S.* Agona (GFP) and *E. coli* O157:H7 (RFP) was subjected to 5 decontamination treatments. Three of the treatments were using *H. sabdariffa* L and the remaining ε-polylysine. *S.* Agona (GFP) was reduced in 1.1 log cycles using 10% of ground *H. sabdariffa* L and *E. coli* O157:H7 (RFP) was reduced 0.9 log cycles using 400 ppm of ε-polylysine. If these natural antimicrobials are combined with current antimicrobial technologies to form a hurdle effect, higher pathogen reductions could be achieved. Reductions in the presence of pathogens in food may lead into reductions in the incidence of foodborne diseases.

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INTRODUCTION

Recent reports indicate that *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes* are responsible for 5.9 billion dollars in economic loses each year. Moreover, the recent emergence of pathogens such as *E. coli* O157:H7, antibiotic resistant *Salmonella* DT104 and *L. monocytogenes* have attracted the attention of federal agencies and researchers. New alternatives to control these microorganisms are being explored.

The worldwide awareness of health risks associated with non-natural additives, added to the current pathogen concerns, may lead to the investigation of natural products such as antimicrobials obtained from plant or microbial sources. Several studies have confirmed the antimicrobial activity of phytochemicals from herbs and spices such as clove, cinnamon, mustard seed, oregano, rosemary, sage, thyme, and vanillin. *Hibiscus sabdariffa* L is a plant that has been used in traditional medicine as an antiseptic, digestive agent and antihypertensive, however, limited information regarding its antimicrobial activity against pathogens is available. Bacteriocins are small peptides that have been shown to exert antimicrobial activity. ε-polylysine, is a new bacto-antimicrobial whose performance against foodborne pathogens within a food matrix has not been thoroughly examined. New antimicrobials are commonly evaluated through the identification of bacteriostatic and bactericidal effects against specific microorganisms.

This thesis follows the style of Journal of Food Science.

The aim of this project was to determine the antimicrobial effectiveness of 2 natural antimicrobials, *H. sabdariffa* L extracts and ε-polylysine. The objectives of this study were to (1) identify the minimum inhibitory concentration (MIC) of *H. sabdariffa* L aqueous extracts against *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes,* (2) determine the minimum bactericidal concentration (MBC) of *H. sabdariffa* L aqueous extracts against *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes,* (3) establish the time-kill for *Salmonella*, *E. coli* O157:H7 and *L. monocytogenes* after exposure to *H. sabdariffa* L aqueous extracts, (4) evaluate *H. sabdariffa* L and ε-polylysine as antimicrobials against *Salmonella* and *E. coli* O157:H7 in ground beef.

LITERATURE REVIEW

Human illness

Underdeveloped and technologically developed countries are struggling with foodborne outbreaks which result in illness, death and large economic losses. Gould and Russell (2003) reported that in underdeveloped countries there are more than one billion cases of gastroenteritis and up to 5 million deaths annually. According to the Centers for Disease Control and Prevention (CDC 2006a), every year in the United States (U.S.) foodborne microbial hazards cause 76 million illnesses, more than 300,000 hospitalizations and 5,000 deaths. Meanwhile, the Economic Research Service of the United States Department of Agriculture (USDA 2006) has estimated that up to 5.9 billion dollars in economic loses are caused by *Salmonella, E. coli* O157:H7 and *L. monocytogenes* each year.

The increasing numbers of foodborne illness outbreaks caused by some pathogens and the antibiotic resistance of some strains of *Salmonella* Typhimurium DT104, *L. monocytogenes* and *Enterococcus faecium*, have captured the attention and concern of regulatory agencies as well as the food safety community. According to reports from the CDC, published in Morbidity and Mortality Weekly Reports (CDC 2006a), foodborne outbreaks still occur frequently, suggesting that new alternatives to fight pathogens are needed (Rota and others 2004; Geornaras and others 2005).

The Food and Drug Administration (FDA 2006a) has estimated that between 2 and 4 million cases of salmonellosis occur in the U.S. annually, and the incidence of

Salmonella infections appears to be increasing in the U.S. and in other developed nations.

E. coli O157:H7 is an emerging pathogen, responsible for approximately 73,000 cases of infection, 2,100 hospitalizations and 61 deaths in the U.S. each year. Children and the elderly are more susceptible to developing complications such as hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), which may lead to kidney failure. Approximately between 2% and 7% of the infections are associated with such complications. In 2004, there was an important decrease of cases to less that one per 100,000 people (CDC 2006b).

L. monocytogenes is also a pathogen of concern. FoodNet active surveillance reported that the incidence of listeriosis was 0.27 cases per 100,000 people in 2004, representing a 40% decrease compared with 1996 and 1998 incidence data, 0.46 and 0.57 respectively. In spite of the significant reduction in the incidence of listeriosis, outbreaks still occur and recently have been associated with the consumption of ready-to-eat meat and unpasteurized fresh cheese (CDC 2006c).

Antimicrobials

Foods are susceptible to biological deterioration and sustain an adequate environment for pathogen growth. Heating, cooling, drying or fermenting have been popular methods to achieve quality and safety goals since prehistoric times. However, it has been only during the last century that the use of chemicals to control spoilage and pathogenic microorganisms in food became extensive. Currently, the use of

antimicrobial agents to preserve the quality characteristics and to assure the safety of foods is a common strategy in the food industry and it is regulated by the FDA under the category of food additives (Branen 1993; FDA 2006b).

Antimicrobial agents are defined as “Substances used to preserve food by preventing growth of microorganisms and subsequent spoilage, including fungistats, mold and rope inhibitors” in the Code of Federal Regulations (CFR) (FDA 2006b). Another term related to this topic is chemical preservative, and it has been defined by the CFR as “Any chemical that, when added to food, tends to prevent or retard deterioration thereof, but does not include common salt, sugars, vinegars, spices, or oil extracted from spices, substances added to food by direct exposure thereof to wood smoke, or chemicals applied for their insecticidal or herbicidal properties.” Even though salt, sugar, vinegars and spices are not considered chemical preservatives in the CFR they provide an important synergistic effect for controlling the growth of microorganisms (Naidu 2000; FDA 2006b).

Recently, the global consumer started to demand high quality foods that are minimally processed and sustain more naturally occurring bioactive ingredients. Moreover, the consumer perception that the use of chemical antimicrobials could have further toxicological consequences has attracted the attention of national food agencies and the food industry. During the last decade, concerns over allergies and carcinogenic compounds developed from chemical additives resulted in an increase in the demand of more naturally processed foods. All these trends are forcing food processors to find new means to extend shelf life and maintain the product safety while decreasing the use of

food additives or severe treatments, such as heat treatments (Gould and Russell 2003; Naidu 2000; Roller 2003).

Natural antimicrobials

These natural components can exhibit antimicrobial activity in the foods in which they are commonly used as ingredients or may be used as additives in other foods. According to some authors, natural antimicrobials can be grouped in 6 categories. In the first group there are the state-of-the art-antimicrobials, which have been used for centuries, such as salt, sugar, phosphate salts and chlorine. The other five categories are lacto, ovo, bacto, acid and phyto-antimicrobials which have been isolated from animals, plants or are microbial by-products and have been investigated thoroughly during the last 20 years. The focus of this project is the investigation of a phyto-antimicrobial and an emerging bacto-antimicrobial, ε-polylysine (Sofos 1998; Naidu 2000; Roller 2003; Davidson 2005).

Plants, flowers, herbs and spices are widely recognized to have antibacterial properties and were used by ancient cultures. Nevertheless most antimicrobial research of herbs and spices has been done recently. Antibacterial compounds of herbs and spices have been isolated and characterized as phenolics and polyphenolics, terpenoids, alkaloids, lectins, and polypeptides which suggested that their commercial use as natural antimicrobials could be expanded (Cowan 1999; Davidson and Naidu 2000).

Curative effect of plants

There are an estimated 250,000 to 500,000 species of plants known to man, of which more than 10% are used for medicinal purposes. The curative effects of plants has been extensively documented among different cultures throughout history. Early civilizations, such as the Chinese, Indian or others in the Middle East have documents defining the use of plants as medicinal remedies five thousand years ago. The recent tendency of the general public to reconsider the alternative medicine has attracted the attention of the food industry and the research community to generate reliable information regarding the claimed therapeutic effects of medicinal plants (Hamburger and Hostettmann 1991; Cowan 1999; Li 2003).

Some plants, herbs and spices are recognized for delivering health benefits from their natural components. These natural components have the potential ability to decrease blood cholesterol levels and protect against osteoporosis and cancer development. These aforementioned characteristics of plants, herbs and spices have caused them to be defined as nutraceuticals (Wildman 2001). According to DeFelice (1995), "A nutraceutical is any substance, food or part of a food, that provides medical or health benefits, including the prevention and treatment of diseases."

Among all the benefits that plants, herbs and spices can contribute to foods, many studies have confirmed their antimicrobial activity as well; such studies have assigned the most potent status to those with a broad spectrum of activity against numerous bacteria and fungi. Clove (*Syzygium aromaticum*), cinnamon (*Cinnamomum zeylanicum*), thyme (*Thymus vulgaris*) oregano (*Origanum vulgare*) and vanilla (*Vanilla

planifola, V. pompona, V. tahitensis) fall into this category, while other spices such as sage (*Salvia officinalis*), rosemary (*Rosmarinus officinalis*), cilantro (*Coriandrum sativum*), tea tree oil (*Melaleuca alternifolia*) and fingerroot extract (*Boesenbergia pandurata*) are considered lower intensity antimicrobials (Cowan 1999; Naidu 2000; Casterton and others 2005; Davidson 2005).

However, in many cases, the concentration of the antimicrobial compound in its natural form is too low to be successfully used without damaging the sensory characteristics of the final product. Therefore, intensive investigations on the bactericidal and bacteriostatic properties of new plants need to be considered. Rota and others (2004) studied the MIC and MBC of ten essential oils from aromatic plants against *S.* Typhimurium, *E. coli* O157:H7, *L. monocytogenes, Yersinia enterocolitica, Shigella flexneri* and *Saphylococcus aureus* (Table 1). The MIC was the lowest concentration at which the pathogens failed to grow in tryptic soy broth (TSB) but grew when 100 µL was transferred to triptic soy agar (TSA). Similarly, the MBC was the lowest concentration at which the bacteria failed to grow in TSB as well as on TSA (Davidson 2005).

Combining the antimicrobial activity of phytochemicals, which may have a different mode of action than conventional preservatives, with current bactericidal technologies, may significantly reduce the risk of foodborne disease by decreasing the risk of exposure to contaminated food. There are numerous plant extracts that have not been tested, among those, *H. sabdariffa* L. This plant has shown promising antimicrobial properties but requires more extensive investigation (Kubo and others 2004; Lin and others 2004).

Table 1 – MIC and MBC of selected plant volatile oils (Rota and others 2004).

Plant	Inhibitory activity (μg/mL)											
	ST		***Ec***		***Lm***		***Ye***		***Sf***		***Sa***	
	MIC	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**	**MIC**	**MBC**
Thymus vulgaris	0.5	0.5	0.8	0.8	0.5	0.8	0.5	0.5	0.8	0.8	0.3	7.0
Salvia officianalis	NT	NT	NT	NT	3.0	7.0	NT	NT	NT	NT	1.0	10.0
Salvia lavandulifolia	NT	NT	NT	NT	4.0	11.0	NT	NT	NT	NT	NT	NT
Lavandula latifolia	1.0	1.0	NT	NT	2.0	10.0	NT	NT	1.5	1.5	0.7	3.0
Lavandin Super	NT	NT	NT	NT	2.0	8.0	2.0	2.0	2.5	2.5	2.0	14.0
Lavandin Abrialis	NT	NT	NT	NT	0.5	5.0	0.8	8.0	1.5	1.5	0.3	4.0
Lavandin Grosso	1.0	1.0	3.5	3.5	1.0	1.0	1.0	1.0	0.5	0.5	2.0	12.0
Rosmarinus officinalis	1.2	1.2	NT	NT	1.0	4.0	1.0	1.0	2.0	2.0	5.0	15.0
Satureja montana	<0.2	<0.2	0.3	0.3	<0.1	<0.1	0.3	0.3	0.3	0.3	<0.1	2.0

ST = *Salmonella* Typhimurium.
Ec = *Escherichia coli* O157:H7.
Lm = *Listeria monocytogenes.*
Ye = *Yersinia enterocolitica.*
Sf = *Shigella flexneri.*
Sa = *Staphylococcus aureus.*
MIC = minimum inhibitory concentration.
MBC = minimum bactericidal concentration.
NT = no tested.

***Hibiscus sabdariffa* L**

H. sabdariffa L belongs to the Malvaceae family. It is an annual, rigid, thick branched, herbaceous shrub, 2.4 m tall, with smooth cylindrical red stems. The green with reddish nervure leaves are from 7.5 to 12.5 cm long. The flowers are as wide as 12.5 cm, yellowish brown with a rose or maroon center, and turn pink as they shrink at the end of the day. The fruits are up to 2.5 cm in length and surrounded by the characteristic red calyxes. The calyxes are formed by 5 large sepals containing between 22 and 34 seeds per pod. The calyxes, steams and leaves have an acid flavor that closely resembles cranberries (Morton 1987; Omemu and others 2006).

This plant is also referred to as Indian sorrel, Jamaican sorrel, Guinea sorrel, Java jute, red sorrel and roselle. It is recognized to be native from India to Malaysia, where it is commonly cultivated, and was brought to Africa at an early date. Currently, it is widely dispersed in both tropics, and in many areas of the West Indies and Central America where it grows as an uncultivated plant (Morton 1987; D'Heureux-Calix and Badrie 2004).

Observations describing this plant were written as early as 1579 by the botanist M. de L'Obel. The use of *H. sabdariffa* L leaves for food purposes was in Java in 1687. Since this plant was extensively cultivated in Africa it is believed that African slaves brought seeds to South America and started growing it in Brazil and Jamaica at the beginning of the 17th century. The use of *H. sabdariffa* L expanded, and by 1840 it was grown for food purposes in Guatemala. In 1899, J.N. Rose noticed large baskets of dried

calyces in the markets of Guadalajara, México and in 1907 Wester affirmed that fresh calyces were sold by the quart in South Florida markets (Morton 1987).

Currently, the thick, bright red and cup-shaped calyces are consumed worldwide to prepare cold or hot beverages, jellies, sauces, wines, food colorants, etc. Moreover, this plant has captured the attention of food and beverage manufacturers who have recognized its possibilities as a natural food product, since according to the CFR it is classified as a generally recognized as safe (GRAS) ingredient. In addition to its attribute as a food ingredient, aqueous extracts have been used in traditional medicine as an antiseptic, digestive agent and antihypertensive (Morton 1987; D'Heureux-Calix and Badrie 2004; Oboh and Elusiyan 2004; Badreldin and others2005; FDA 2006b).

Chemical composition

Phytochemicals are aromatic substances synthesized by plants which in many cases have been suggested to be an inherent defense mechanism to microbial infection, insect and herbivore predation. Phenolics is one of the groups of phytochemicals which comprise simple phenols, phenolic acids, quinones, flavonoids, flavones, flavonols, tannins and coumarins. Aqueous extracts of *H. sabdariffa* L calyces have been analyzed and the results attribute the bright red color to the presence of anthocyanins, allowing the extract to fit in a phenolic and flavonoid profile. Eighty-five percent of these anthocyanins was identified as delphinidin 3-sambubioside and cyaniding 3-sambubioside. Moreover, Badreldin and others (2005) examined the chemical composition of aqueous extracts of *H. sabdariffa* L and reported the presence of

hibiscitrin, gossypitrin, sabdaritrin, flavonol glycosides and some organic acids such as citric, malic, tartaric and ascorbic (Cowan 1999; Tsai and others 2002).

Antimicrobial activity

H. sabdariffa L extracts have been investigated as antimicrobial agents for at least 40 years. The first investigations were made by Sharaf and others (1966), who found that aqueous extracts of *H. sabdariffa* L prevented the growth of *Pasteurella, Pseudomonas, Proteus* and *Streptococcus.* More recently, Oboh and others (2004) studied the effect of aqueous extracts of *H. sabdariffa* L for inhibiting the growth of *P. aeruginosa, Lactobacillus* sp.*, Bacillus* sp.*,* and *Corynebacterium* sp. and found the extract appropriate for the inhibition of all bacteria present. *Lactobacillus* sp. was the test organism that exhibited the largest zone of inhibition.

Bacto-antimicrobials

These substances are bacteriocins produced as by-products of the natural metabolism of certain bacteria. The biotechnology industry has been able to increase their production in large-scale. In the market, the more commonly seen are nisin, pediocins, reuterin, sakacins and recently in the Japanese market the use of ε-polylysine has been a success (Naidu 2000, Chisso Corporation 2006).

ε-polylysine

ε-polylysine is a homopolymer of L-lysine formed by 25 to 35 residues of the amino acid L-lysine. It is an emerging bacto-antimicrobial with important inhibiting and bactericidal properties that has not been studied thoroughly within food systems. It is produced by a mutant strain of *Streptomyces albulus* and as an antimicrobial it shows a wide range of activity. Moreover, it is stable at high temperatures as well as acidic and alkaline conditions. In *in vitro* studies, ε-polylysine at 25 ppm has been found to inhibit the growth of *L. monocytogenes* at refrigeration temperature. At 24 ^{o}C the MIC of ε-polylysine against *E. coli* O157:H7 and *L. monocytogenes* is 100 ppm whereas for *S.* Typhimurium is 300 ppm. However, this new antimicrobial agent has not been tested in food systems, therefore additional research in this area is necessary to ensure the effectiveness of this agent (Geornaras and others 2005).

MATERIALS AND METHODS

Bacterial cultures

MIC experiments

The MIC of *H. sabdariffa* L aqueous extracts against one strain of *Salmonella, E. coli* O157:H7 or *L. monocytogenes* was determined using cultures from the culture collection of the Food Microbiology Laboratory of the *Universidad Autónoma del Estado de Hidalgo* (Hidalgo State University) (Hidalgo, México). These cultures were maintained on tryptic soy agar (TSA; Difco, Detroit, Mich., U.S.A.) slants at 4-7 ^{o}C. For each analysis, each microorganism was activated in tryptic soy broth (TSB; Difco) and incubated at 35 ^{o}C for 24 h.

MBC and time-kill effect experiments

Mixtures of different strains (cocktail) of each microorganism were used for this study. The *Salmonella* cocktail consisted of *S.* Michigan (isolated from cantaloupe), *S.* Poona and *S.* Montevideo (both human isolates) provided by Dr. Linda Harris from the University of California (Davis, Calif., U.S.A.). The *E. coli* O157:H7 cocktail consisted of 3 strains obtained from the bacterial culture collection of the Food Microbiology Laboratory at Texas A&M University (College Station, Tex., U.S.A.). The *Listeria* cocktail consisted of a mixture of two strains of *L. monocytogenes* obtained from the National Animal Disease Center (NADC, Ames, Iowa, U.S.A.) and *L. monocytogenes* Scott A (serotype 4b), a human isolate from the Food Microbiology Laboratory at Texas

A&M University culture collection. Individual stock cultures were stored in cryopellets (Key Products, Round Rock, Tex., U.S.A.) at -80 ^{o}C. Each strain was activated by transferring a cryopellet into TSB and incubated at 35 ^{o}C for 24 h. All strains were streaked on TSA slants and maintained at 20-25 ^{o}C until needed.

Pathogen reduction in ground beef by use of natural antimicrobials

E. coli O157:H7 originally isolated from cattle by the Food Microbiology Laboratory at Texas A&M University and one *S.* Agona isolated from alfalfa sprouts and provided by Dr. Linda Harris from the University of California were used in this study. Both microorganisms were transformed by insertion of a plasmid containing a gene to express a red or green fluorescent protein and ampicillin resistance. Plasmid insertion was achieved by electroporation following the technique described by O'Callaghan and Charbit (1990). The plasmid vector for *E. coli* O157:H7 expressed a red fluorescent protein (RFP) and for *S.* Agona a green fluorescent protein (GFP). Individual stock cultures were maintained in cryopellets (Key Products) at –80 ^{o}C. Each strain was activated by transferring a cryopellet into TSB supplemented with ampicillin (Sigma-Aldrich Corp. St. Louis, Mo. U.S.A.) (100μg/ml) (TSB+amp). The broth was incubated at 35 ^{o}C for 24 h. Then, all the strains were streaked on TSA slants supplemented with ampicillin (Sigma-Aldrich Corp.) (100μg/mL) (TSA+amp) and maintained at 20-25 ^{o}C until needed.

Inoculum preparation

MIC experiments

From TSA slants, *Salmonella, E. coli* O157:H7 or *L. monocytogenes* isolates were individually transferred to tubes containing 9 mL of TSB and incubated at 35 ^{o}C for 18-24 h. The inoculum reached a level of approximately 8.0 log CFU/mL. This level was confirmed by preparing serial dilutions of the inoculum in sterile 0.1% peptone water (PW) (Difco Laboratories, Detroit, Mich., U.S.A.), spread plating onto TSA and incubating at 35 ^{o}C for 24 h before colony enumeration.

MBC and time-kill effect experiments

From TSA slants, the three *Salmonella*, *E. coli* O157:H7 or *L. monocytogenes* cultures were individually transferred twice into 9-mL TSB and incubated at 35 ^{o}C for 18 to 24 h. The day of the experiment, 3 mL of each strain grown in TSB were combined in a sterile tube to make up a 9 mL cocktail of each microorganism. The suspension then was washed by centrifuging at 1,792 x *g* for 15 min and the resulting pellet was resuspended in 9 mL of sterile 0.85% (w/v) saline solution. The expected level of inoculum in the washed suspension was 8.0 log CFU/mL of each microorganism. To confirm this level, serial dilutions of the inoculum were prepared in sterile 0.1% PW, spread plated onto TSA and incubated at 35 ^{o}C for 24 h before colony enumeration.

Pathogen reduction in ground beef by use of natural antimicrobials

From TSA+amp slants, *E. coli* O157:H7 (RFP) and *S.* Agona (GFP) were individually transferred into 9-mL TSB+amp and incubated at 35 ^{o}C for 18-24 h. The day before the experiment, each microorganism was transferred from a fresh TSB culture into a flask containing 200 mL of TSB+amp and incubated at 35 ^{o}C for 18-24 h. Ten mL of each culture were combined and mixed in a 50-mL sterile tube to make up a total of 20 mL of inoculum. The number of viable cells in this inoculum was verified by spread plating 0.1-mL aliquots of serial dilutions of sterile 0.1% PW onto TSA+amp plates and incubated at 35 ^{o}C for 24 h before colony enumeration.

Preparation of *H. sabdariffa* L

Aqueous extract

H. sabdariffa L dried sepals (Frontera Produce LTD, Edinburg, Tx., U.S.A.) were purchased from a local grocery store and stored at 20-25 ^{o}C until needed. A fresh extract was prepared by adding 200 mL of distilled water to 25 g of sepals into a beaker, heated for 10 min on a hot plate (VWR International, Inc., West Chester, Pa., U.S.A.) until the liquid reached 95 °C. Subsequently, the mixture was allowed to cool down for 10 min to increase extraction of active compounds. The resulting water extract was put through a 49 squares/cm^2 cheesecloth, then poured into a screw-cap sterile container and stored at 4-7 ^{o}C for 24 h. Fifteen different treatments were prepared with appropriate volumes of the refrigerated extract and TSB. The *H. sabdariffa* L content ranged from 3, 893 to 58,400 µg/mL (Table 2).

Table 2 – Total *H. sabdariffa* L extract content in solutions tested.

***H. sabdariffa* L extract (µg/mL)**	***H. sabdariffa* L extract (mL)**	**TSB (mL)**	**Total volume (mL)**
58,400	60.0	0	60.0
29,200	30.0	30	60.0
19,467	30.0	60	90.0
14,600	20.0	60	80.0
11,680	15.0	60	75.0
9,733	12.0	60	72.0
8,343	10.0	60	70.0
7,300	8.5	60	68.5
6,489	7.5	60	67.5
5,840	6.5	60	66.5
5,309	6.0	60	66.0
4,867	5.5	60	65.5
4,492	5.0	60	65.0
4,171	4.5	60	64.5
3,893	4.0	60	64.0

TSB = tryptic soy broth.

Powder

Two hundred g of *H. sabdariffa* L dried sepals were finely ground using a blender (Hamilton Beach, Washington, N.C., U.S.A.). The powder was put through a sieve of 0.1 cm aperture and stored in plastic bags for 24 h at room temperature (20-25 ^{o}C) until needed within the same day of grinding.

Extraction yield

The *H. sabdariffa* L extract was dried in a freeze-dryer (FTS Systems, Inc. Stone Ridge, N.Y., U.S.A.) at -65 ^{o}C for 48 h under vacuum. The extraction yield was calculated by weight difference between the liquid and the dried sample.

Phytochemical analysis

The total phenol content of the water extract was colorimetrically tested by an adaptation of the Folin-Ciocalteau reagent method described below (Hahn 1984). A 1:10 distilled water dilution of the original water extract was prepared and combined with 0.4 mL of in-house-prepared Folin-Ciocalteau reagent and 0.9 ml of 0.5M ethanolamine (Fisher Scientific Company, Fair Lawn, N.J., U.S.A.). After thorough mixing, the solution was allowed to stand for 20 min and absorbance was then measured with a spectrophotometer (BioMate^{TM} 3, Thermo Electron Corporation, Madison, Wis., U.S.A.) set at 600 nm. This assay was performed in triplicate. Simultaneously, a blank and a standard curve were determined. For the standard curve, solutions of 0, 5, 10, 15 and 20 mg/L of gallic acid (GA) were prepared. Standard solutions were prepared with GA

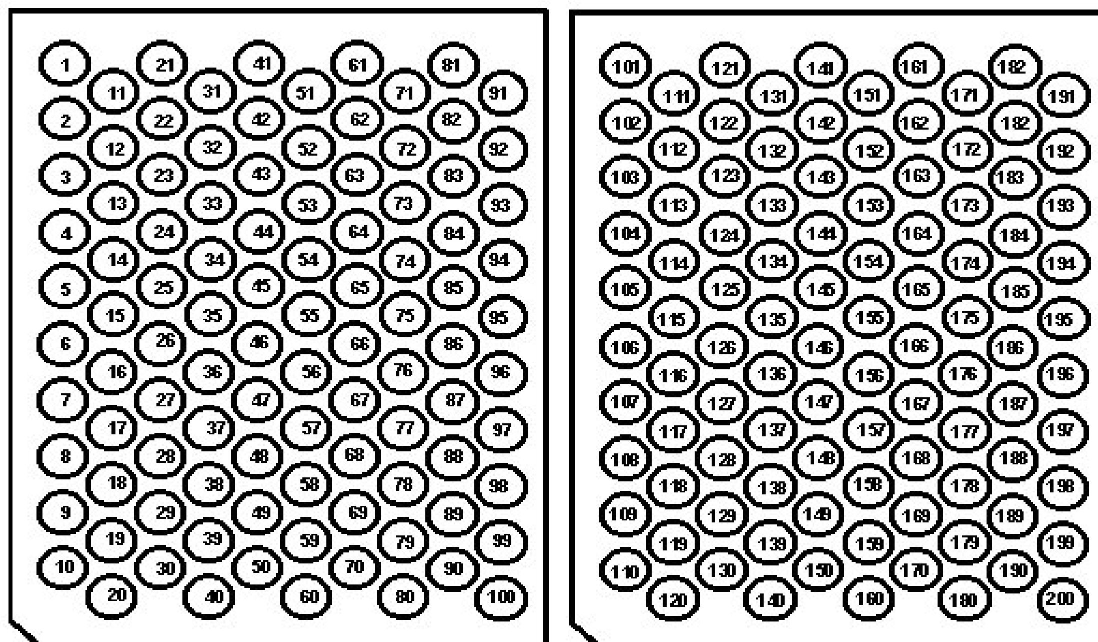
diluted with a mixture of methanol-water (50:50, v/v), Folin-Ciocalteau reagent and 0.5M ethanolamine. The absorbance of each solution was measured after a 20 min dwell time. Using linear regression of the standard curve, the concentration of total phenols in the sample was determined and reported as mg of GA per g of sample.

Identification of minimum inhibitory concentration (MIC)

The methodology for this experiment is based on the work of Lambert and others (2001) and was performed at the *Universidad Autónoma del Estado de Hidalgo* using a Bioscreen C Microbiology Reader (Oy Growth Curves AB Ltd, Helsinki, Finland). From the 15 *H. sabdariffa* L dilutions previously prepared, 400 µL of each were dispensed along a 100-well honeycomb plate (Growth Curves USA, Piscataway, N.J., U.S.A.) (Figure 1). Ten µL of inoculum were dispensed in each well. After inoculation, the plate was incubated at 25 ^{o}C for 24 h and the optical density (O.D.) of each well was recorded at 600 nm every 20 min after shaking. Calculation of growth characteristics was accomplished by fitting the O.D. data in a modified Gompertz function as analyzed by Lambert and Pearson (2000) using the software GraphPad Prism version 4.03 (GraphPad Software, Inc. 2006).

Identification of minimum bactericidal concentration (MBC)

The methodology described below is an adaptation of the Minimum Bactericidal Concentration Testing from the Clinical Microbiology Procedures Handbook (Hindler 1992). The five dilutions with higher content of *H. sabdariffa* L extract were used for



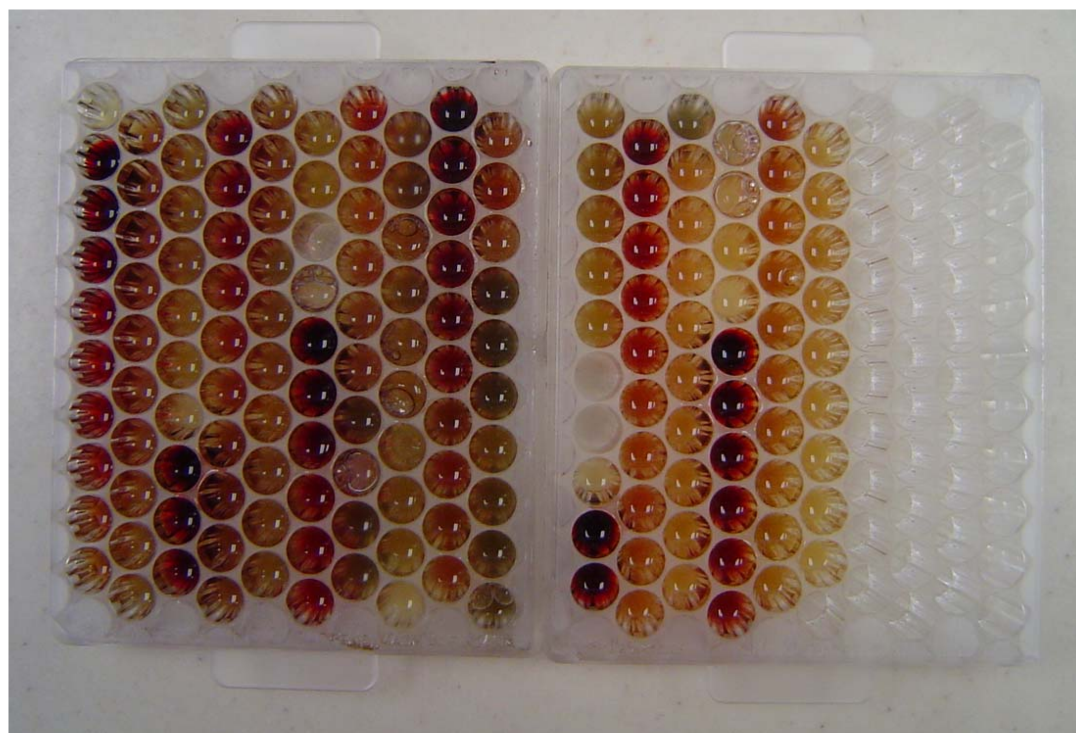


Figure 1 – Distribution of dilutions in honeycomb plate.

this study. *H. sabdariffa* L content ranged from 11,680 to 58,400 µg/mL. Four sets of each dilution were dispensed into 15-mL sterile tubes, one set for each microorganism and one as a negative control. For all sets, each tube was inoculated with 0.1 mL of the corresponding pathogen suspension and then incubated for 24 h at 35 °C.

After the 24-h contact time at 25 °C, 1 mL from each dilution was transferred to 10 mL of TSB and mixed. One mL aliquot of the mixture was poured into a 1.5 mL cuvette (VWR International, Inc.) where initial O.D. was read at 600 nm. The TSB mixtures were incubated for 48 h at 35 °C with O.D. readings at 24 and 48 h. O.D. values at 0, 24 and 48 h were used for further comparison and MBC calculation. In order to confirm the O.D. readings, after the 24-h contact time, 0.1 mL of the inoculated dilutions were spread plated on TSA and incubated at 35 °C for 24 h before counts were performed and reported as CFU/mL.

Time-kill assay

The methodology described below is an adaptation of the Clinical Microbiology Procedures Handbook (Hindler 1992). To evaluate the concentration-dependent activity, 2 *H. sabdariffa* L extract dilutions were selected; one with high and one with low extract concentration (dilution 1:1 and 1:10). Each dilution and a control, consisting of sterile TSB, were inoculated with 0.1 mL of the corresponding pathogen and kept in a water bath (VWR International, Inc.) at 25 °C. The behavior of the three pathogens was followed by determining the initial and remaining viable counts at 0, 1, 2, 4, 6, 12 and 24 h of contact with the extract dilutions.

For the enumeration of surviving cells, serial dilutions with 0.1% sterile PW (Difco) were prepared and plated on TSA using an automatic spiral plater (DWS, West Yorkshire, England). Plates were incubated at 35 ^{o}C for 24 h, colonies were counted using an automatic colony counter (Protos, Bioscience International, Frederick, Md., U.S.A.) and reported as CFU/mL. All experiments were performed in triplicate. Death curves were fitted to the modified Gompertz function as analyzed by Linton and others (1995) using the software GraphPad Prism version 4.03 (GraphPad Software, Inc. 2006).

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Sample preparation and inoculation

Three kg of GB containing 93% lean and 7% fat were purchased from a local grocery store and maintained at 4 ^{o}C until use within the same day of purchase. Twelve mL of the *E. coli* O157:H7 (RFP) and *S.* Agona (GFP) inoculum were mixed with the GB using a standard mixer KitchenAid -KSM90 (KitchenAid, St. Joseph, Mich., U.S.A.) for 2 min at speed #2.

Application of treatments

The inoculated GB was separated into five 500-g portions, each portion assigned to one of the following 5 treatments: (1) ground *H. sabdariffa* L calices at 5 % (w/w); (2) ground *H. sabdariffa* L calices at 10% (w/w); (3) *H. sabdariffa* L aqueous extract at 5% (w/w); (4) ε-polylysine (Chisso Coorporation, Tokyo, Japan) at 200 ppm; and (5) ε-polylysine (Chisso Coorporation) at 400 ppm.

For treatment 1, 475 g of inoculated GB were mixed with 25 g of the *H. sabdariffa* L powder. Treatment 2 consisted of adding 50 g of *H. sabdariffa* L powder to 450 g of inoculated GB, treatment 3 consisted of adding 25 g of *H. sabdariffa* L aqueous extract to 475 g of inoculated GB. Finally, for treatments 4 and 5, 100 and 200 mg of ε-polylysine (Chisso Coorporation), respectively, were dissolved in 5 mL of distilled-sterile water and mixed with 500 g of inoculated GB. In all treatments, the inoculated GB and natural antimicrobials were mixed using a standard mixer KitchenAid-KSM90 (KitchenAid) for 2 min at speed #2. After mixing, meat of each treatment and the positive control was divided in six 11-g portions, then individually bagged in stomacher bags (25×28 cm) and stored at 4-7 ^{o}C for 2 and 24-h sampling times.

Enumeration

Samples were analyzed by homogenizing each 11-g sample with 99 mL of sterile 0.1% PW in a Stomacher 400 Lab Blender (Seward, London, U.K.) for 1 min. Serial dilutions in sterile 0.1% PW were prepared and spread plated onto TSA+amp. The plates were incubated at 35 ^{o}C for 24 h. Colonies of *E. coli* O157:H7 (RFP) and *S.* Agona (GFP) were counted using an ultraviolet (UV) light. All experiments were performed in triplicate.

Data analysis

Microbial counts (CFU/ml) were transformed into log values. MIC, MBC and microbial counts, were analyzed by analysis of variance (ANOVA) to find differences

among treatments. If differences were found, mean comparisons were performed by Duncan's multiple range test. These analyses were performed using Statistical Package for the Social Sciences (SPSS 12.0.1) (2006).

RESULTS AND DISCUSSION

Extraction yield and phytochemical analysis

Quantification of total solids and total phenols extracted during the preparation of the aqueous extract was determined by the extraction yield and phytochemical analysis. When 50 g of calyces were placed in 400 mL of distilled water the extraction yield of the *H. sabdariffa* L extract was 467 mg/g of dry sample.

The relationship between phenols and their antimicrobial activity has been extensively documented in the literature. Indeed, some authors have intended to elucidate the antimicrobial mechanism of phenols. It has been suggested that oxidized phenols inhibit metabolic enzymes leading to the inactivation of the reproductive activity of the cell. Other phenolic structures, reported to be present in the *H. sabdariffa* L extracts (anthocyanins), were proposed to complex with nucleophilic amino acids of the cell wall, following loss of function (Cowan 1999; Tsai and others 2002). However, phenols may not be responsible for all the antimicrobial activity of *H. sabdariffa* L. According to results observed by Roman (2006), rifamycine, a precursor of the antibiotic rifampicin was isolated from *H. sabdariffa* L extracts.

Herbs and spices contain phenolic compounds which are known for exhibiting antioxidant properties. The total phenol content of herbs and spices is usually estimated according to the Folin-Cicalteu method. Following this method, different researchers have found the total phenol content of basil, laurel, cumin, parsley, ginger and fig leaves to be 147.0, 92.0, 37.4, 29.2, 23.5 and 6.9 mg of gallic acid (GA)/g, respectively (Hahn 1984; Konyahoglu and others 2005; Bamdad and others 2006; Hinneburg and others

2006). In this study, *H. sabdariffa* L was found to have 15.8 mg GA/g , which suggests that *H. sabdariffa* L extracts may have lower antioxidant activity than most spices for which an antioxidant profile was found in the literature; but higher than fig leaves.

The increase of concerns regarding the carcinogenic issues associated with the use of synthetic antioxidants has promoted the investigation of alternative antioxidants of plant origin. Their application in food would decrease food deterioration as well as increase the delivery of beneficial health effects. Antiseptic, digestive and antihypertensive, are some of the health properties that *H. sabdariffa* L has been recognized for. *H. sabdariffa* L extracts should be considered effective food additives within a holistic approach due to the antimicrobial activity reported in this study as well as documented antioxidant and health-related properties (D'Heureux-Calix and Badrie 2004; Sivakumar and Richards 2006; Chatterjee and others 2007).

MIC identification

MIC is defined as "The lowest concentration which resulted in maintenance or reduction of inoculum viability." This parameter is used to semiquantitatively measure the *in vitro* activity of potential antimicrobial agents and its determination provides an estimate of the lowest concentration of an antimicrobial needed to prevent microbial growth. Previously, the most common method used was the broth microdilution test which provided the minimum concentration of the antimicrobial that resulted in no visual growth. Other methods such as turbidimetry are used to measure growth of a

bacterial population in real time (Hindler 1992; McMeeking and others 1993; Lambert and Pearson 2000; McKellar and Lu 2004).

Growth curves at 25 ^{o}C of *Salmonella, E. coli* O157:H7 and *L. monocytogenes* isolates were carried out using 15 different concentrations of *H. sabdariffa* L extracts (Figures 2-4). The initial levels of *Salmonella, E. coli* O157:H7 or *L. monocytogenes* in these solutions were 7.9, 7.5 and 6.1 log CFU/ml respectively. The MIC of each strain was identified as the concentration where no increase in O.D. was observed over 24h of incubation. Statistical comparison of O.D. readings at times 0 and 24 was conducted. No difference was observed between O.D. readings at 0 and 24 h ($p < 0.05$), indicating lack of growth (data not shown in tabular form) at the MIC. In the case of *Salmonella*, the MIC of *H. sabdariffa* L extract was 6,489 µg/mL. Kim and others (1995), Rota and others (2004) and Dupont (2006) have reported MIC for *Salmonella* at 0.5, 62.5, 250 and 500 µg/mL for *Thymus vulgaris* and *Eucaliptus straigerana* extracts, carvacrol and eugenol, respectively. The MIC of *H. sabdariffa* L extracts against *E. coli* O157:H7 was 6,489 µg/mL. Kim and others (1995), Rota and others (2004) and Dupont (2006) have reported MIC for *E. coli* at 0.8, > 500, 500 and 1000 µg/mL for *Thymus vulgaris* and *Eucaliptus straigerana* extracts, carvacrol and eugenol, respectively. To inhibit the growth of *L. monocytogenes* 5,309 µg/mL of *H. sabdariffa* L extract were needed. Kim and others (1995), Rota and others (2004) and Dupont (2006) have observed bacteriostatic effect against *L. monocytogenes* with *Thymus vulgaris, Eucaliptus straigerana,* carvacrol and eugenol at concentrations of 0.5, 500, 500 and >1,000 µg/mL.

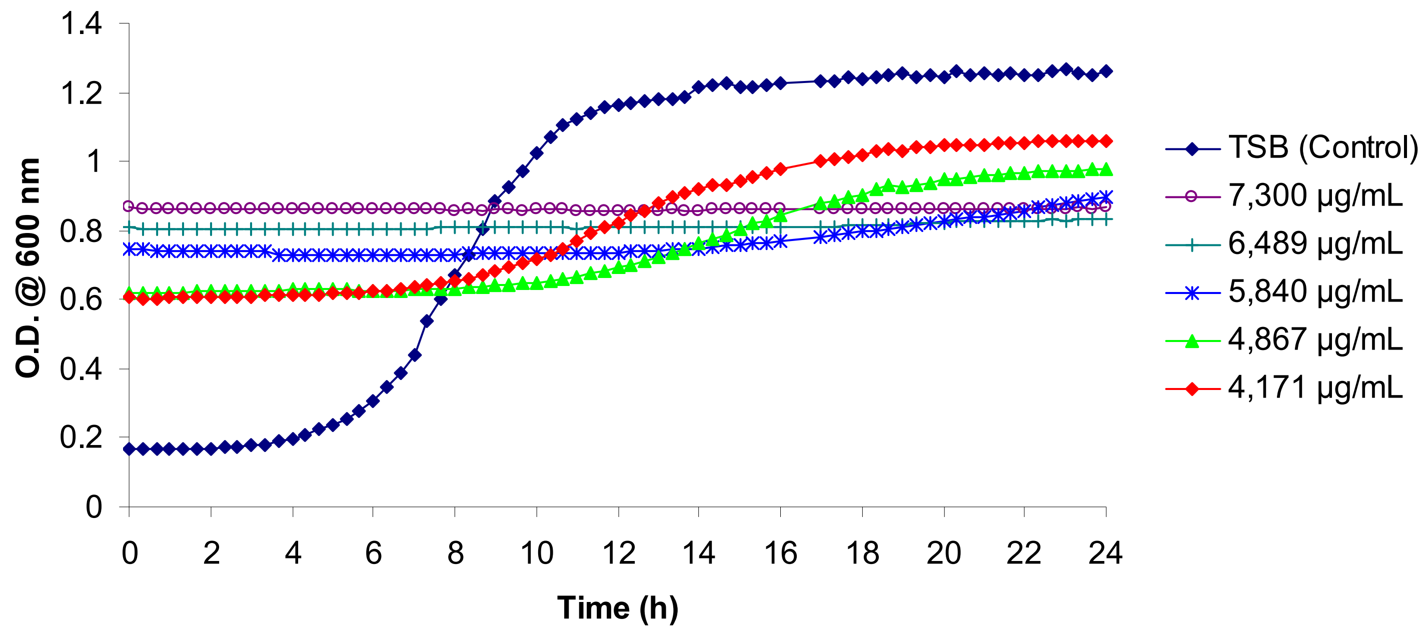

Figure 2 – O.D. at 600 nm for *Salmonella* collected every 20 min from different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h.

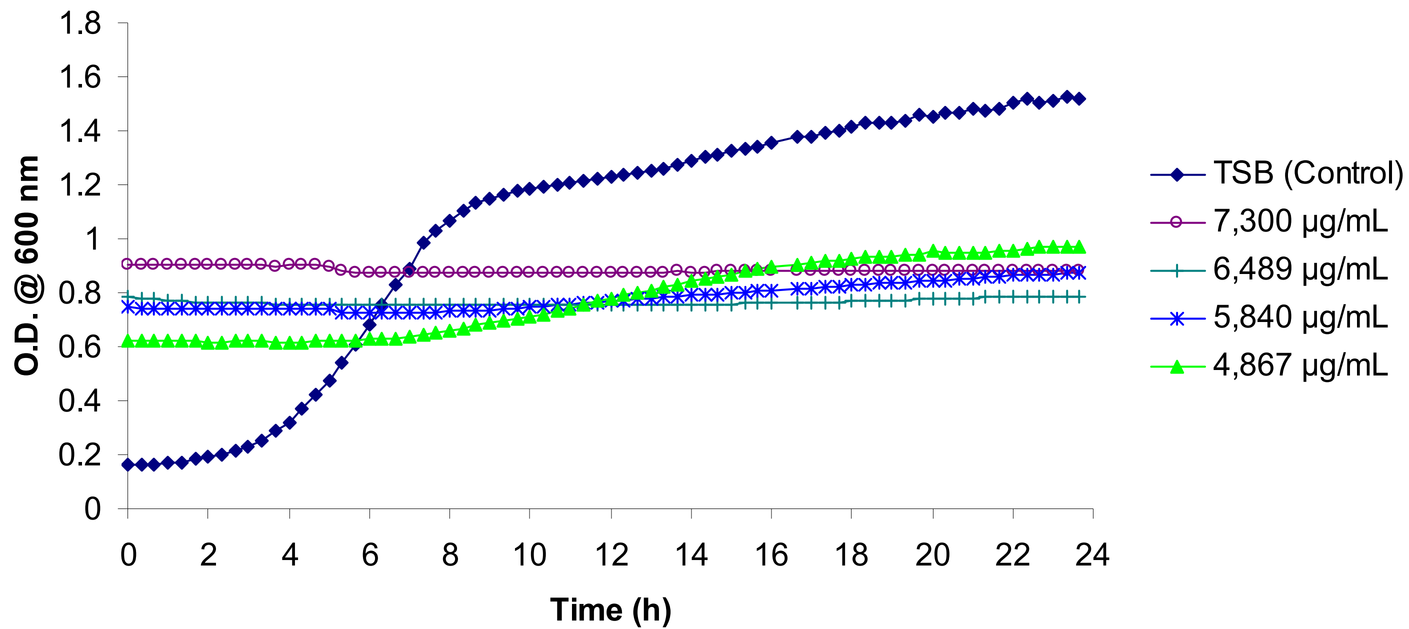

Figure 3 – O.D. at 600 nm for *Escherichia coli* O157:H7 collected every 20 min from different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h.

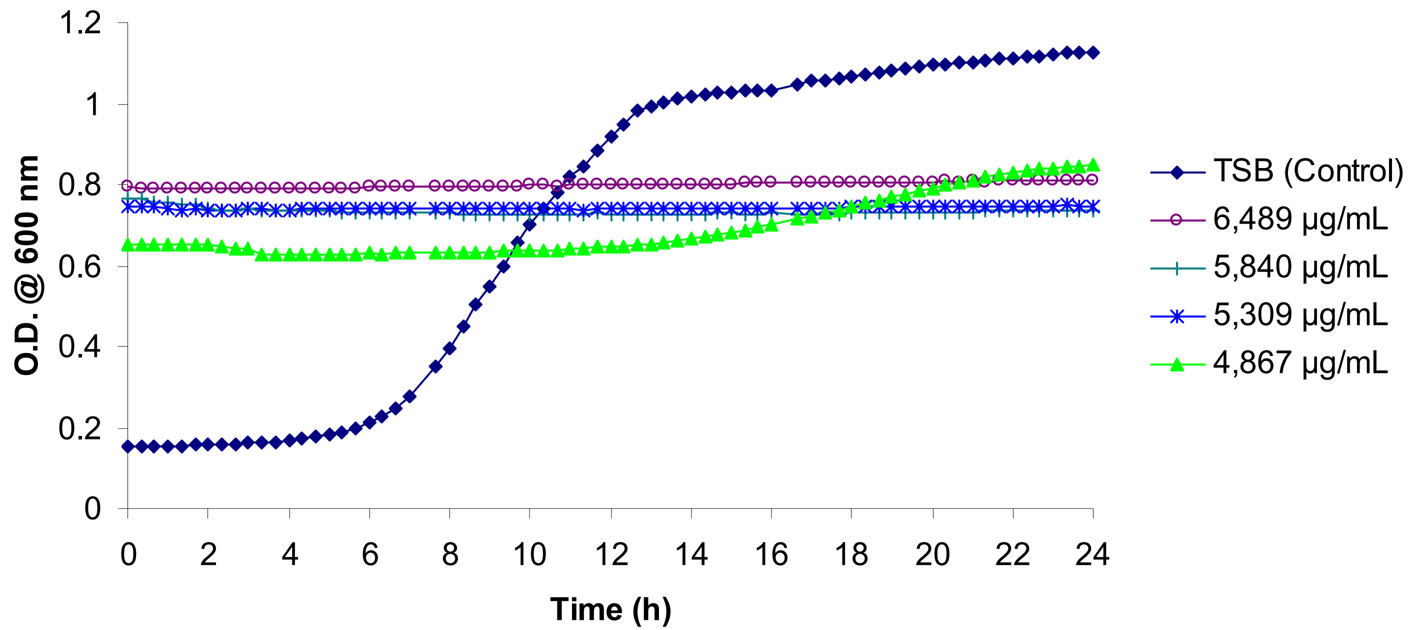

Figure 4 – O.D. at 600 nm for *Listeria monocytogenes* collected every 20 min from different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h.

The bacteriostatic effect of *H. sabdariffa* L extracts demonstrated to be concentration dependant. Similar observation was described in studies done by Sharaf and others (1966) when investigating the MIC of *H. sabdariffa* L extracts against *P. aeruginosa, E. coli* and *S. aureus*. Results of this study indicate the MIC of *H. sabdariffa* extract against the *Salmonella, E. coli* O157:H7 and *L. monocytogenes* to be greater than the MIC reported elsewhere for other plant extracts, suggesting a weaker bacteriostatic effect of *H. sabdariffa* L in comparison to other natural antimicrobials such as eugenol or carvacrol, among others. The effect of *H. sabdariffa* extract against the 3 pathogens tested in this study may be inferior to that of other natural antimicrobials (Kim and others 1995; Rota and others 2004; Dupont 2006).

Growth curves description

Lag phase and generation time (GT) are parameters that allow for quantitative interpretations of growth curves. Lag phase represents the time a microorganism needs to adapt to a new environment and generation time is the time required by a cell to divide into 2. Their quantity relates to the behavior of the microorganisms at certain environmental conditions. The more stringent the environment, the longer the lag phase and generation time (Lim 2002).

The growth of *Salmonella* in TSB (Figure 2), described a sigmoidal curve and showed a lag phase of 4 h at 25 ^{o}C. Juneja and Marks (2006) found that *Salmonella* spp. grown in brain heart infusion broth (BHIB) at 25 ^{o}C, had a lag phase of 3.6 h. In the case of *E. coli* O157:H7, the growth curve showed a sigmoidal form and a lag phase of 2.5 h

at 25 ^{o}C, (Figure 3). Skandamis and others (2001) inoculated *E. coli* in BHIB and incubated it at 27 ^{o}C, and the resulting lag phase lasted for 4.2 h. The growth curve of *L. monocytogenes* in TSB (Figure 4) at 25 ^{o}C, showed a sigmoidal curve and a lag phase of 5 h, which corresponds to the studies of Robinson and others (1998) who described that the duration of the lag phase was 5.5 h when incubated at 25 ^{o}C. Data in Figures 2-4 demonstrate that as the concentration of *H. sabdariffa* L extract increases, the lag phase is delayed to a point where the exponential phase is never reached.

The modified Gompertz equation has been used in food microbiology to model bacterial growth curves with sigmoidal shape (Linton and others 1995). To determine GT from growth curves determined from O.D. data, McMeekin and others (1993) recommend to transform O.D. to percent transmittance [%T] by the use of Eq 1. Using the software GraphPad Prism version 4.03 (GraphPad Software, Inc. 2006), the modified Gompertz function (Eq. 2) was fitted to the growth curves. The software provided the parameters B and D to calculate GT using Eq. 3.

$$[\%T] = -\log_{10}(O.D. + 2) \tag{1}$$

$$\log N_{(t)} = A + D\exp\{-\exp[-B(t - M)]\} \tag{2}$$

$$[GT] = \frac{66.6}{BD} \tag{3}$$

According to Eq. 3, the GT for *Salmonella* when grown in TSB was 0.14 h, however, when grown at the MIC (6,489 μg/mL of *H.sabdariffa* L), the GT significantly

increased ($p < 0.05$) to 7.42 h. In the case of *E. coli*, the control growth curve had a GT of 0.23 h whereas when grown at the MIC (6,489 µg/mL of *H. sabdariffa* L), the GT was 5.14 h. The growth curve of *L. monocytogenes* had a GT of 0.26 h, which was delayed to 34.6 h at the MIC (5,309 µg/mL of *H. sabdariffa* L). MIC assays were carried out at 25 ^{o}C for all the microorganisms.

MBC identification

MBC is an accepted parameter to evaluate new antimicrobial agents and it is commonly used as a research tool. It is defined as "The lowest concentration of antimicrobial agent that kills ≥ 99.99% of the test inoculum." In other words, MBC is the lowest concentration that yields a 4 log CFU/mL reduction from the initial level of inoculum (Hindler 1992). Cocktails with inoculum levels of 8.5, 8.2 and 8.0 log CFU/ml of *Salmonella, E. coli* O157:H7 and *L. monocytogenes* respectively, were inoculated into 5 *H. sabdariffa* L solutions and TSB as a control. Each cocktail was exposed to the 5 different concentrations of *H. sabdariffa* L for a period of 24 h at 25 ^{o}C. From each concentration of inoculated *H. sabdariffa* L extract a 0.1-mL aliquot was plated onto TSA, incubated at 35 ^{o}C and colonies were counted after 24 h. An additional 0.1-mL aliquot from each of the concentrations of *H. sabdariffa* L was transferred to TSB and the initial O.D. was read. The TSB and *H. sabdariffa* L mixtures were then incubated for 48 h at 35 ^{o}C with following O.D. readings taken at 24 and 48 h.

A decrease in O.D. values through time was found in the mixtures of TSB and *H. sabdariffa* L during preliminary experiments. These changes were attributed to chemical

degradation of *H. sabdariffa* L components and TSB ingredients. To account for these inherent changes, the O.D. was transformed to O.D.^{T}, which is the ratio of the O.D. of inoculated solution divided by the O.D. of uninoculated solution. O.D.^{T} values at 0, 24 and 48 h were compared by the Duncan's multiple range test. When changes in O.D.^{T} throughout the 48-h period were not found, they were related to absence of growth, then, the concentration tested was considered bactericidal.

Tables 3-5 show the O.D.^{T} means of *Salmonella, E. coli* O157:H7 and *L. monocytogenes* at time 0, 24 and 48 h. In the case of *Salmonella,* the MBC was identified at 19,467µg/mL of *H. sabdariffa* L since no significant differences ($p < 0.05$) in O.D.^{T} were found throughout the 48-h incubation period. For *E. coli* O157:H7 and *L. monocytogenes*, the MBC was identified at 58,400 µg/mL of *H. sabdariffa* L because there were not significant differences ($p < 0.05$) during the 48-h period.

The confirmation of the MBC by spread plating is shown in Table 6 which exhibits mean counts and reductions achieved after exposing the 3 pathogens to different concentrations of *H. sabdariffa* L extracts for 24 h at 25 ^{o}C. The MBC of *Salmonella* and *E. coli* O157:H7 by the turbidimetric method was supported by the MBC obtained by the plate method because at the MBC, reductions of more than 4 log cycles were obtained. In contrast, when investigating the MBC of *L. monocytogenes*, the TSB solutions had O.D. increases in all the *H. sabdariffa* L solutions, except for the 58,400 µg/mL. These increases were statistically significant ($p < 0.05$). This result might lead to the conclusion that a reduction of 4 log cycles was reached only at this high concentration (58,400 µg/mL). Meanwhile, spread plating results showed that a 4 log cycle decrease was

Table 3 – Mean of the O.D. values of TSB and *H. sabdariffa* L extract solutions inoculated with *Salmonella*.

***H. sabdariffa* L extract (μg/mL)**	**Time (h)**		
	0	**24**	**48**
58,400	0.96±0.05[a]	0.87±0.05[a]	0.85±0.07[a]
29,200	1.05±0.04[a]	0.97±0.03[a]	1.56±0.61[a]
19,467	0.97±0.04[a]	3.46±0.14[a]	3.76±0.13[a]
14,600	0.98±0.04[a]	2.53±0.56[b]	2.57±0.45[b]
11,680	0.97±0.01[a]	3.25±0.39[b]	3.46±0.35[b]
0*	5.94±0.29[a]	19.28±0.69[b]	20.44±0.28[b]

[a, b] Means in the same row with different superscript are significantly different ($p < 0.05$).
*TSB free of *H. sabdariffa* L extract.

Table 4 – Mean of the O.D. values of TSB and *H. sabdariffa* L extract solutions inoculated with *Escherichia coli* O157:H7.

H. sabdariffa L extract (μg/mL)	Time (h)		
	0	**24**	**48**
58,400	0.89±0.04[a]	0.85±0.05[a]	0.83±0.06[a]
29,200	0.99±0.06[a]	3.23±0.06[b]	3.74±0.05[c]
19,467	0.96±0.04[a]	3.93±0.14[b]	4.51±0.12[c]
14,600	0.98±0.06[a]	4.05±0.15[b]	4.42±0.18[b]
11,680	0.97±0.03[a]	3.59±0.62[b]	3.90±0.71[b]
0[*]	5.46 ±0.09[a]	17.04±0.61[b]	16.60±1.02[b]

[a, b, c] Means in the same row with different superscript are significantly different ($p < 0.05$).
[*]TSB free of *H. sabdariffa* L extract.

Table 5 – Mean of the O.D. values of TSB and *H. sabdariffa* L extract solutions inoculated with *Listeria monocytogenes*.

H. sabdariffa L extract (µg/mL)	Time (h)		
	0	24	48
58,400	0.92 ± 0.04^{a}	0.92 ± 0.05^{a}	0.87 ± 0.06^{a}
29,200	1.01 ± 0.05^{a}	2.57 ± 0.34^{b}	2.22 ± 0.06^{b}
19,467	0.95 ± 0.02^{a}	3.09 ± 0.41^{b}	2.78 ± 0.06^{b}
14,600	0.95 ± 0.04^{a}	2.58 ± 0.89^{b}	1.51 ± 0.53^{b}
11,680	0.95 ± 0.03^{a}	1.42 ± 0.46^{b}	0.97 ± 0.01^{c}
0*	3.26 ± 0.09^{a}	10.48 ± 0.33^{b}	8.36 ± 0.18^{c}

[a, b, c] Means in the same row with different superscript are significantly different ($p < 0.05$).
*TSB free of *H. sabdariffa* L extract.

Table 6 – Mean populations (log CFU/mL) and estimated reductions of *Salmonella, Escherichia coli* O157:H7 and *Listeria monocytogenes* after exposed to different concentrations of *H. sabdariffa* L. extract for 24 h.

H. sabdariffa L. extract (μg/mL)	*Salmonella*		*E. coli* O157:H7		*L. monocytogenes*	
	Mean±SEM	**ER**	**Mean±SEM**	**ER**	**Mean±SEM**	**ER**
Control	8.5±0.11	NA	8.2±0.10	NA	8.0±0.04	NA
58,400	0.7±0.00	7.8	0.7±0.00	7.5[*]	1.6±0.50	6.4
29,200	0.7±0.00	7.8	4.4±0.04	3.8	2.7±0.11	5.3[*]
19,467	3.8±0.21	4.7[*]	5.2±0.65	3.0	4.7±0.36	3.3

Mean = Average of three repetitions.
SEM = Standard error of the mean.
Control = Initial inoculum.
ER = Estimated reduction = (Log CFU/mL of control) - (Log CFU/mL after 24 h of exposure to the treatment).
NA = No applicable.
[*]MBC since > 4 log CFU/mL reduction was achieved.

already attained at 29,200 µg/mL of *H. sabdariffa* L. These differences between O.D. and viable numbers may be caused by *L. monocytogenes* cells behavior explained by Bereski and others (2002) and Francois and others (2005). Their work showed that *L. monocytogenes* cells elongate when they are exposed to stressful environments. One of the limitations of turbidimetric methods is that bacteria absorb or scatter light depending on their concentration, size, and shape (McKellar and Lu 2004). Therefore, the O.D. increases found in the *L. monocytogenes* TSB solutions may be attributable to enlargement of the cells, given that they were exposed to a stressful environment, and not to viable cells growth. Therefore, due to the interference of *L. monocytogenes* stressed cells, the MBC against *L. monocytogenes* was determined only by the spread plating method, 29,200 µg/mL of *H. sabdariffa* L extract.

Time-kill

The survival curves of *Salmonella, E. coli* O157:H7 and *L. monocytogenes* (Figures 5-7) were fitted to the modified Gompertz function (Eq. 2). Adequate fits, R^2 ranging from 0.71 to 0.99 provided the estimation parameters (Table 7) that allow for further predictions of the survival of bacterial populations.

According to the American Society for Microbiology (ASM) (Hindler 1992), new biocides are usually tested for concentration and time dependency. This assay was used to investigate the rate at which two concentrations of a *H. sabdariffa* L extract kill a bacterial isolate; understanding that bacterial kill is the permanent loss of reproductive

activity. In general, an antimicrobial agent which enables a reduction of 4 log cycles is considered effective.

Figure 5 indicates that *H. sabdariffa* L at 58,400 µg/mL exerted a marked bactericidal effect against *Salmonella.* This effect developed rapidly yielding a reduction of 5.1 log cycles within the first hour. In contrast, the effect at 5,840 µg/mL was essentially static within the first 2 h but, after an incubation of 4 h, growth was observed. Figure 6 demonstrates that when *E. coli* O157:H7 is exposed to a concentration of 58,400 µg/mL of *H. sabdariffa* L extract, a lethality of 2.9 log CFU/mL occurred within the first hour. After this rapid decrease, a bacteriostatic effect was observed within the next 4 h, but finally, cell numbers were reduced to the detection limit (≤ 1.5 log CFU/mL) within 12 h. When using one-tenth of the previous concentration (5,840 µg/mL) the growth of *E. coli* O157:H7 was promoted. Cell numbers of *L. monocytogenes* declined slowly over 12 h (Figure 7), followed by rapid growth when exposed to 58,400 µg/mL of *H. sabdariffa* L extract. The same type of death pattern was also shown at 5,840 µg/mL but after 24 h of incubation, bacterial recovery was observed. These phenomena may be attributed to an injury-recovery process. According to the 3.0 log reduction criteria of the ASM, a concentration of 58,400 µg/mL of *H. sabdariffa* L is an effective antimicrobial for the 3 pathogens tested.

Pathogen reduction in ground beef by use of natural antimicrobials

The effectiveness of *H. sabdariffa* L in food systems was tested using GB as a model. The same model was used to explore the value of another potential food

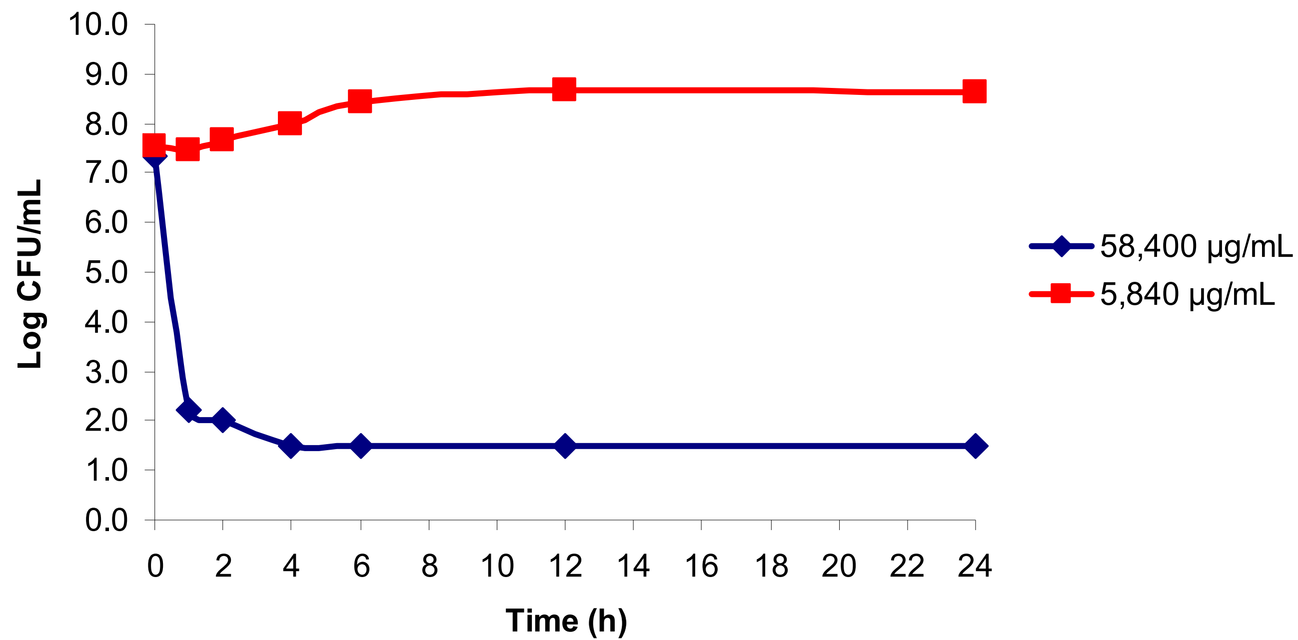

Figure 5 – Viable counts for *Salmonella* cocktail collected from 2 different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h to determine time-kill effect.

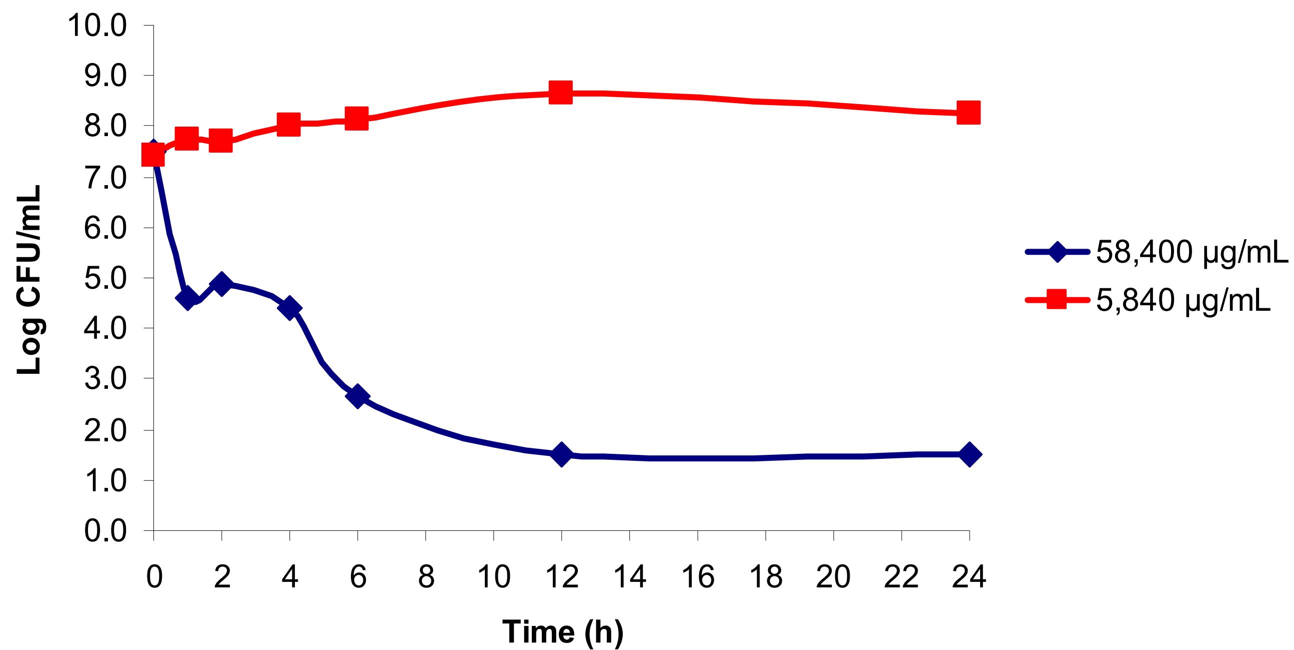

Figure 6 – Viable counts for *Escherichia coli* O157:H7 cocktail collected from 2 different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h to determine time-kill effect.

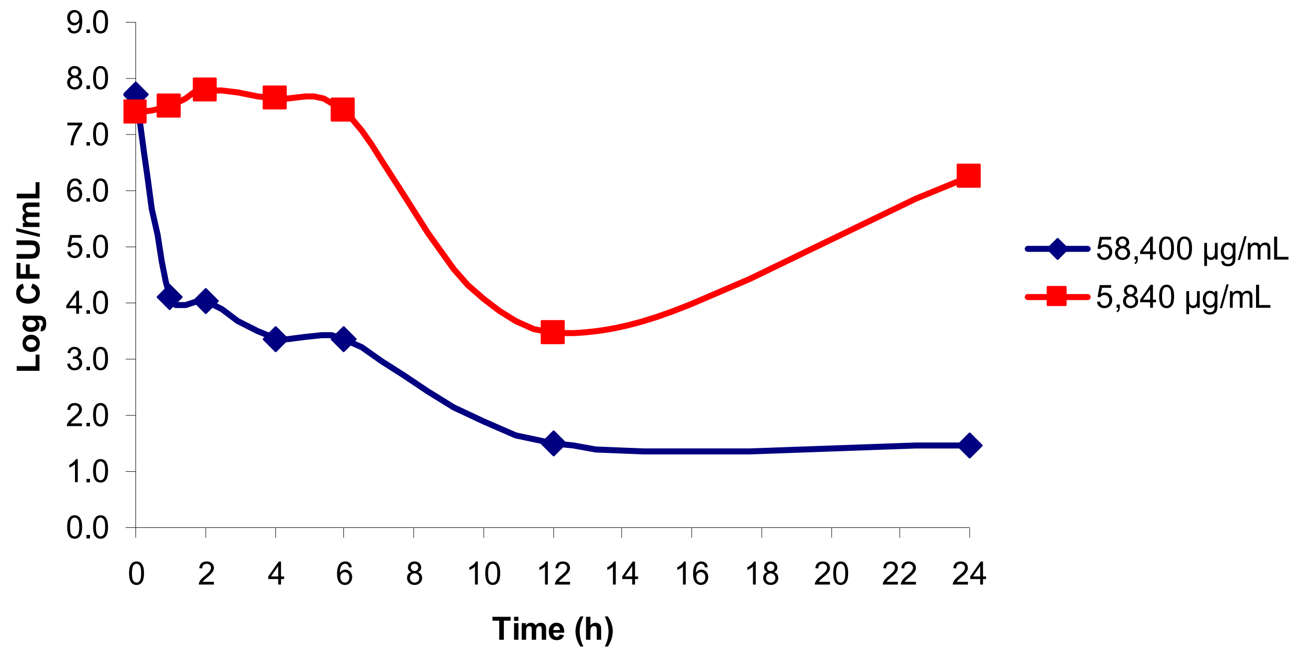

Figure 7 – Viable counts for *Listeria monocytogenes* cocktail collected from 2 different dilutions of *H. sabdariffa* L incubated at 25 °C during 24 h to determine time-kill effect.

Table 7 – Parameters obtained from fitting time-kill survival curves into Gompertz equation.

Parameters	***Salmonella***		***E. coli* O157:H7**		***L. monocytogenes***	
	58,400 μg/mL	**5,840 μg/mL**	**58,400 μg/mL**	**5,840 μg/mL**	**58,400 μg/mL**	**5,840 μg/mL**
A	1.57	7.42	2.08	4.90	1.39	7.15
D	196.03	1.24	988.72	2.70	237.81	1.33
M	-1.67	4.08	-10.12	6.49	-16.46	2.79
B	-0.91	0.30	-0.23	-2.22	-0.10	0.18
R^2	0.99	0.99	0.86	0.72	0.91	0.89

A = Lower asymptote.

D = Distance between upper and lower asymptote.

M = Log of the inflexion point.

B = Slope parameter.

R^2 = Coefficient of determination.

antimicrobial that has not been studied in food systems before, ε-polylysine. The antimicrobial activity of the tested natural antimicrobials against *S.* Agona (GFP) and *E. coli* O157:H7 (RFP) is presented in Tables 8 and 9.

After 2 h of exposure, ground *H. sabdariffa* L at 5 and 10% (w/w) significantly reduced ($p < 0.05$) *S.* Agona (GFP) by 0.3 and 0.5 log cycles, respectively. *H. sabdariffa* L aqueous extract and ε-polylysine at 200 or 400 ppm did not show any antimicrobial effect against *S.* Agona (GFP) within 2 h of application of the treatment. Similarly, after 24 h of exposure only ground *H. sabdariffa* L at 5 and 10% (w/w) significantly reduce ($p < 0.05$) the *S.* Agona (GFP) counts in 1.1 and 0.9 log CFU/mL, respectively. Other natural antimicrobials have been similarly studied in the attempt to control *S.* Typhimurium in ground beef. Uhart and others (2006) tested the bactericidal effect of 5% garlic, 7% ginger and 5% turmeric in ground beef inoculated with *S.* Typhimurium. Only garlic and turmeric treatments showed significantly reductions.

In the present study, some treatments were found to be effective against *S.* Agona (GFP) and others to *E. coli* O157:H7 (RFP). After 2 h of exposure, the populations of *E. coli* O157:H7 (RFP), were reduced by 0.6 log CFU/mL by 5% *H. sabdariffa* L aqueous extract. Although this reduction was significant ($p < 0.05$), it is doubtful that less than 1 log reduction would be of biological relevance in food safety. The remaining treatments did not produce any bactericidal effect. Surprisingly, after 24 h of exposure and storage at 4-7 ^{o}C, all the treatments promoted a reduction in *E. coli* O157:H7 counts ($p < 0.05$). Again, the reductions, albeit significant, were < 1 log cycle. The highest decrease (0.9 log CFU/mL), with ε-polylysine was obtained at 400 mg/L, followed by *H. sabdariffa* L

Table 8 – Mean counts (log CFU/mL) of *Salmonella* (GFP) after 2 and 24-h contact time with different natural antimicrobials at different concentrations.

	Time (h)	
	2	24
Treatment	**Mean±SEM**	**Mean±SEM**
Control	$6.0 \pm 0.03^{a,y}$	$6.2 \pm 0.03^{a,z}$
1	$5.7 \pm 0.15^{b,y}$	$5.3 \pm 0.39^{bc,y}$
2	$5.5 \pm 0.03^{b,y}$	$5.1 \pm 0.40^{c,y}$
3	$5.8 \pm 0.13^{ab,y}$	$5.7 \pm 0.06^{abc,y}$
4	$6.0 \pm 0.09^{a,y}$	$6.2 \pm 0.03^{a,y}$
5	$6.0 \pm 0.03^{a,y}$	$6.0 \pm 0.20^{ab,y}$

Control = Ground beef inoculated and free from antimicrobials.
1 = Ground *H. sabdariffa* L sepals at 5 % (w/w).
2 = Ground *H. sabdariffa* L sepals at 10 % (w/w).
3 = *H. sabdariffa* L aqueous extract at 5% (w/w).
4 = ε-polylysine at 200 ppm.
5 = ε-polylysine at 400 ppm.
Mean = Average of three replicates.
SEM = Standard error of the mean.
^{a,b,c} Means in the same column with different superscript are significantly different ($p < 0.05$).
^{y,z} Means in the same row with different superscript are significantly different ($p < 0.05$).

Table 9 – Mean counts (log CFU/mL) of *Escherichia coli* O157:H7 (RFP) after 2 and 24-h contact time with different natural antimicrobials at different concentrations.

	Time (h)	
	2	24
Treatment	Mean±SEM	Mean±SEM
Control	$6.2\pm0.00^{a,y}$	$6.2\pm0.07^{a,y}$
1	$6.0\pm0.33^{ab,y}$	$5.9\pm0.09^{b,y}$
2	$5.8\pm0.03^{ab,y}$	$5.6\pm0.06^{b,y}$
3	$5.6\pm0.27^{b,y}$	$5.8\pm0.09^{b,y}$
4	$6.0\pm0.12^{ab,y}$	$5.8\pm0.09^{b,y}$
5	$6.1\pm0.06^{ab,y}$	$5.3\pm0.13^{c,z}$

Control = Ground beef inoculated without antimicrobials.
1 = Ground *H. sabdariffa* L sepals at 5 % (w/w).
2 = Ground *H. sabdariffa* L sepals at 10 % (w/w).
3 = *H. sabdariffa* L aqueous extract at 5% (w/w).
4 = ε-polylysine at 200 ppm.
5 = ε-polylysine at 400 ppm.
Mean = Average of three replicates.
SEM = Standard error of the mean.
^{a,b,c} Means in the same column with different superscript are significantly different ($p < 0.05$).
^{y,z} Means in the same row with different superscript are significantly different ($p < 0.05$).

extract at 10% (w/w), *H. sabdariffa* L aqueous extract 5% (w/w), ε-polylysine at 200 ppm and ground *H. sabdariffa* L extract at 5% (w/w) with reductions of 0.6, 0.4, 0.4, 0.3 log CFU/mL, respectively. Ceylan and others (1998), inoculated ground beef with *E. coli* O157:H7, treated it with 7.5% of clove, cinnamon, garlic, oregano and sage. Immediate enumerations showed reductions of 2.1, 0.7, 0.6, 0.3 and 0.2 log CFU/g, respectively. These experiments showed an increase in the bactericidal effect through time. Indeed, this effect has been extensively reported in the literature (Tassou and others 1996; Skandamis and Nychas 2001; Careaga 2003; Yin and Cheng 2003; Uhart and others 2006).

Differences in the bactericidal strength of natural antimicrobials are observed between *in vitro* studies and when they are incorporated into a food matrix. It has been suggested that fat and/or protein interactions may be responsible for this shortage of strength (Uhart and others 2006). Farbood and others (1976) suggested three reasons to explain such effect. The lipid fraction of the matrix may absorb the spice, decreasing its concentration in the aqueous phase and thus, the bactericidal effect. Proteins may bind the active components of the natural antimicrobials reducing their availability in the aqueous phase. A layer of fat surrounding the bacterial cell may protect the microorganisms from the bactericidal ability of the *H. sabdariffa* L and ε-polylysine. These three conditions may have affected the bactericidal power of the natural antimicrobials. However, if these natural antimicrobials are combined with other antimicrobial systems to form a hurdle effect, they may achieve larger reductions of microorganisms.

CONCLUSIONS

According to the phytochemical analysis of *H. sabdariffa* extracts it was observed to have a lower phenolic content than other herbs and spices cited in the literature. However, *H. sabdariffa* L may be considered an effective food additive since phenols can exert antioxidant activity and provide health-related effects. Further investigations regarding these effects should be conducted.

Determination of MIC, MBC and time-kill were *in vitro* studies to investigate *H. sabdariffa* L characteristics as a potential food antimicrobial ingredient. Unveiling the antimicrobial potency of *H. sabdariffa* L extracts may contribute to an increase in its use as a food additive. The antimicrobial effect of *H. sabdariffa* L extracts was found to be dose and time dependent. *H. sabdariffa* L extracts were found to be effective antimicrobials in the inhibition or reduction of *Salmonella, E. coli* O157:H7 and *L. monocytogenes*. Reductions of more than 6.0 log cycles were achieved when the pathogens were in contact with 58,400 µg/mL of *H. sabdariffa* L extracts at 25 ^{o}C for 12 h.

When the natural antimicrobials were tested in GB, the greatest reduction in *S.* Agona (GFP) was 1.1 log CFU/g, using 10% of ground *H. sabdariffa* L and of 0.9 log CFU/g in *E. coli* O157:H7 (RFP) using 400 ppm of ε-polylysine. Fat and protein content of the food matrix were suggested to be factors affecting the potency of natural antimicrobials. Natural antimicrobials were able to decrease pathogen populations, increase the phytochemical content and provide different organoleptic properties to the

food. However, further studies are needed to determine how natural antimicrobials and concentrations will affect organoleptic attributes.

Regardless of the low antimicrobial reductions achieved, if these natural antimicrobials are used as a part of a hurdle system, higher pathogen reductions could be met. However, this research needs further investigation.

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