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Effects of nisin and buffered sodium citrate supplemented with sodium diacetate against listeria monocytogenes on commercial beef frankfurters formulated without antimicrobials stored at 4 and 10{deg}c in vacuum packages

Shweta Kumari

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EFFECTS OF NISIN AND BUFFERED SODIUM CITRATE SUPPLEMENTED WITH

SODIUM DIACETATE AGAINST *LISTERIA MONOCYTOGENES* ON

COMMERCIAL BEEF FRANKFURTERS FORMULATED

WITHOUT ANTIMICROBIALS STORED AT

4 AND 10°C IN VACUUM PACKAGES

By

Shweta Kumari

A Thesis
Submitted to the Faculty of Mississippi State University
in Partial Fulfil*Lm*ent of the Requirements for the
Degree of Master of Science in Food Science
in the Department of Food Science,
Nutrition and Health Promotion

Mississippi State, Mississippi

August 2008

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Title of Study: EFFECTS OF NISIN AND BUFFERED SODIUM CITRATE
SUPPLEMENTED WITH SODIUM DIACETATE AGAINST
LISTERIA MONOCYTOGENES ON COMMERCIAL BEEF
FRANKFURTERS FORMULATED WITHOUT
ANTIMICROBIALS STORED AT 4 AND 10°C IN VACUUM
PACKAGES

Pages in Study: 79

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This study evaluated the antilisterial effects of externally applied solutions of

nisin, buffered solution of sodium citrate supplemented with sodium diacetate (SCSD)

and a combined solution of the two antimicrobials on commercial beef frankfurters

formulated without antimicrobials. Autoclaved frankfurters were inoculated (10^4-10^5

CFU/g), dipped (5 min, 25 ± 2°C) in treatments consisting of 2000; 4000;or 6000 IU/ml

nisin, and buffered solutions of 2.5; 3.0; or 3.5% SCSD (Study I), and 6000 IU/ml nisin

followed by 3.5% SCSD, or 3.5% SCSD followed by 6000 IU/ml nisin, or a combined

solution containing both 6000 IU/ml nisin and 3.5% SCSD(Study II). Treated hot dogs

were vacuum packaged and stored at 4 and 10°C. *L. monocytogenes* counts were

determined on Modified Oxford Agar on 0, 14, 28, and 42 days at 4°C and 0, 4, 8, 12, 16,

and 20 days at 10°C. Nisin (6000 IU/ml) initially reduced *L. monocytogenes* population

by 2.1 and 2.5 logs (at 4 and 10°C, respectively on day 0), buffered SCSD (3.5%) by 1.1 and 0.2 logs (at 4 and 10°C, respectively on day 0) and the combined solution by 1.7 and 2.0 logs (at 4 and 10°C, respectively on day 0). The combined solution was the most effective treatment compared to nisin and buffered SCSD, used in sequence or alone, since it inhibited the growth of *L. monocytogenes* during 28 days at 4°C. The buffered SCSD was effective against *L. monocytogenes* at 4°C but not at 10°C. The results of this study may be useful for further research on combinations of antimicrobials.

Key words: *Listeria monocytogenes*, commercial beef frankfurters, antimicrobials

DEDICATION

      I dedicate this manuscript to my mother and father, Mrs. Indira Singh and Mr. Jaikant Singh. They have been by my side when the times have been tough. Mom and Dad, I would have never reached this far without you, I love you both dearly and can never thank you enough! I also dedicate this work to my sisters and brothers, Sushmita Singh and Swati Singh, Shivendra Singh and Suhanshanu Singh for being there at every step of my life. I can not think of myself without you guys.

ACKNOWLEDGEMENTS

TABLE OF CONTENTS

vi

LIST OF TABLES

LIST OF FIGURES

CHAPTER I

INTRODUCTION

Recontamination of cooked ready-to-eat (RTE) meat products with *L. monocytogenes* has been a major food safety concern. *L. monocytogenes* causes an estimated 2500 cases of food borne illnesses and 500 deaths (~ 20% case fatality rate) annually in the United States *(46)*. In addition, *L. monocytogenes* causes significant annual loss in the food industry. In 2005 alone, 300,424 pounds of RTE meat products were recalled for probable *L. monocytogenes* contamination *(23)*. *L. monocytogenes* can survive under normally limiting, extreme physiochemical conditions such as temperature ranges of -1.5 to 45°C, pH ranges of 4.4-9.4, high salt concentration and is capable of growth in highly acidic foods *(63)*. *L. monocytogenes* is ubiquitous in nature and in spite of its being susceptible to cooking temperature (70°C) *(43)*, post processing recontamination during slicing and packaging is difficult to avoid. Therefore, hurdles are required to inhibit the growth of pathogens during storage.

Organic acids and salts have been used for thousands of years as food preservatives *(7, 39)*. The salts of organic acids such as lactates, acetates, diacetates and other chemical compounds have been studied as antilisterial agents on meat products *(5-7, 26-28, 31, 39, 44, 53, 67, 68, 75, 77-80)*, however there is uncertainty regarding their optimum concentration. The efficacy of antimicrobials are dependent on pH, water

1

activity, fat, nitrite and salt content of product, storage temperature and packaging

atmosphere of the food product *(13, 27, 45, 51)*. The level of contamination of the food

surface with *L. monocytogenes* is another factor which influences the activity of these

antimicrobials *(7, 93)*. The effectiveness of buffered sodium citrate supplemented with

sodium diacetate (SCSD) has not yet been studied against *L. monocytogenes* on meat

products. However, this antimicrobial has been shown to be effective in controlling

germination and outgrowth of *Clostridium perfringens* during cooling of cooked meat

and poultry products *(80)*. Therefore it is possible that use of buffered sodium citrate

supplemented with sodium diacetate, if used at optimized concentrations and with other

antimicrobials, may prove to be effective against *L. monocytogenes* on RTE meat

products such as beef frankfurters.

Nisin is a bacteriocin produced by *Lactococcus lactic* subsp. *lactis*, which is

added to a variety of foods throughout the world and generally regarded as safe. In the

United States, nisin has been approved for use only in processed cheeses *(73)*. Studies

have shown that nisin is effective in reducing *L. monocytogenes* counts on RTE meat

products. However, the use of nisin alone in food products may cause the emergence of

nisin-resistant strains. To overcome this problem, several studies have combined nisin

with other treatments to achieve antilisterial effects at lower concentrations *(5, 6, 18)*.

The specific objective of this research was to investigate the combined effects of

nisin and buffered SCSD against *L. monocytogenes* on beef frankfurters. In order to

evaluate the effects of different concentrations of nisin and buffered SCSD used

individually and used in combination, the experiment was split into two studies. The

objective of the first study was to determine the antilisterial effects of different concentrations of nisin and buffered SCSD on commercial beef frankfurters formulated without antimicrobials in vacuum packages stored at 4 or 10°C. The second objective aimed to evaluate the potential application of combined nisin with buffered SCSD against surface inoculated *L. monocytogenes* on frankfurters stored at 4 or 10°C.

CHAPTER II

LITERATURE REVIEW

Listeria monocytogenes

History and Taxonomy

Listeria monocytogenes was first identified in an outbreak of a disease among laboratory rabbits at Cambridge University by Murray et al. *(50)* in 1926. The organism was isolated from the blood of infected rabbits and was able to reproduce the disease in healthy animals, thereby establishing the organism's pathogenicity. The next year, a similar outbreak was noticed by Pirie *(54)* in South Africa involving wild gerbils. The disease was termed "Tiger River Disease". In 1940 the organism was named *Listeria monocytogenes (55)* after previously being called *Listerella hepatolytica (54)* and *Bacterium monocytogenes (50).* In 1929, Gill *(30)* for the first time described an illness in sheep called "circling disease", which is still used to characterize listerial encephalitis, encephalomyelitis, and meningoencephalitis. For many years, the organism was thought to be associated commonly with animals, mostly in sheep causing abortion, and less frequently in humans until the widely publicized food borne outbreaks of the mid 1980s *(63).*

The genus *Listeria* at present contains six species: *L. monocytogenes, L. innocua, L. ivanovii, L. welshimeri, L. seeligeri* and *L. grayi,* as evidenced by DNA homology values, 16S rRNA sequencing homology, chemotaxonomic properties and multilocus enzyme electrophoresis *(15, 58, 59, 65). L. monocytogenes* is pathogenic to humans and animals. *L. ivanovii* mostly causes disease in animals, while occasional human infections due to *L. ivanovii* and *L. seeligeri* have been reported *(16).* All other species are non-pathogenic *(83).*

Morphology

L. monocytogenes is a short, gram positive, non-spore forming, rod-shaped bacterium which can appear coccoidal or filamentous *(57).* Broth cultures typically become turbid within 8-24 hrs of incubation at 35°C. When *Listeria* is grown on nutrient agar the colonies are smooth, bluish gray, slightly raised and measure about 0.2-0.8 mm in diameter after 24 hrs of incubation *(63). L. monocytogenes* is catalase positive, oxidase negative, ferments carbohydrates to acid without gas, methyl red positive, produces ammonia from arginine, and shows negative reactions for hydrogen sulphide production, indole, nitrate reductase, starch and urea hydrolysis *(37).* The differentiation of *Listeria* species is partly based on the lysis of red blood cells (i.e., hemolysis) which differentiates between pathogenic or hemolytic *L. monocytogenes* and non-pathogenic species *(83).*

Disease

Listeria monocytogenes causes a disease called listeriosis. Listeriosis is an asymptomatic disease with symptoms ranging from a mild influenza-like disease to meningitis and/or meningoencephalitis. Pregnant women, neonates, elderly and immunocompromised individuals are especially susceptible to infection. In pregnant women, infection of the fetus is common which leads to abortion, stillbirth or delivery of an acutely ill infant.

There are two types of disease associated with the organism; invasive and non-invasive (Figure 1). The invasive disease has an incubation time of 1-90 days and occurs in people with weakened immune systems. Flu-like symptoms, diarrhea, vomiting, meningitis, septicaemia and spontaneous abortions have been reported *(81)*. The non-invasive disease can occur in individuals who consume a high number of cells. The estimate of the number of cells to cause the disease is debatable. In general 100- 1000 cells are required. Outbreaks can be produced with foods containing > 10^5 cells/g *(38)*.
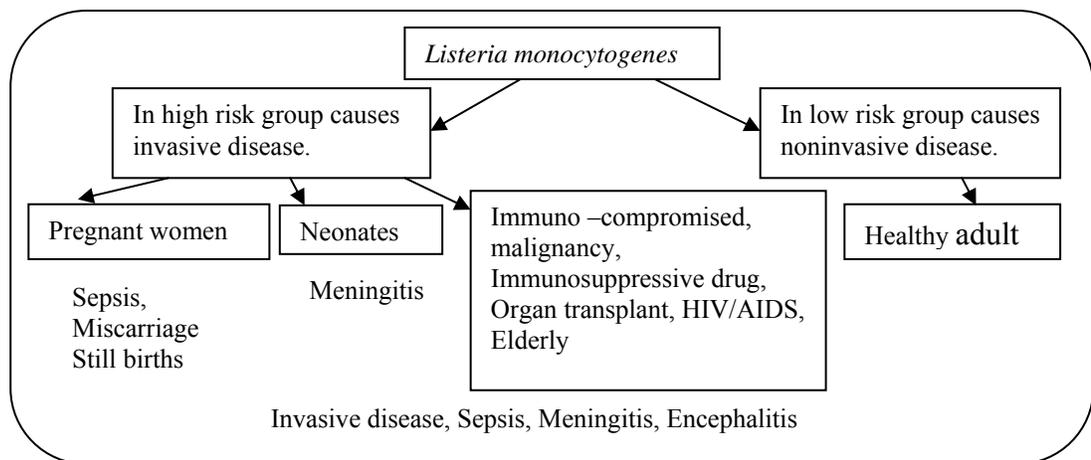
FIGURE 1. Types of disease associated with *L. monocytogenes* (81).

6

L. monocytogenes is ingested with food and penetrates the epithelial lining of the intestine with the help of an enzyme, invasin, secreted by bacteria *(81)*. The invasion and spread of *L. monocytogenes* is shown in Figure 2. *Listeria monocytogenes* multiplies not only extracellularly but also intracellularly. The disease can be treated if it is diagnosed early, which is very difficult because the first signs of infection are reports of still births or serious infections *(81)*.
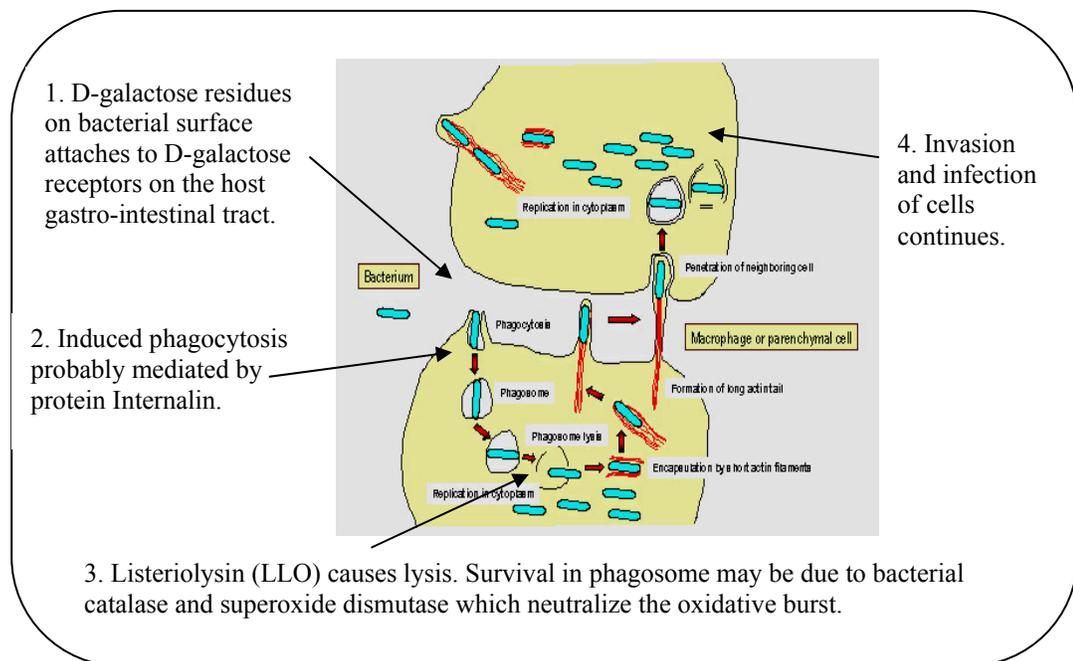
FIGURE 2. Intracellular invasion by *L. monocytogenes (81)*.

Growth limits

     Listeria monocytogenes grows and multiplies at temperatures ranging from -1.5 to 45°C with optimum growth at 37°C. *L. monocytogenes* is regarded as a psychotropic food-borne pathogen. *Listeria monocytogenes* can grow at a pH ranging from 4.4-9.4,

with an optimum pH of 7.0. The organism is acid tolerant and can survive in foods with

higher acidity for several days and weeks. Microaerophilic conditions are optimum but

the organism grows well both aerobically and anaerobically. The organism can grow in

relatively high (30%) CO_2, but is inhibited under 100% CO_2. Additionally *Listeria* can

grow in foods having a lower water activity such as 0.92. In the laboratory it can grow in

media containing up to 10% NaCl *(63)*.

Sources of *Listeria monocytogenes* contamination

Listeria monocytogenes is ubiquitous in nature (Figure 3) and has been widely

found in soil, mud, silage, decaying vegetation, water, sewage, and feces *(63)*. *L.*

monocytogenes is carried asymptomatically in the feces of 2-6% of the human

population. Person-to-person spread (other than mother to fetus) is uncommon *(38)*.

Disease in animals can spread to humans by way of manure and feed, however food

borne transmission is the primary source of human infections. All raw foods, cooked

foods with post-cooking contamination and ready-to-eat cooked foods with long shelf

lives are major sources of infection. Examples are raw milk, meat, poultry and seafood,

processed dairy foods and fish. In addition coleslaw, chocolate milk, various soft and

surface-ripened cheeses, jellied pork tongue, cooked chicken, smoked mussels and

smoked sausages have been associated with outbreaks *(38, 63)*. However, there could be

other sources of outbreaks. For instance, in Costa Rica, a neonatal outbreak involved the

use of contaminated mineral oil for cleaning infants after delivery *(71)*.
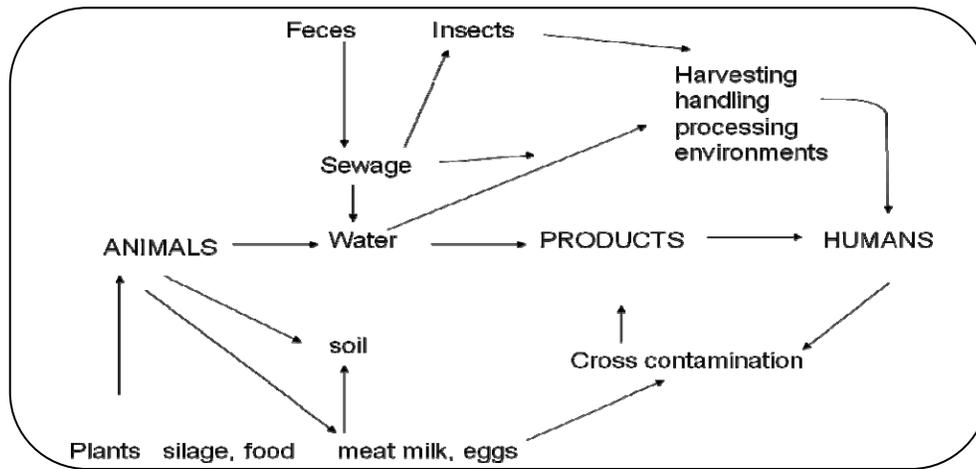
FIGURE 3. Cross-contamination of *L. monocytogenes* in the environment (*38*).

Listeria monocytogenes **and Ready-To-Eat (RTE) meat products**

Food borne outbreaks related to *Listeria monocytogenes*

There are an estimated 76 million cases of food borne illnesses each year in the United States. The listeriosis cases averages 2,500 as compared to the more common infections from other food borne pathogens such as *Escherichia coli O157:H7 (E. coli), Campylobacter spp*. and *SaLmonella spp.* (Table 1). However, Listeriosis accounts for as high as 20-30% mortality, causing 500 deaths, and $200 million of monetary loss per year *(32)*.

9

TABLE 1. Comparison of the mortality rate among *L. monocytogenes* and other common food borne pathogens *(32)*.

Pathogen	Mortality Rate (%)	Hospitalization Rate (%)	Total Cases
Campylobacter spp.	<1	17.3	~ 1.9 million
E. coli O157:H7	<1	3.0	~ 62,500
SaLmonella spp.	<1	25.6	~ 1.3 million
L. monocytogenes	20.0	3.8	2500

Ready to eat (RTE) meat products are at maximum risk of contamination with *L. monocytogenes (8).* This is due to three reasons: 1. RTE meats have various shelf-lives and may be consumed directly without re-heating; 2. *L. monocytogenes* can grow to an extent during refrigerated storage that can cause listeriosis; 3. The emergence of multiple resistance in *L. monocytogenes (40).* There have been various listerial outbreaks in spite of the precautions taken by food processors (Table 2) *(23).* In 1998, a major *Listeria monocytogenes* outbreak occurred in 22 states where hotdogs and deli meats were implicated, causing 101 illnesses, 15 deaths, and 6 stillbirths. In 2000, a *Listeria* outbreak spread to 10 states leading to 29 illnesses, 4 deaths and 3 miscarriages from turkey deli meat contamination. In 2002, another multi-state outbreak from turkey deli meat products occurred. In this incident 46 illnesses, 7 deaths, and 3 miscarriages were reported *(24).*

TABLE 2. Outbreaks of *L. monocytogenes* in the United States (1970-2005) with known food vehicle *(12, 25)*.

Year	Food Vehicle	State	Cases	Perinatal cases (% of total)	Deaths (% of total)
1979	Raw vegetables or cheese	Massachusetts	20	0(0)	3 (15)
1983	Pasteurized fluid milk	Massachusetts	32	7 (21.9)	14 (43.8)
1985	Mexican-style cheese (raw milk)	California	142	93 (65.5)	48 (33.8)
1986-1987	Ice cream, salami, brie cheese	Pennsylvania	36	4 (11.1)	16 (44.4)
1986-1987	Raw eggs	California	2	Unknown	Unknown
1987	Butter	California	11	Unknown	Unknown
Not specified	Frozen vegetables	Texas	7	3 (42.9)	Unknown
1998-1999	Hot dogs, deli meats	22 states	101	Unknown	21 (20.8)
1999	Pâté	Connecticut, New York, MD, Maryland	11	2 (18.2)	Unknown
2000	Deli turkey meat	10 states	29	8 (27.6)	7 (24.1)
2000-2001	Homemade Mexican-style cheese (raw milk)	North Carolina	12	10 (83.3)	5 (41.7)
2002	Deli turkey meat, sliceable	8 North Eastern states	63	3 (4.8)	7 (11.1)
2003	Queso fresco (unpasteurized)	Texas	12	-	Unknown
2005	Deli turkey meat	Multi-State	13	-	Unknown
2005	Queso fresco (unpasteurized)	Texas	12	-	Unknown
Total			503		

Frankfurters

The earliest record of a hot dog type product dates back to 1500 B.C. in Babylonia. The exact origin is still debatable. Some historians report it was first developed in Frankfurt, Germany in 1484. While others claim it originated in Vienna, Austria, and suggests that the term 'weiner' represents this view. In 1852, spiced and smoked frankfurters were brought to America by Charles Feltman on New York's Coney Island *(61)*. In 1893 sausages became a popular food at baseball parks. In 1901, the name 'hot dog' was coined by a sports cartoonist named Tad Dorgan. He was at the New York Polo Grounds, when he drew a cartoon of a red hot dog dachshund. Dachshund was the name used during that time by sellers of hot dogs. Facing difficulty in spelling the word dachshund, he wrote on the caption "get your hot dogs" and thereafter the name persisted *(61)*. According to the National Hot Dog and Sausage Council current hot dog production is approximately two billion pounds with 64 percent comprised of pork/meat combination hot dogs, 24 percent all beef hot dogs and 12 percent poultry hot dogs. This equates to approximately 60 hot dogs per person per year *(2)*.

Hot dogs/ frankfurters/ wieners/ bologna or red hots are cooked and/or smoked sausages with not less than 15% of one or more kinds of raw skeletal muscle meat and raw meat byproducts (heart, kidney, or liver for example), according to the Code of Federal Regulations, volume 9, section 319.180 (CFR) standards of identity *(14)*. The standard requires that hot dogs made from beef, pork, turkey, chicken, or a combination should be comminuted (reduced to minute particles) and made into semi-solid products.

Smoking and curing ingredients contribute to flavor, color, and preservation of the product. Frankfurters are link-shaped and are available in various sizes- short, long, thin, and chubby. The finished product may not contain more than 30% fat or no more than 10% water, or a combination of 40% fat and added water. Up to 3.5% non-meat binders and extenders (such as nonfat dry milk, cereal or dried whole milk) or 2% isolated soy protein may be used, but must be shown in the ingredients statement on the product's label by its common name *(14)*.

Frankfurters may become contaminated with *Listeria monocytogene*s during post lethality processing steps such as packaging. The data for presence of *L. monocytogenes* in frankfurters manufactured at several different processing plants is presented in Table 3. The bacteria can grow to a level sufficient to cause illness during refrigerated and retail market storage, and in instances of temperature abuse *(25, 89)*.

TABLE 3. Presence of *L. monocytogenes* in frankfurters from different commercial
processing plants *(77)*.

Plant #	Size	Product	Season	Positive tested/Total tested/%
42	Large	Pork/Beef	Spring	0/ 2,900
94	Large	Turkey	Spring	2/ 2,700/ 0.07
105	Large	Beef	Fall	0/ 2,800
133	Large	Turkey	Spring	437/ 2, 800/ 16
172	Large	Beef	Winter	3/ 2,700/ 0.11
236	Small	Pork/ Beef	Winter	0/ 2,900
344	Large	Pork/ Beef/ Combination	Fall	4/ 2,500/ 0.16
367	Small	Pork	Summer	44/ 2,900/ 1.5
385	Small	Pork/ Beef	Fall	2/ 2,600/ 0.08
399	Large	Pork/ Beef	Summer	0/ 2,800
439	Large	Pork/ Beef	Spring	51/ 2,300/ 2.2
443	Large	Pork/ Turkey	Winter	0/ 2,900
Total				543/ 32,800/ 1.65

Government regulations

Outbreaks of *L. monocytogenes* require government regulatory intervention to be
dynamic not static. At present, RTE meat products under the Food Safety and Inspection
Service (FSIS) operate under "zero tolerance" policy, which means any food
contaminated with detectable levels of *L. monocytogenes* is deemed adulterated *(76)*. As
a result, in the United States, between 1999 and 2004 there were five major recalls that
affected 127 million pounds of RTE meat products *(24)*. FSIS has issued regulations that
all RTE product processors should develop written programs, such as Hazard Analysis
and Critical Control Point (HACCP) systems, Sanitation Standard Operating Procedures
(Sanitation SOPs) and other prerequisite programs. FSIS has also mandated the final
interim rule requiring RTE food processors to choose among 3 alternatives *(88)*:

Alternative 1. Use both a post-lethality treatment and a growth inhibitor for *Listeria* on RTE products. These facilities will be subject to FSIS verification activity that focuses on post-lethality treatment effectiveness. Sanitation is necessary but is built into the degree of lethality requirement for safety.

Alternative 2. Use either a post-lethality treatment or a growth inhibitor. These facilities will be subject to more frequent FSIS verification than those using Alternative1.

Alternative 3. Use sanitation measures only. The facilities will have most frequent verification activity. Under this, FSIS will place increased scrutiny on operations that produce hotdogs and deli meats.

Control of *Listeria monocytogenes* in RTE meat products

Listeria monocytogenes' ability to survive in adverse conditions in food processing environments for periods of several months to several years demands the application of hurdle technologies that inactivate, reduce or inhibit its growth during storage *(82)*. Selection of hurdles is based on the regulatory requirements of United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) *(87)*.

Listeria monocytogenes is heat labile and it can be eliminated at cooking steps of RTE products. Therefore, the foremost concern is the control of post-lethality, also referred to as post-process recontamination. *L. monocytogenes* can adhere to stainless steel food contact surfaces and can be easily aerosoled as vectors of contamination in the food processing area that require due attention *(43, 60)*. Established cleaning and

15

sanitation programs must be strictly implemented and followed for maximum effectiveness to prevent product recontamination *(9)*.

Post-process control of *L. monocytogenes* in RTE meat products could be physical, chemical, biological, or a combination of methods. Physical post-package decontamination methods are in-package thermal pasteurization, irradiation, and high pressure process (HPP). Biological control methods include use of lactobacilli, probiotic bacteria, plant products (like grape seed extract *(75)*, clove oil *(47)*) and bacteriocins *(82)*. Bacteriocins are ribosomally synthesized polypeptides produced by bacteria capable to kill or inhibit the growth of similar bacterial strain(s). Chemical treatments include the use of organic acids and their salts, nisin, pediocin, lysozyme and essential oils *(95)*. Technologies like irradiation are not yet approved for RTE packaged food, therefore the use of chemicals and biological antimicrobial compounds remain effective choices to control *L. monocytogenes* in the food industry *(68)*

Buffered Sodium Citrate and Sodium Diacetate

The salts of organic acids like lactates, diacetates, acetates, propionates, benzoates, sorbates, citrates and sulphates have been reported to be effective antimicrobial agents. The antimicrobial effects of these acids are due to the depression of pH below the growth range and metabolic hindrance by un-dissociated acid molecules *(39)*. Salts of organic acids that are commonly used in the manufacture of RTE meat products do not need further regulatory approval as they are traditional ingredients and most are listed as generally recognized as safe (GRAS). Sodium and potassium salts of

16

lactate, acetate and diacetate have been shown to provide listeriostatic effects in RTE

meat products *(35, 70)*. USDA-FSIS has approved these salts for use in RTE meat

products at concentrations up to 4.8% for sodium or potassium lactate and 0.25 % for

sodium diacetate by weight of total formulation *(86)*. The antimicrobials can be added to

meats as an ingredient in formulations, dipping or spraying solutions, package fi*Lm*s,

edible fi*Lm*s, coatings or in combinations. Post-process antimicrobial dipping or spraying

solutions are not currently utilized in industry, however various studies have shown the

potential effectiveness of this technique in controlling *Listeria monocytogenes (5, 26, 31,*

33, 66).

Samelis *(66)* in 2002 reported that sodium lactate used alone inhibited growth of

L. monocytogenes for 35 to 50 days . Sodium acetate, and sodium diacetate used in

combination inhibited growth throughout a storage period of 120 days at 4°C. It has been

reported that when cut pieces of beef were treated with sodium lactate, sodium acetate,

sodium diacetate, or combinations, the most effective treatment to delay the growth of

microorganisms was the combination of sodium lactate and sodium diacetate at 4°C *(72)*.

Stopforth *(79)* in 2005 showed that frankfurters formulated with 0.25% sodium diacetate

treated with dipping solution of 2.5% acetic acid, proved more efficient than lactic acid

dipping solution. Potassium lactate combined with sodium diacetate has been proven to

inhibit growth of *L. monocytogenes* in vacuum-packaged cold-smoked sa*Lm*on with no

adverse sensory effects *(90)*. However, salts of citric acid have not been investigated as

an antilisteriostic agent, and in limited studies have been discounted as ineffective.

Thippareddi *(80)* in 2003 showed buffered sodium citrate and buffered sodium citrate

supplemented with sodium diacetate to be effective in controlling germination and outgrowth of *Clostridium perfingens* during chilling of cooked meat and poultry products. Additionally, buffered sodium citrate can enhance the flavor of meat products *(85)*. Further studies could be done to investigate the efficacy of a buffered solution of sodium citrate with sodium diacetate against *L. monocytogenes.*

Nisin

At present, demand for natural biopreservatives by consumers has increased due to their safety and availability. Bacteriocins are natural antibiotics produced by bacteria which inhibit the growth of other related bacteria *(95)*. Nisin belongs to Class I bacteriocins that are called lantibiotics which contains the rare amino acids, meso-lanthionine and 3-methyl-lanthionine *(48, 64)*. Nisin was first discovered in the late 1920s and early 1930s when it was described as a toxic substance in milk which adversely affected the performance of cheese starter cultures *(94)*. Nisin is a low molecular weight antimicrobial protein produced by certain strains of *Lactococcus lactis* subsp. *lactis (19).*

Mode of action

Figure 4. shows the structure of nisin. Nisin is a flexible, elongated, amphipathic molecule which inserts itself into the cytoplasmic membrane of Gram-positive bacteria, forming pores resulting in the killing or inhibition of bacteria. Nisin is not effective against Gram-negative bacteria because of its relatively large size (approximately 1800-

18

4600 Dalton). This prevents it from penetrating the outer membrane of Gram-negative cell walls *(11)*. Nisin activity can be increased if bacterial cells are treated to make the cell membrane more permeable *(18)*. Nisin activity against spores has not been reported frequently in the literature.
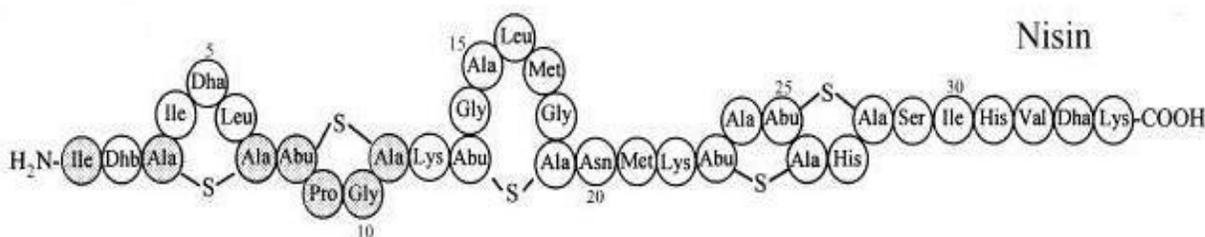
FIGURE 4. Structure of nisin *(11)*.

Nisin was approved as an antimicrobial for use in food in 1969 by the Joint Food and Agriculture Organization of the United Nations (FAO)/ World Health Organization (WHO) Expert Committee on Food Additives (JECFA). Currently it is used as a food preservative in over 50 countries. Nisin has characteristics which make it a suitable food preservative: it is non-toxic; it is naturally produced by bacteria; it is heat stable and retains its functionality over long storage periods (over two years when stored in the dark below 25°C); it is destroyed by digestive enzymes; and contributes no off-flavor. Nisin has been given GRAS status under 21 CFR 184.1538 *(56)*. Nisin has been available in the market by the name of *Nisaplin*® since 1953. Nisaplin contains 2.5% pure nisin and milk and milk solids derived from fermented milk by nisin producing strains of *L. lactis (18)*.

Nisin has been used in dairy products, egg products, pasteurized soups, flour based

products, canned foods and meat products in more than 50 countries. However in the

United States, it has been approved for use only in processed cheeses *(18)*.

Nisin activity in food depends on three factors: incubation temperature; length of

storage and pH. Nisin solubility is pH dependent and is most active between pH ranges of

3.0-3.5. At a higher pH, solubility and activity of nisin decreases. This fact can be

problematic in using nisin in certain food processing applications. Another challenge is

related to the amount of nisin used. Nisin action is concentration dependent with

increasing amounts of nisin being required to kill larger numbers of bacteria. However,

the emergence of nisin-resistant bacteria with use of higher concentrations has limited its

application. A high concentration of nisin ($\sim 10^4$ IU/ml) is needed in most food matrices

to produce bacteriostatic activity against *L. monocytogenes (22)*. It has also been reported

that development of nisin resistance increases when storage temperature increases above

4°C *(41)*. To overcome the problem , nisin is often combined with other treatments, like

modified atmospheric packaging *(52, 66)*, CO_2, heat, low storage temperature and other

antimicrobials *(13, 27, 45, 51)*.

Combination of nisin with organic acids

Various studies have been conducted with nisin in combination with salts of

organic acids like sodium diacetate, and sodium lactate. Samelis *(67)* reported 5000

IU/ml of nisin with 3 g/100 ml sodium diacetate showed maximum inhibition as a

dipping solution to control *Listeria* on sliced cured pork bologna as compared to nisin in

combination with sodium acetate, potassium benzoate or sorbate. Geornaras *(28)* in 2006 studied antilisterial effects on commercial frankfurters formulated with and without antimicrobials using dipping solutions of nisin 500 IU/ml with lactic acid, acetic acid and potassium benzoate at 10°C for 48 days. It was reported that all treatments inhibited the growth initially, however significant growth occurred by the end of storage. Geornaras *(27)* reported acetic acid, lactic acid or potassium benzoate applied alone reduced initial *L. monocytogenes* population by 0.4-1.5 log CFU/cm^2, while treatments including nisin caused reductions of 2.1-3.3 log CFU/cm^2. These findings indicate the need for future research to find effective combinations and concentrations of these antimicrobials for food processors to achieve safety, shelf-life and to retain the quality necessary for food products. Therefore, the present study was conducted to investigate the combined effect of nisin with a buffered solution of sodium citrate and sodium diacetate on surface inoculated *Listeria monocytogenes* on commercial beef frankfurters formulated without antimicrobials stored at 4°C (39.2° F) and 10°C (50.0° F) in vacuum packages.

CHAPTER III

MATERIALS AND METHODS

Study I: Individual antilisterial effects of nisin and buffered sodium citrate

supplemented with sodium diacetate (SCSD) as dipping solutions on commercial

beef frankfurters formulated without antimicrobials stored at 4 and 10°C in

vacuum packages

Bacterial strains and preparation of inocula

A cocktail for inoculation was made using five strains of *L. monocytogenes.* The

strains used were American Type Culture Collection (ATCC, Manassas, VA) 15313,

51414, 43256, 19115, and 7644. All cultures were obtained from the Department of Food

Science Nutrition and Health Promotion at Mississippi State University (FSNHP at

MSU). Strains were obtained from trypticase soy agar slants with 0.6 % yeast extract

(TSAYE; Becton, Dickinson Co., MD, USA) which were maintained at 4°C. The *L.*

monocytogenes strains were maintained by transferring to fresh TSAYE slants every

month. A loop of each strain was streaked on a TSAYE plate and incubated for 24 hours

at 35°C. A colony of *L. monocytogenes* from a TSAYE plate was transferred into 30 ml

of trypticase soy broth with 0.6% yeast extract and incubated for 24 hours at 35°C with

no agitation. The initial concentration of *L. monocytogenes* in each broth was

approximately 10^8 CFU/ ml. Two ml of each broth were combined together in a sterile test tube and vortexed for 10 seconds. Two ml of this cocktail were then dispersed into 2 L of 0.1% peptone water (Difco Laboratories, Detroit, MI) to get a final inoculum of 10^5 to 10^6 CFU/ml of *L. monocytogenes.*

Preparation of antimicrobials

Nisin (1000 IU/mg) was received from Profood International, Inc., Chicago, IL. Three dipping solutions of nisin 2000 IU/ml, 4000 IU/ml and 6000 IU/ml were prepared by dissolving 4 g, 8 g, and 12 g of newly purchased and opened nisin in 2 L of sterilized distilled water by stirring for 2 minutes.

Buffered sodium citrate and sodium diacetate (SCSD) was obtained from World Technology Ingredients, Inc. (WTI, Jefferson, GA, USA). The antimicrobial treatments were prepared by dissolving 50, 60 and 70 g of buffered SCSD in 2 L of sterilized distilled water to obtain 2.5, 3.0 and 3.5% weight/volume (w/v) of dipping solutions respectively. Freshly (15 minutes before) prepared antimicrobial solutions were used for each replicate.

Frankfurter inoculation

Beef frankfurters formulated without added antimicrobials (such as sodium diacetate, sodium lactate, potassium diacetate, potassium citrate, potassium benzoate, and salts of acetate) were obtained from a commercial distributor (Applegate Farms,

Bridgewater, NJ) and transported in a cooler on ice packs directly to FSNHP, MSU. The frankfurters were immediately frozen and stored at -20°C until used.

The day before treatment, the appropriate number of frankfurters (8 treatments x 6 sampling days at10°C; and 8 treatments x 4 sampling days at 4°C) were taken from the freezer, and allowed to temper at ambient temperature (25 ± 2°C) for sufficient time (approximately 30 minutes) and cut in half perpendicular to the longitudinal axis of the frankfurters (calculated: 2.5 cm diameter; 6 cm length; 57 cm^2 surface area; 22±2 g). The halved frankfurters were vacuum packaged (Prime Source, Vacuum Pouches, 40.64 cm x 52.07 cm, oxygen transmission properties of 3 to 6 cc per m^2/24 hrs at 4.4°C, Kansas City, MO) using Multivac model A300/16 (Kansas City, MO) at 999 mbar vacuum for 1 second with a 2.5 second seal. The vacuum packaged frankfurters were autoclaved (Consolidated, Stills & Sterilizers, Boston, MA) for 15 minutes at 121°C to destroy or inactivate any indigenous microorganisms *(20)* and then allowed to equilibrate at 25 ± 2°C in the laminar flow hood before inoculation.

Prior to dipping into inoculating solutions, autoclaved frankfurters were aseptically removed from their vacuum bags under a laminar flow hood by thoroughly wiping the bag with 70% ethanol and cutting horizontally along the seal with a sterile scalpel. Sterile plastic steamer containers with strainers (4 L, Progressive International, Kent, WA) were used for inoculation. A solution of 0.1 % peptone water with no *L. monocytogenes* was used for control. The frankfurters were placed in the strainers and then dipped in containers with either control or inoculum solutions for 5 minutes with constant agitation. The strainers containing the inoculated frankfurters were drained 10

24

minutes into empty containers. Not more than 20 frankfurters were used at a time in a steamer basket for inoculations. The non-inoculated and inoculated frankfurters were aseptically transferred to plastic storage bags with a zip closure (Ziploc, 26.8 cm x 27.3 cm; S. C. Johnson & Son Inc., Racine, WI, USA) and stored over night at 4°C to allow for maximum adherence of *L. monocytogenes*.

Treatment with antimicrobials

Twelve hours post storage at 4°C, inoculated frankfurters were aseptically removed from the zip lock bags under a laminar flow hood. Treatments of non-inoculated and inoculated frankfurters were performed using 8 dipping solutions. The 8 treatments were as follows: 1) non-inoculated control dipped in 2 L of sterile 0.1% peptone water as negative control; 2) inoculated control dipped in 2 L sterile 0.1% peptone water as positive control (*Lm* control); 3) inoculated frankfurters dipped in 2000 IU/ml nisin; 4) inoculated frankfurters dipped in 4000 IU/ml nisin; 5) inoculated frankfurters dipped in 6000 IU/ml; 6) inoculated frankfurters dipped in 2.5% buffered SCSD; 7) inoculated frankfurters dipped in 3.0% buffered SCSD and 8) inoculated frankfurters dipped in 3.5% buffered SCSD. The frankfurters were immersed and agitated in treatment solutions for 5 minutes and then allowed to drain for 10 minutes, as previously described in the inoculation procedure.

Packaging

Each of the drained frankfurters were aseptically placed into separate vacuum
bags (Prime Source, 15.24 cm x 21.59 cm, Kansas City, MO) and packaged using
Multivac model A300 /16 (Kansas City, MO) at 999 mbar vacuum for 1 second with a
2.5 second seal. The packages from each treatment were randomly selected and stored at
4 or 10°C. The frankfurters stored at 4°C were analyzed on day 0, 14, 28 and 42, and
those stored at 10°C were analyzed on day 0, 4, 8, 12, 16 and 20. Twelve hours post
treatment storage was designated as day 0.

Bacterial enumeration

On each sampling day two packages from each treatment were randomly selected
and analyzed for microbial counts. Twenty three ml of 0.1% peptone water were added to
each frankfurter sample in a stomacher bag (Fisher Scientific, Sterile Filtra Bag, Two
chamber filter bag, 3 mil thick, 7 x 12 inc.; capacity 1650 ml, Fits stomacher model: 400)
The samples were homogenized for 2 minutes using a stomacher (Seward Medical,
Stomacher 400 Lab Blender; London, UK). Serial dilutions of the homogenized 1 to 1
frankfurters and 0.1% peptone water were made in 0.1% peptone water. An aliquot (0.1
ml) was removed from each dilution, and spread plated on duplicate Modified Oxford
Agar (MOX) (Oxford Medium Base plus Modified Antimicrobic Supplement; Becton,
Dickinson, Sparks, MD) plates. The MOX plates were then incubated at 35°C for 48
hours to allow for the growth of *L. monocytogenes* colonies. The colonies were 2-3 mm
in diameter and appeared grayish black with black halo on MOX plates *(91)*.

pH measurements

A piece of frankfurter (2.5-5 g) was cut and placed into a two chamber stomacher bag (Sterile Filtra Bag, Fischer Scientific), diluted to 10-fold (wt/wt) with distilled water and homogenized using a stomacher for 2 minutes (Seward Medical, London, UK). The filtered slurry from one chamber of the bag was used to take pH measurements (Accumet® Research, AR60 Fisher Scientific, Waltham, MA). The pHs of the antimicrobial solutions were taken on each treatment day prior to application.

The pH of frankfurters was 6.05 ± 0.07 (means ± standard deviation). The pH of nisin at 2000 IU/ml was 3.85 ± 0.02, 4000 IU/ml was 3.52 ± 0.42, 6000 IU/ml was 3.70 ± 0.07, buffered SCSD 2.5% was 5.77 ± 0.03, SCSD 3.0% was 5.79 ± 0.01 and SCSD 3.5% was 5.56 ± 0.19.

Statistical analysis

Three replicates of each treatment were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Least-square treatment means in bacterial counts were estimated and significance of differences were determined using the analysis of variance in Proc combined model procedure of SAS 9.1 *(69)*. All differences were reported significant at $P \leq 0.05$.

In addition, the logarithm of the *L. monocytogenes* counts from each of the two storage temperatures were modeled as a function of time using the Baranyi model *(4)*.

For curve fitting, the program DMFit *(3)* (provided by Dr. J. Baranyi) of IFR (Intitute of Food Research, Reading, UK) was used. The Baranyi model is based on four parameters: a parameter which expresses the lag phase; μ_{max}, which expresses the maximum specific growth rate (per day); Y_0, which represents the lower asymptote, corresponding to the initial bacterial counts (log CFU/g); and Y_{end}, represents the upper asymptote, corresponding to the maximum bacterial counts (log CFU/g) when the growth curve forms an upper plateau at the stationary phase of growth. The lag phase is formally separated from the exponential and the stationary phase, which can be regarded as part of the potential growth curve defined by the model. The main difference between this model and other sigmoid curves is the mid-phase curve is similar to a linear curve, unlike other classical sigmoid curves which have a pronounced curvature.

Study II: Combined effects of nisin and buffered sodium citrate supplemented with sodium diacetate (SCSD) as dipping solutions against surface inoculated *Listeria monocytogenes* on commercial beef frankfurters formulated without antimicrobials stored at 4 and 10°C in vacuum packages

Bacterial strains and preparation of inocula

All bacterial strains and inocula used in study II were prepared according to the procedure followed in Study I.

Preparation of antimicrobials

Preliminary data analyzed from Study I were used to determine the most effective treatments. Nisin 6000 IU/ml and buffered SCSD 3.5% were chosen for combined study. A combined solution (2 L) was prepared using nisin 6000 IU/ml and 3.5% buffered SCSD in sterilized distilled water. Nisin 6000 IU/ml and buffered SCSD 3.5% were also prepared according to the protocol followed in study I, for sequence treatments.

Frankfurter inoculation

The method of frankfurter inoculation in study II followed the protocol utilized in study I.

Treatment with antimicrobials

In study II, treatment of inoculated frankfurters with nisin 6000 IU/ ml and 3.5% buffered SCSD, in various combinations, was performed using 5 immersion treatments. All were done in 4 L steamer baskets containing 2 L of antimicrobial treatments with a maximum of 20 halved frankfurters at a time. The 5 treatments were as follows: 1) non-inoculated control dipped in 0.1% peptone water; 2) inoculated control dipped in 0.1% peptone water; 3) inoculated frankfurters dipped in nisin 6000 IU/ml for 5 minutes, drained for 10 minutes and then dipped in 3.5% buffered SCSD for 5 minutes; 4) inoculated frankfurters dipped in 3.5 % buffered SCSD for 5 minutes, drained for 10 minutes and then dipped in nisin 6000 IU/ml for 5 minutes; and 5) inoculated frankfurters dipped in combined solution of nisin 6000IU/ ml and 3.5% buffered SCSD. The frankfurters were immersed and agitated in each of the dip treatments for 5 minutes and drained for 10 minutes before packaging.

Packaging

Treated frankfurters were vacuum packaged (1 per bag) as described in our previous study. Samples were stored at 4°C for 42 days and at 10°C for 20 days. Storage of frankfurters at 10°C served as a model to represent "temperature abused" products.

Bacterial enumeration

The procedures used for bacterial enumeration in this study followed protocols previously described in study I.

pH measurements

A piece of frankfurter (2.5-5 g) was cut and placed into a two chamber stomacher bag (Sterile Filtra Bag, Fischer Scientific), diluted to 10-fold (wt/wt) with distilled water and homogenized using a stomacher for 2 minutes (Seward Medical, London, UK). The filtered slurry from one chamber of the bag was used to take pH measurements (Accumet® Research, Waltham, MA). The pHs of the antimicrobial solutions were taken on each treatment day prior to application.

The pH of frankfurters was 6.04 ± 0.07 (means ± standard deviation). The pH of nisin 6000 IU/ml was 3.70 ± 0.06, buffered SCSD 3.5% was 5.55 ± 0.19 and the combined solution (nisin 6000 IU/ml and buffered SCSD 3.5%) was 5.56 ± 0.11.

Statistical analysis

Three replicates of each treatment experiments were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Least-square treatment means in bacterial counts were estimated and significance of differences were determined using the analysis of variance in Proc combined model procedure of SAS 9.1 *(69)*. The data for treatments was also expressed as the difference in bacterial counts from *L. monocytogenes* control and least-square mean were analyzed using Proc combined model

procedure of SAS 9.1 *(69)*. All differences were reported at a significance level of $P \leq$ 0.05.

In addition, the logarithm of the *L. monocytogenes* counts from each of the two storage temperatures were modeled as a function of time using the Baranyi model *(4)*. For curve fitting, the program DMFit *(3)* (provided by Dr. J. Baranyi) of IFR (Intitute of Food Research, Reading, United Kingdom) was used. The Baranyi model is based on four parameters: a parameter which expresses the lag phase; μ_{max}, which expresses the maximum specific growth rate (per day); Y_0, which represents the lower asymptote, corresponding to the initial bacterial counts (log CFU/g); and Y_{end}, represents the upper asymptote, corresponding to the maximum bacterial counts (log CFU/g) when the growth curve forms an upper plateau at the stationary phase of growth. The lag phase is formally separated from the exponential and the stationary phase, which can be regarded as part of the potential growth curve defined by the model. The main difference between this model and other sigmoid curves is the mid-phase is similar to a linear curve, unlike other classical sigmoid curves which have a pronounced curvature.

CHAPTER IV

RESULTS AND DISCUSSION

Study I: Individual antilisterial effects of nisin and buffered sodium citrate

supplemented with sodium diacetate (SCSD) as dipping solutions on commercial

beef frankfurters formulated without antimicrobials stored at 4 and 10°C in

vacuum packages

Sampling of non-inoculated autoclaved beef frankfurters indicated no indigenous

L. monocytogenes present. Antilisterial effects of individual dipping solutions of nisin

(2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) on surface inoculated beef

frankfurters stored at 4°C for 42 days and at 10°C for 20 days are presented in Tables 4

and 5, respectively. The inoculation levels of *L. monocytogenes* on control samples were

4.4 and 4.7 logs for 4 and 10°C. The control samples supported *L. monocytogenes* growth

to populations exceeding 8 logs CFU/g after 28 and 8 days of storage at 4 (Table 4) and

10°C (Table 5), respectively.

Nisin reduced *L. monocytogenes* initial levels by 2.2 (2000 IU/ml), 2.3 (4000

IU/ml) and 2.1 (6000 IU/ml) CFU/g compared to controls at 4°C.The initial log

reductions of 1.8, 1.9, 2.5 log CFU/g (nisin 2000, 4000, 6000 IU/ml) were observed at

10°C. The initial antilisterial effect of nisin was significantly ($P < 0.05$) higher than

33

buffered SCSD at 10°C (Tables 4). Nisin dipped treatments inhibited ($P < 0.05$) the

growth of *L. monocytogenes* populations on beef frankfurters during 28 days of storage at

4°C compared to the control. However at 10°C, nisin effectiveness declined by day 8,

when no significant difference ($P > 0.05$) in *L. monocytogenes* counts was observed

between nisin treated and control samples. These results indicate effectiveness of nisin

against *L. monocytogenes* is dependant on growth temperature. Similar results *(6, 74, 75)*

were reported by Barampalia et al. 2005 *(6)* and Theivendran et al. 2006 *(74)* utilizing

pork bologna and commercial turkey frankfurters, respectively, when nisin was more

effective at 4 than 10°C. Samelis et al. (2005) *(67)* inoculated sliced pork bologna

samples with *L. monocytogenes* at 10^2-10^3 CFU/cm^2 and immersed them in nisin solution

(5000 IU/ml) and found that nisin reduced the count of *L. monocytogenes* by 1.0-1.5 log

CFU/cm^2. In a similar study, Geornaras et al. *(28)* investigated the antilisterial effects of

nisin on commercial frankfurters. Samples were inoculated at 3-4 log/cm^2 and dipped in

nisin solution (0.5%) and found that nisin reduced the count of *L. monocytogenes* by 2.4-

3.5 log CFU/cm^2. Hampikyan *(34)* studied the effect of different nisin concentrations (0,

5, 10, 25, 50 and 100 µg/g) against *L. monocytogenes* on Turkish fermented sausages and

determined that the degree of listerial inhibition increased with increasing concentrations

of nisin. There are several other studies published on the antilisterial properties of nisin in

meat products such as beef frankfurters, raw beef, turkey frankfurters and pork bologna

(5, 26-28, 53, 66-68, 79). Moreover nisin has been studied in regard to reducing the

outgrowth of spoilage bacteria and other pathogenic bacteria in addition to *L.*

monocytogenes in RTE meats *(17, 29)*.

The mode of action of nisin involves pore formation in the cytoplasmic membrane, which leads to rapid removal of free amino acids, ATP and cations from the cell *(1)*. The antilisterial effect of nisin occurs immediately after cells are exposed to the bacteriocin resulting in cell death *(21)*. Our results are in agreement with previous studies *(26, 51, 66, 67)* showing high initial reductions of *L. monocytogenes* following treatment with nisin and subsequent cell recovery and growth during storage. The inability of nisin to maintain its antilisterial activity has previously been observed in RTE meat products *(42, 67)*. Some apparent reasons for loss of nisin activity include insufficient quantity of nisin to interact with all the target cells, inactivation by food components (such as pH), and increased resistance of some strains *(10, 36, 49, 67)*.

The dipping treatments of buffered SCSD had no significant effect on initial *L. monocytogenes* populations as compared to the control either at 4 or 10°C, except 3.5% buffered SCSD. However, buffered SCSD (2.5 and 3.5%) significantly inhibited the growth of *L. monocytogenes* during 28 days of storage at 4°C compared to control (Table 4). At 10°C SCSD did not provide an effective hurdle against the growth of *L. monocytogenes,* as compared to controls probably due to temperature abuse *(6, 74, 75)*.

There has been limited work investigating the efficacy of buffered SCSD against *L. monocytogenes* on meat products. Theraapidi (2003) *(80)* reported that SCSD (buffered sodium citrate supplemented with sodium diacetate) of ≥ 1.0 % was effective in reducing *C. perfringes* populations in roast beef or injected pork. Sodium diacetate is considered to be an effective organic salt against *L. monocytogenes* by various studies *(6, 67, 78, 79, 90)*. In contrast, Patel et al. found that when commercial beef frankfurters

were immersed in a 5% sodium diacetate solution, no significant inhibition of *L. monocytogenes* populations occurred during 28 days of storage at 4°C *(53)*. In such a situation, further investigation of the effects of buffered SCSD along with other antimicrobials against *L. monocytogenes* on meat products is warranted.

The growth behavior of *L. monocytogenes* on inoculated frankfurters treated with antimicrobials is analyzed using Baranyi model*(4)* and presented in Tables 6 and 7. The model is based on four parameters: a lag phase; μ_{max}, which represents the maximum specific growth rate per day, Y_0 (log CFU/g), which represents the initial bacteria counts and Y_{end}, which corresponds to the maximum bacterial counts (log CFU/g) when the growth curve forms an upper plateau at the stationary phase. There was no lag phase growth observed with any treatment at 4 or 10°C based on Baranyi model, which could be explained by less frequent sampling days compared to other studies. Using the same Baranyi model, Barampalia et al.*(6)* observed a lag phase of 13.78 days and 5.02 days at 4 and 10°C , respectively, in pork bologna formulated with sodium lactate (1.8 %) and sodium diacetate (0.125%) in combination. No lag phase growth was observed in samples where sodium lactate (1.8 %) and sodium diacetate (0.125%) was used as single ingredient. Geornaras et al. *(27)* evaluated the antilisterial effect of post-processing chemical solutions on commercial smoked sausage formulated with or without 1.5% potassium lactate plus 0.05% sodium diacetate, inoculated to level 3-4 log CFU/cm^2 stored at 10°C. Their data revealed a lag phase duration for *L. monocytogenes* of 10.2 days on sausage formulated with antimicrobials and no lag phase on samples that did not contain antimicrobials. Other explanations of differences in results could be due to

36

variations in experimental protocol, type of product, presence or absence of indigenous bacteria which could be inhibitory to *L. monocytogenes*, or differences in absorption rates of antimicrobial solutions due to specific surface characteristics *(5, 28, 66-68)*.

The Y_0 values of control samples were 4.7 and 4.4 logs CFU/g at 4 and 10°C, respectively. The 12 h post-treatment storage of samples, considered as day 0, may have affected the magnitude of initial reductions at different temperatures.

The maximum specific growth rate (μ_{max}) of nisin ranges from 0.339-0.368 day^{-1} while that of buffered SCSD was 0.230-0.274 day^{-1} and control was 0.263 day^{-1} at 4°C, inferring that recovered nisin treated *L. monocytogenes* grew faster than non-stressed populations. Rutherford *(62)* and others *(42, 67)* found similar results of higher μ_{max} values on treated samples than control samples. Those researchers postulated nisin could have slowed the growth of other bacteria thus reducing the bacterial competition against injured or stressed *L. monocytogenes*, which permitted it to grow at a faster rate than when in the presence of competitor organisms. In this study frankfurters were autoclaved before inoculation with *L. monocytogenes* and treatment with antimicrobials to avoid possible confounding effects of any indigenous microflora. However some surface contamination by environmental bacteria may have occurred immediately prior to inoculation treatments.

TABLE 4. Inhibitory effects of various concentrations of nisin and buffered sodium citrate and sodium diacetate (SCSD) against *L. monocytogenes* on beef frankfurters stored at 4°C.

Treatment[1]	*L. monocytogenes* population (mean ± SE) (log CFU/g)[2]			
	Time (day)			
	0	14	28	42
Lm control	4.4 a	8.0 a	8.5 a	9.0 a
Nisin (2000 IU)	2.2 b	7.0 b	8.2 c	9.0 a
Nisin (4000 IU)	2.1 b	7.2 b	8.1 d	9.0 a
Nisin (6000 IU)	2.3 b	7.0 b	8.2 c	9.0 a
SCSD (2.5%)	4.0 a	7.3 b	8.2 c	8.9 a
SCSD (3.0%)	3.2 a	7.3 b	8.4 ab	8.8 a
SCSD (3.5%)	3.3 ab	7.1 b	8.3 b	8.7 a

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials.
[2]All means are measurements of three separate experiments in duplicate. Values are means of three determinations. Means within a column followed by the same letter(s) are not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.

TABLE 5. Inhibitory effects of various concentrations of nisin and buffered sodium
citrate and sodium diacetate (SCSD) against *L. monocytogenes* on beef
frankfurters stored at 10°C.

Treatment[1]	*L. monocytogenes* population (mean ± SE) (log CFU/g)[2]					
	Time (day)					
	0	4	8	12	16	20
Lm control	4.7 a	7.7 a	8.6 a	8.7 a	8.8 a	9.0 a
Nisin (2000 IU/ml)	2.9 b	5.6 b	7.9 a	8.3 a	8.4 ab	8.6 b
Nisin (4000 IU/ml)	2.8 b	6.5 ab	7.9 a	8.3 a	8.5 ab	8.7 ab
Nisin (6000 IU/ml)	2.2 b	6.1b	8.0 a	8.0 b	7.9 c	8.5 b
SCSD (2.5%)	4.2 a	7.5 a	8.9 a	9.0 a	8.6 ab	9.1 a
SCSD (3.0%)	4.5 a	7.4 a	8.5 a	9.0 a	8.6 ab	9.3 a
SCSD (3.5%)	4.5 a	7.4 a	8.8 a	8.8 a	8.6 ab	9.0 a

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone
water with no antimicrobials.
[2]All means are measurements of three separate experiments in duplicate. Values are
means of three determinations. Means within a column followed by the same letter(s) are
not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.

TABLE 6. Growth kinetics of *L. monocytogenes* inoculated on the surface of beef
frankfurters treated with nisin, SCSD, alone or in sequence or combination,
then vacuum packaged and stored at 4°C for 42 days.

Treatment	Maximum specific growth rate (μ_{max}; per day ±standard error)	$Y_0{}^a$ (log CFU/g)	$Y_{end}{}^b$ (log CFU/g)	R^2
Lm control	0.263±0.026	4.4	8.7	0.976
Nisin (2000 IU/ml)	0.348±0.045	2.2	8.6	0.931
Nisin (4000 IU/ml)	0.368±0.046	2.1	8.5	0.929
Nisin (6000 IU/ml)	0.339±0.041	2.2	8.6	0.940
SCSD (2.5%)	0.230±0.025	4.0	8.5	0.955
SCSD (3.0%)	0.300±0.057	3.1	8.6	0.859
SCSD (3.5%)	0.274±0.059	3.2	8.6	0.840
Nisin + SCSD[1]	0.275±0.039	3.1	8.0	0.908
SCSD + Nisin[1]	0.341±0.046	2.7	8.2	0.899
Combined[1]	0.140±0.046	3.3	-[c]	0.893

[1]Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin +
SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) and a combined
solution containing 6000 IU/ml nisin and 3.5% SCSD.
[a]Lower asymptote estimated by the Baranyi model *(4)*.
[b]Upper asymptote estimated by the Baranyi model *(4)*.
[c]No value could be estimated when the upper part of the growth curve ceased without
forming an upper asymptote that corresponds to the stationary phase.

TABLE 7. Growth kinetics of *L. monocytogenes* inoculated on the surface of beef frankfurters treated with nisin, SCSD, alone or in sequence or combination, then vacuum packaged and stored at 10°C for 20 days.

Treatment	Maximum specific growth rate (μ_{max}; per day ±standard error)	$Y_0{}^a$ (log CFU/g)	$Y_{end}{}^b$ (log CFU/g)	R^2
Lm control	0.773±0.020	4.7	8.8	0.950
Nisin (2000 IU/ml)	0.661±0.017	2.9	8.4	0.980
Nisin (4000 IU/ml)	0.951±0.021	2.8	8.4	0.972
Nisin (6000 IU/ml)	0.996±0.040	2.1	8.1	0.915
SCSD (2.5%)	0.837±0.037	4.2	8.9	0.886
SCSD (3.0%)	0.698±0.037	4.6	8.9	0.870
SCSD (3.5%)	0.741±0.027	4.5	8.8	0.922
Nisin + SCSD[1]	0.788±0.031	3.0	8.2	0.929
SCSD + Nisin[1]	0.891±0.027	2.7	8.4	0.955
Combined[1]	0.828±0.290	2.7	8.1	0.942

[1]Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin + SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.
[a]Lower asymptote estimated by the Baranyi model *(4)*.
[b]Upper asymptote estimated by the Baranyi model *(4)*.

Study II: Combined effect of nisin and buffered sodium citrate supplemented with sodium diacetate (SCSD) as dipping solutions against surface inoculated *Listeria monocytogenes* on commercial beef frankfurters formulated without antimicrobials stored at 4 and 10°C in vacuum packages

According to the results from study I which were used as a basis of selection, nisin 6000 IU/ml and 3.5% (w/v) of buffered SCSD were selected to further investigate combined effects against *L. monocytogenes* on surface inoculated frankfurters. At these individual concentrations of nisin and buffered SCSD, *L. monocytogenes* growth was slowed, though it was not inhibited completely (Tables 4 and 5). The hypothesis that the singular inhibitory effect of nisin and buffered SCSD, when combined may lead to synergistic or additive effects against *L. monocytogenes* on beef frankfurters was the rationale for study II.

The combination of antimicrobials at the post-process application step can be achieved by either dipping the product in individual antimicrobial solutions sequentially or in one combined solution. There are few studies *(53, 67)* where antimicrobials were combined in a solution and others *(27, 28)* where two separate solutions in sequence were used. Samelis et al. (2005)*(67)* studied the combined effect of nisin (5000 IU/ml) with either lactic acid (5 g/100ml), acetic acid (3 or 5 g/100ml) or sodium diacetate (3 or 5 g/100ml) against *L. monocytogenes* on pork bologna slices. The inoculated bologna samples were immersed in organic acid or salt solutions supplemented with nisin. It was

42

theorized that the combined solution of nisin and organic acids or salts was effective

against *L. monocytogenes*, as they inhibited growth for 90-120 days at 4°C. Patel et al.

(53) evaluated the effectiveness of dipping frankfurters in sodium lactate (10%), sodium

diacetate (5%) and nisin (5000 IU/ml) in combined solutions. While Geornaras et al. *(27,*

28) treated frankfurters with Nisaplin (0.5%, pH 1.70± 0.02), lactic acid (2.5%, pH 2.04±

0.07), acetic acid (2.5%, pH 2.51± 0.04) and potassium benzoate (2.5%, pH 7.32± 0.06)

as individual solutions in sequence. Nisin is most effective as an antimicrobial, between a

pH range of 3.0-3.5 *(22)*, and has better functionality at pH 5.5 or below than pH 6.0 or

above especially in the presence of sodium chloride and nitrite in meat products *(45, 84)*.

In our study it was hypothesized that the sequential dipping treatment of nisin 6000 IU/ml

(pH 3.70± 0.07) followed by dipping in 3.5% (w/v) SCSD (pH 5.56± 0.19) would be

most effective as compared to treatments of buffered SCSD followed by nisin or the

combined solution (pH 5.56± 0.11). Nisin applied at the lowest pH possible was expected

to cause high initial mortality of *L. monocytogenes* followed by supplemental antilisterial

effects of buffered SCSD during storage at 4 or 10°C.

Data presented in Tables 8 and 9 illustrates antilisterial effects of combination

treatments on beef frankfurters stored in vacuum packages at 4 and 10°C, respectively.

All sequential and combined dipping treatments had significant ($P < 0.05$) inhibitory

effect on *L. monocytogenes* populations for 16 days at 10°C and 28 days at 4°C compared

to the control (Tables 8 and 9). There was no significant difference in initial log reduction

among any single sequential treatments, however antilisterial effectiveness during storage

was enhanced by treatments applied as a single combined solution compared to single

43

sequential immersion in separate solutions on certain sampling days (Tables 8 and 9) at both temperatures. The combined solution appeared to induce simultaneous damage to *L. monocytogenes*. This evidence could lead us to deduce that combining antimicrobials is a more effective technique of treatment application both scientifically and practically compared to individual solutions applied in sequence. Further research is needed to standardize the pH of solutions in order to reduce pH effect and investigate the efficiency of antimicrobial combinations (combined and separate solutions) irrespective of pH influence.

Samelis et al. *(67)* did not observe significant differences in the pH of homogenized pork bologna when treated with dipping solutions of nisin, acetic acid, lactic acid, and sodium acetate, used alone or in combination. Patel et al. *(53)* noted no significant difference in the initial pH of commercial beef frankfurters dipped in sodium lactate, sodium diacetate or nisin used alone or in combinations on day 0 or throughout the storage period of 28 days at 4°C. Bedie et al. *(7)* also did not observe significant change in the pH of frankfurters when sodium acetate, sodium diacetate or sodium lactate was added in the formulation. In addition Bedie et al. *(7)* indicated pH, in synergistic action with other antimicrobials can suppress the growth of *L. monocytogenes* during storage at 4°C for 120 days. Other studies have suggested that the increased inhibition of *L. monocytogenes* in meat products was due to the product itself or its interaction with other antimicrobials, or both and not just due to pH alone *(7, 67, 70, 73)*. In contrast, other researchers stated that the pH of the surroundings occupied by pathogens on the surface of a food influence their survival *(79, 92)*. Geornaras et al. *(26)* noted a pH

reduction of 0.29 to 0.85 for frankfurters dipped in acetic acid or lactic acid, and a reduction of 0.04 to 0.17 pH units for samples dipped in Nisaplin or Nisaplin followed by potassium benzoate.

The antilisterial activity of nisin 6000 IU/ml and buffered SCSD 3.5% used individually and in a combined solution on frankfurters stored at 4°C is presented in Table 10 (and Figure 5). Initially (day 0) there was no significant difference ($P > 0.05$) in the log reduction of *L. monocytogenes* with any treatment at 4°C. However the combined solution was more effective in inhibiting the growth of bacteria during 28 days of storage at 4°C, compared to antimicrobials used alone (Table 10).

The possible causes for similar initial reduction with nisin (2.1 log CFU/g, pH 3.70± 0.07), SCSD (1.1 log CFU/g, pH 5.56± 0.19) and combined solutions (1.7 log CFU/g, pH 5.56± 0.11) could be that pH increase was not sufficient enough to reduce the activity of nisin in combined treatments, or nisin activity was reduced by higher pH although compensated by the antilisterial effect of buffered SCSD. Samelis et al. (2000) reported lowered concentration of lactic acid, acetic acid, potassium benzoate or sorbate resulted in minimization of their residual antilisterial effects after 20-35 days of storage at 4°C even though nisin was present. Further, it was also suggested that the long-term antilisterial effect of dipping treatments on sliced pork bologna was primarily dependent on the type and concentration of the acid or salt, rather than on the inclusion of nisin (66-68) However, nisin might have contributed to lowering of cell numbers on the pork bologna samples initially during treatment and probably caused injury and lag phase extension. Further studies with different concentrations of SCSD could be conducted with

the intent to observe possible additional antilisterial effects. Lungu and Johnson *(44)*

inoculated commercial turkey frankfurters and treated them by dipping in solutions of

sodium diacetate (6%) and nisin (640 Activity Unit/ml) individually and in combination.

The treated samples were vacuum packaged and stored for 28 days at 4°C. These

antimicrobials used alone or in combination, had similar positive antilisterial effects.

There was no synergistic effect observed between nisin and sodium diacetate or sodium

lactate when used in combination.

Nisin used alone and in combined solution with SCSD provided greater ($P <$

0.05) initial reduction (2.5 and 2.0 log CFU/g) of *L. monocytogenes* as compared to

buffered SCSD used alone (0.2 CFU/g) at 10°C (Table 11and Figure 6). Nisin and

combined solutions were more ($P < 0.05$) effective in inhibiting the growth of *L.*

monocytogenes compared to buffered SCSD throughout the storage period of 16 days.

Geornaras et al. *(26)* reported that frankfurters dipped in solutions of acetic acid (2.5%),

lactic acid (2.5%) or potassium benzoate (2.5%) led to initial log reduction of 1.0 to 1.8

CFU/cm^2, and treatments that included Nisaplin (5000 IU/ml) reduced initial levels by

2.4 to > 3.8 log CFU/cm^2.

The growth behavior of *L. monocytogenes* on treated samples at 4 and 10°C is

illustrated in Tables 6 and 7. The maximum specific growth rate of the combined solution

was 0.140 day^{-1}, of nisin 6000 IU/ml was 0.339 day^{-1}and buffered SCSD 3.5% was 0.274

day^{-1}at 4°C (Table 6). For frankfurters stored at 10°C the maximum specific growth rate

for each treatment was > 0.828 day^{-1} (Table 7).The Y_{end} values were < 8.2, 8.6 and 8.6

log CFU/g in samples treated with combined, nisin 6000 IU/ml and 3.5% buffered SCSD,

respectively at 4°C (Table 6). The μ_{max} of 0.154 day^{-1} and 0.485 day^{-1} was obtained by

Geornaras et al.*(28)* for frankfurters formulated with and without antimicrobials stored at

10°C. They also reported lower Y_{end} values for *L. monocytogenes* on products containing

antimicrobials than on products formulated without antimicrobials. Barampalia *(6)* found

μ_{max} values generally higher at 10°C compared to 4°C.

TABLE 8. Combined effects of nisin and buffered sodium citrate and sodium diacetate (SCSD) on the inhibition of *L. monocytogenes* on beef frankfurters stored at 4°C.

Treatment[1]	*L. monocytogenes* population (mean ± SE) (log CFU/g)[2]			
	Time (day):			
	0	14	28	42
Lm control	4.4 a	8.0 a	8.5 a	9.0 a
Nisin + SCSD	3.1 b	7.3 b	7.5 b	8.7 b
SCSD + Nisin	2.7 b	6.9 c	7.5 b	9.0 a
Combined	2.7 b	6.3 d	7.4 b	8.9 a

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials. Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin + SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.
[2]All means are measurements of three separate experiments in duplicate. Values are means of three determinations. Means within a column followed by the same letter(s) are not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.

TABLE 9. Combined effects of nisin and buffered sodium citrate and sodium diacetate (SCSD) on the inhibition of *L. monocytogenes* on beef frankfurters stored at 10°C.

Treatment[1]	*L. monocytogenes* population (mean ± SE) (log CFU/g)[2]					
	Time (day)					
	0	4	8	12	16	20
Lm control	4.7 a	7.7 a	8.6 a	8.7 a	8.7 a	9.0 a
Nisin + SCSD	3.0 b	6.3 b	7.7 b	7.9 bc	8.4 b	8.8 b
SCSD + Nisin	2.7 b	6.3 b	7.9 b	8.0 b	8.6 bc	8.9 a
Combined	2.7 b	6.1 b	7.5 b	7.7 c	8.3 c	8.5 c

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials. Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin + SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) or a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.
[2]All means are measurements of three separate experiments in duplicate. Values are means of three determinations. Means within a column followed by the same letter(s) are not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.

TABLE 10. Combined effects of nisin and buffered sodium citrate and sodium diacetate (SCSD), used alone and in combination on the inhibition of *L. monocytogenes* on beef frankfurters stored at 4°C, expressed as difference in bacterial counts from *Lm* control[1].

| Treatment[2] | *L. monocytogenes* population (mean ± SE) (log CFU/g)[3] | | | |
| | Time (day) | | | |
	0	14	28	42
Nisin (6000 IU)	2.1 a	1.1 b	0.22 b	0.05 a
SCSD (3.5%)	1.1 a	0.97 b	0.12 b	0.26 a
Combined	1.7 a	1.7 a	1.07 a	0.10 a

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials.
[2]Inoculated beef frankfurters were dipped in 6000 IU/ml nisin, 3.5% buffered SCSD, and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.
[3]All means are measurements of three separate experiments in duplicate. Values are means of three determinations. Means within a column followed by the same letter(s) are not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.

TABLE 11. Combined effects of nisin and buffered sodium citrate and sodium diacetate (SCSD), used alone and in combination on the inhibition of *L. monocytogenes* on beef frankfurters stored at 10°C, expressed as difference in bacterial counts from *Lm* control[1].

Treatment[2]	*L. monocytogenes* population (mean ± SE) (log CFU/g)[3]					
	Time (day)					
	0	4	8	12	16	20
Nisin (6000 IU/ml)	2.5 a	1.6 a	0.50 a	0.69 a	0.86 a	0.5 a
SCSD (3.5%)	0.20 b	0.32 b	-0.15 b	-0.10 b	0.20 c	0.05 a
Combined	2.0 a	1.6 a	1.0 a	0.97 a	0.43 b	0.47a

[1]*Lm* control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials.
[2]Inoculated beef frankfurters were dipped in 6000 IU/ml nisin, 3.5% buffered SCSD, and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.
[3]All means are measurements of three separate experiments in duplicate. Values are means of three determinations. Means within a column followed by the same letter(s) are not significantly different ($P > 0.05$). Minimum detection limit was 100 CFU/g.
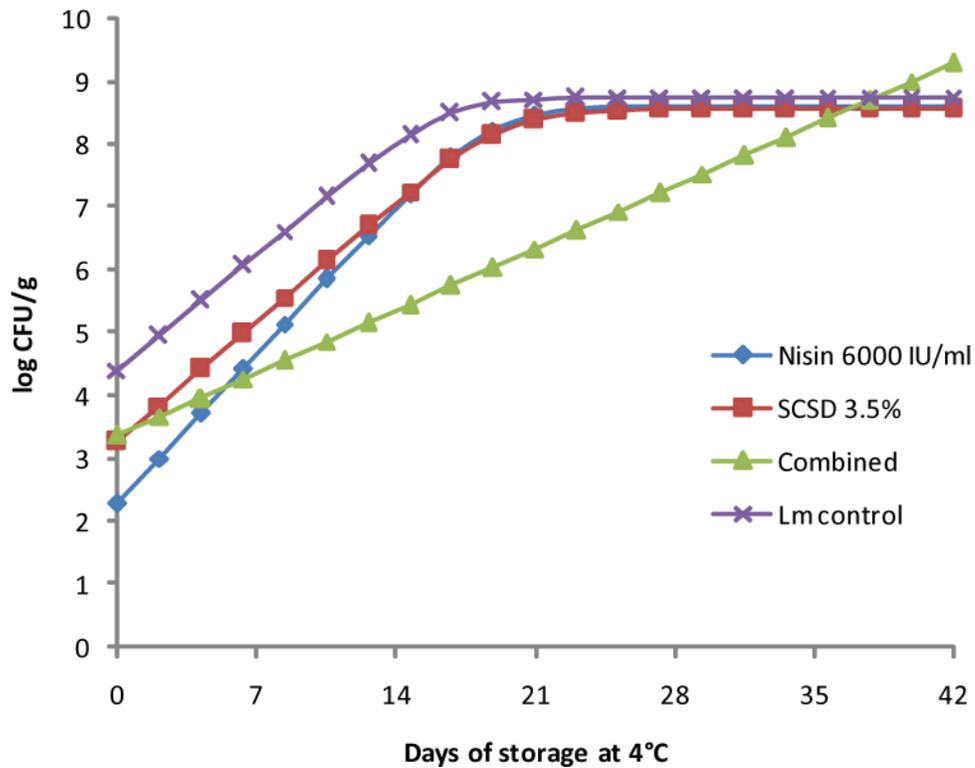
FIGURE 5. Survival/growth of inoculated *L. monocytogenes* on beef frankfurters dipped in solutions of nisin (6000 IU/ml), SCSD (3.5%), and combined solution of both, vacuum packaged and stored at 4°C.

Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials.
Values represent means of three determinations.
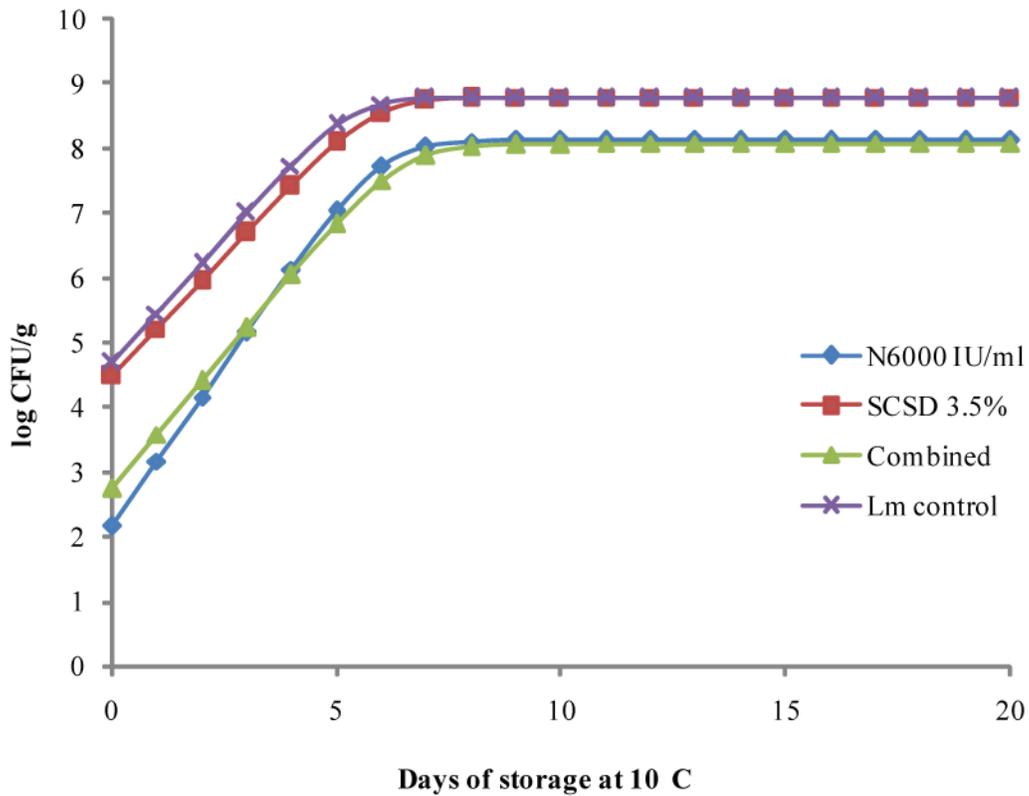
FIGURE 6. Survival/growth of inoculated *L. monocytogenes* on beef frankfurters dipped in solutions of nisin (6000 IU/ml), SCSD (3.5%), and combined solution of both, vacuum packaged and stored at 10°C.

Lm control, *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water with no antimicrobials.
Values represent means of three determinations.

CHAPTER V

SUMMARY AND CONCLUSIONS

The combination of nisin and buffered SCSD on beef frankfurters addressed two objectives. The first objective was to choose an optimum concentration of each antimicrobial. The second objective was to evaluate the efficacy of antimicrobial combinations. The present study addressed these two issues in two separate investigations. Study I: to determine the individual antilisterial effects of various concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) against *L. monocytogenes* on frankfurters formulated without antimicrobials stored at 4 and 10°C in vacuum packages. Study II: to evaluate the effect of predetermined concentrations of nisin and buffered SCSD, separately and in combination, against *L. monocytogenes* on frankfurters stored at 4 and 10°C.

The inoculated frankfurters (10^4 -10^5 CFU/g) in study I were immersed in 2000, 4000 and 6000 IU/ml nisin and 2.5, 3.0, 3.5% buffered SCSD, and then vacuum packaged and stored at 4 and 10°C. The growth of *L. monocytogenes* was monitored throughout the storage period of 42 days at 4°C and 20 days at 10°C. Nisin inhibited the additional growth of *L. monocytogenes* during 28 days of storage at 4°C and 4 days of storage at 10°C compared to control. Buffered SCSD controlled *L. monocytogenes*

54

population growth up to 28 days of storage at 4°C while it was not effective at 10°C compared to the controls. The antimicrobials showed bactericidal as well as bacteriostatic properties against *L. monocytogenes*, thus the combined effects of low temperature (4°C) environments and judicious use of antimicrobials can provide an effective food safety hurdle against *L. monocytogenes.*

In the combined study, antimicrobials nisin (6000 IU/ml) and SCSD (3.5% w/v) were applied in sequence and in a combined solution to inoculated frankfurter surfaces. The treated frankfurters were vacuum packaged and stored for 42 days at 4°C and 20 days at 10°C, to simulate ideal and abusive storage temperatures, respectively. Under the conditions of this study, it was found that the combination of antimicrobials applied in a single solution was more effective as compared to sequential treatment of single antimicrobials in separate solutions. The combination of antimicrobials in dipping solutions could be the most promising barrier against *L. monocytogenes* on beef frankfurters in a post-process application step.

In conclusion, the combination treatment was more effective than antimicrobials used alone at 4°C. As expected, temperature and antimicrobial combinations proved to be an effective hurdle against *L. monocytogenes.* The results obtained from this study suggest that *L. monocytogenes* proliferation can be controlled by using topical post-processing antimicrobial treatments, which substantially reduce initial population levels and suppress the growth of the pathogen during storage.

A limited number of studies have been done on assessing possible advantages of using combined antimicrobial strategies to control *L. monocytogenes* on RTE meat

products. Nisin and buffered SCSD at lower concentrations with optimized pH should be studied for their optimum conditions to be used as a combined antimicrobial solution.

Other antimicrobials or unique antibacterial substances may also be applied to ready to eat meats, either alone or in combination. The mechanism of action of the combined antimicrobials used in this study and their specific mode of action on the microbial cell needs further investigation. Variables like concentration of antimicrobials, combination technique and various methods of application (such as introducing in steam) should be studied. We suggest that nisin and buffered SCSD in a combined solution can be a highly potent antilisterial agent during storage conditions. This study reveals some interesting possibilities in the quest for antilisterial agents applied individually or in combination and these warrant further investigation.

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APPENDIX A

STUDY I

L. MONOCYTOGENES POPULATION OBTAINED FROM FRANKFURTERS

TREATED WITH DIFFERENT CONCENTRATIONS OF NISIN

(2000, 4000, 6000 IU/ML) AND BUFFERED SCSD

(2.5, 3.0, 3.5%) STORED AT 4 AND10°C

A1: *L. monocytogenes* population obtained from frankfurters treated with different concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 4°C

	L. monocytogenes population log CFU/g²											
	Day 0						**Day 14**					
Treatments[1]	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	4.20	4.23	4.38	4.30	4.79	4.83	8.04	8.30	7.90	8.01	7.94	7.92
Nisin 2000IU	1.00	1.00	1.00	2.90	4.70	3.72	6.83	6.95	-	-	7.20	7.16
Nisin 4000IU	1.00	2.00	1.00	1.30	4.70	3.68	6.92	6.88	-	-	7.72	7.11
Nisin 6000IU	1.00	2.00	1.00	2.30	4.70	3.70	6.93	7.05	-	-	7.05	6.98
SCSD 2.5%	4.12	3.45	3.60	3.90	4.70	4.81	7.40	7.35	-	-	7.06	7.26
SCSD 3.0%	1.00	1.00	3.60	4.38	4.70	4.43	7.39	7.29	-	-	7.37	7.29
SCSD 3.5%	1.00	1.00	4.34	4.08	4.70	4.39	7.20	6.95	-	-	6.94	7.21

A1 (Cont.): *L. monocytogenes* population obtained from frankfurters treated with different concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 4°C

Treatments[1]	*L. monocytogenes* population log CFU/g[2]											
	Day 28						Day 42					
	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	8.03	8.70	8.53	8.48	8.30	8.33	8.39	9.02	8.98	9.06	8.89	9.12
Nisin 2000IU	7.06	8.29	8.19	8.29	8.20	8.25	8.20	8.75	8.68	9.09	9.33	9.12
Nisin 4000IU	7.31	8.02	8.33	8.06	8.06	7.98	8.19	8.96	8.83	8.87	8.92	9.10
Nisin 6000IU	7.01	8.23	8.28	8.23	8.18	8.26	8.21	8.78	8.82	8.99	9.00	9.16
SCSD 2.5%	7.29	8.24	8.15	8.15	8.14	8.16	8.17	8.64	8.96	9.21	9.34	8.68
SCSD 3.0%	7.34	8.31	8.44	8.38	8.38	8.43	8.41	8.91	8.78	8.82	9.03	8.96
SCSD 3.5%	7.10	8.33	8.31	8.33	8.32	8.35	8.36	8.56	8.76	8.93	9.24	8.75

[1]Treatments, non-inoculated frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Negative control), *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Positive control).

[2]Three replications of each treatment were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Values are *L. monocytogenes* population in terms of log CFU/g.

A.2: *L. monocytogenes* population obtained from frankfurters treated with different concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 10°C

L. monocytogenes population log CFU/g^2

Treatments[1]	Day 0						Day 4					
	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.30	0.00	0.00	0.00	0.00	0.00
Positive control	3.81	4.17	4.73	5.60	4.87	4.88	7.24	7.33	7.54	7.49	8.27	8.58
Nisin 2000IU/ml	3.15	2.00	3.15	3.08	3.15	3.15	5.35	5.46	5.58	5.61	5.71	5.71
Nisin 4000IU/ml	2.90	3.20	2.90	2.78	2.60	2.30	6.78	6.58	6.47	6.59	6.53	6.64
Nisin 6000IU/ml	1.00	2.60	1.00	1.00	3.68	3.73	7.03	6.98	5.03	5.06	6.37	6.35
SCSD 2.5%	2.60	3.08	4.38	4.66	5.29	4.98	6.34	6.68	8.25	8.25	7.70	7.73
SCSD 3.0%	3.72	4.02	4.76	4.66	5.07	5.03	6.35	6.80	8.03	8.05	7.08	7.84
SCSD 3.5%	4.45	3.90	4.00	4.58	4.92	4.97	6.38	6.90	7.99	7.95	7.60	7.70

A.2 (Cont.): *L. monocytogenes* population obtained from frankfurters treated with different concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 10°C

| | *L. monocytogenes* population log CFU/g² | | | | | |
| | **Day 8** | | | **Day 12** | | |
Treatments[1]	rep1	rep2	rep3	rep1	rep2	rep3
Negative control	0.85	0.00	0.00	0.00	0.00	0.00
Positive control	8.83	8.60	8.62	8.66	8.58	8.73
Nisin 2000IU/ml	8.38	7.10	8.39	8.08	8.41	8.45
Nisin 4000IU/ml	8.30	7.48	8.27	8.56	8.51	8.15
Nisin 6000IU/ml	8.24	7.87	8.04	8.00	7.78	7.85
SCSD 2.5%	7.90	9.36	9.43	9.11	8.85	9.24
SCSD 3.0%	7.56	9.70	9.04	9.16	8.75	9.08
SCSD 3.5%	7.78	9.34	8.75	9.15	8.38	8.81

A.2 (Cont.): *L. monocytogenes* population obtained from frankfurters treated with different concentrations of nisin (2000, 4000, 6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 10°C

L. monocytogenes population log CFU/g[2]

Treatments[1]	Day 16 rep1	Day 16 rep1	Day 16 rep2	Day 16 rep2	Day 16 rep3	Day 20 rep1	Day 20 rep1	Day 20 rep2	Day 20 rep2	Day 20 rep3	Day 20 rep3
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	8.58	8.72	8.82	8.87	8.88	8.81	9.17	9.09	9.02	9.17	9.02
Nisin 2000IU	8.51	8.26	8.08	8.28	8.45	8.73	8.51	8.68	8.51	8.56	8.58
Nisin 4000IU	8.53	8.64	8.78	8.18	8.09	8.53	8.64	8.81	8.93	8.68	8.83
Nisin 6000IU	8.03	7.85	7.72	7.68	8.48	8.86	9.06	8.38	8.34	8.08	8.53
SCSD 2.5%	8.41	8.56	8.60	8.83	8.45	9.19	9.18	9.25	9.20	9.01	8.72
SCSD 3.0%	8.48	8.38	8.60	8.58	8.61	9.50	9.34	9.46	9.50	8.86	9.02
SCSD 3.5%	8.30	8.56	8.75	8.75	8.58	9.40	9.18	8.92	8.93	8.70	8.86

[1] Treatments, non-inoculated frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Negative control), *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Positive control).

[2] Three replications of each treatment were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Values are *L. monocytogenes* population in terms of log CFU/g.

74

APPENDIX B

STUDY II

L. MONOCYTOGENES POPULATION OBTAINED FROM FRANKFURTERS

TREATED WITH COMBINED SOLUTIONS OF NISIN (6000 IU/ML) AND

BUFFERED SCSD (3.5%) STORED AT 4 AND 10°C

B.1. *L. monocytogenes* population obtained from frankfurters treated with combined solutions of nisin (6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 4°C

	L. monocytogenes population log CFU/g^2											
	Day 0						**Day 14**					
	rep1		rep2		rep3		rep1		rep2		rep3	
Treatments[1]												
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	4.20	4.23	4.38	4.30	4.79	4.83	8.04	8.30	7.90	8.01	7.94	7.92
Nisin + SCSD	2.78	3.30	3.20	3.70	4.70	2.90	7.62	6.30	6.98	7.03	6.97	6.76
SCSD +Nisin	2.78	1.00	2.90	2.90	4.70	3.41	7.34	7.96	7.15	7.20	7.31	7.29
Combined	3.08	3.20	2.60	1.00	4.70	2.60	6.90	6.30	6.03	6.19	6.25	6.08

76

B.1 (Cont.): *L. monocytogenes* population obtained from frankfurters treated with combined solutions of nisin (6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 4°C

| Treatments[1] | *L. monocytogenes* population log CFU/g[2] | | | | | | | | | | | |
| | Day 28 | | | | | | Day 42 | | | | | |
	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	8.03	8.70	8.53	8.48	8.30	8.33	8.39	9.02	8.98	9.06	8.89	9.12
Nisin + SCSD	7.12	8.46	8.46	7.25	6.90	6.81	6.85	8.64	8.81	8.66	8.48	8.62
SCSD +Nisin	7.49	8.29	8.32	7.15	7.04	7.02	7.13	9.12	8.95	8.87	8.68	9.03
Combined	6.42	7.76	8.02	7.62	7.08	6.93	6.86	9.08	8.98	8.78	8.87	8.97

[1] Treatments, non-inoculated frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Negative control), *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Positive control). Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin + SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.

[2] Three replications of each treatment were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Values are *L. monocytogenes* population in terms of log CFU/g.

B.2. *L. monocytogenes* population obtained from frankfurters treated with combined solutions of nisin (6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 10°C

L. monocytogenes population log CFU/g[2]

Treatments[1]	Day 0						Day 4					
	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	4.17	3.81	4.73	5.60	4.87	4.88	7.24	7.33	7.54	7.49	8.27	8.58
Nisin + SCSD	2.90	2.60	2.30	2.60	4.01	3.64	6.25	6.20	5.68	5.98	7.36	6.26
SCSD +Nisin	2.00	2.30	2.41	2.48	3.53	3.38	6.41	6.26	5.88	6.57	6.41	6.45
Combined	2.30	2.30	2.30	2.30	3.68	3.45	5.75	6.00	5.93	5.68	6.45	6.96

L. monocytogenes population log CFU/g[2]

Treatments[1]	Day 8						Day 12					
	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	8.83	8.60	8.60	8.64	8.62	8.43	8.66	8.60	8.82	8.58	8.87	8.73
Nisin + SCSD	7.19	7.20	7.70	7.81	8.26	7.99	8.19	8.28	7.30	7.85	7.93	7.68
SCSD +Nisin	7.62	7.42	7.75	7.98	8.20	8.28	8.30	7.99	8.03	8.25	8.01	7.96
Combined	7.64	7.42	7.64	7.62	7.45	7.51	7.81	7.64	7.58	7.99	7.68	7.75

B.2 (cont.): *L. monocytogenes* population obtained from frankfurters treated with combined solutions of nisin (6000 IU/ml) and buffered SCSD (2.5, 3.0, 3.5%) stored at 10°C

	L. monocytogenes population log CFU/g[2]											
	Day 16						**Day 20**					
Treatments[1]	rep1		rep2		rep3		rep1		rep2		rep3	
Negative control	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00	0.00
Positive control	8.58	8.72	8.82	8.73	8.87	8.88	8.81	9.17	9.09	9.02	9.17	9.02
Nisin + SCSD	8.29	8.33	8.42	8.37	8.55	8.53	8.88	8.83	8.81	8.92	8.73	8.62
SCSD +Nisin	8.35	8.37	8.45	8.81	8.85	8.64	8.92	8.95	8.93	8.99	8.64	9.14
Combined	8.18	8.19	8.19	8.19	8.75	8.53	8.60	8.66	8.58	8.51	8.41	8.68

[1]Treatments, non-inoculated frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Negative control), *L. monocytogenes* inoculated on beef frankfurters dipped in 0.1% peptone water which contained no antimicrobials (Positive control). Beef frankfurters were dipped in 6000 IU/ml nisin followed by 3.5% SCSD (Nisin + SCSD), 3.5% SCSD followed by 6000 IU/ml nisin (SCSD + Nisin) and a combined solution containing 6000 IU/ml nisin and 3.5% SCSD.

[2]Three replications of each treatment were conducted with two samples tested per treatment at each sampling time in each replicate. The data obtained by plating the liquid from homogenized samples were converted to log CFU/g, based on the weight (23 g) of the halved frankfurter before being analyzed. Values are *L. monocytogenes* population in terms of log CFU/g.