ABSTRACT

SHEFET, SARID M. Development of nisin-based treatments to control pathogenic and spoilage microorganisms associated with poultry products. (Under the direction of Dr. Brian W. Sheldon).

More than 10% of the U.S. population experience at least one incident of foodborne disease annually (Todd, 1989). From 1983 to 1987, *Salmonella* species were the most often identified bacteria responsible for foodborne disease (Bean *et al.*, 1990) causing an estimated 2 million cases of foodborne salmonellosis annually in the United States at an estimated annual cost of one billion dollars (Cohen, 1978; Roberts, 1988). The Centers for Disease Control estimate that *Salmonella* infections contribute to at least 1,000 deaths per year in the United States.

Poultry products are considered to be the single most important food source of *Salmonella*. For example, approximately one third of the foodborne salmonellosis outbreaks for which a mode of transmission was identified have been linked to poultry consumption. Salmonellae contamination rates for live chickens can vary from about 13% to 80% of the flock and are invariably higher after processing (Mead, 1976; Roberts, 1988; Budnik, 1990). In 1992, the U.S. was ranked first in the world in poultry consumption with 94.8 pounds per capita, followed by Israel with 83.7 pounds, and Hong Kong with 79.3 pounds (Brown, 1993). In 1993 over 27.6 billion pounds of ready-to-cook poultry products were produced in the U.S. Per capita consumption of poultry products has increased substantially over the last two decades relative to other meat products; therefore, exposure of the consumer to poultry product-associated microorganisms including

pathogens has correspondingly increased and no doubt contributes to these foodborne disease statistics.

Besides bacterial pathogens, poultry products are also contaminated with a variety of spoilage microorganisms which can contribute to the development of strong off odors and/or slime formation and shortened product shelf life. These organisms, however, are not generally associated with human illness. A reduction in the population of these microorganisms or suppression of their growth often results in increased product shelf life and greater consumer acceptability. Some reports have estimated that the presence of pathogenic and spoilage microorganisms on poultry may cost the American public over two billion dollars annually in foodborne disease-related expenditures and spoiled products (Roberts, 1988; Todd, 1989).

The bacteriocin nisin was approved by the United States Food and Drug Administration in 1988 as a GRAS (general recognized as safe) substance for use in pasteurized cheese spreads to control outgrowth and toxin production by *Clostridium botulinum*. Blackburn *et al.* (1990) and Stevens *et al.* (1991) reported that nisin's spectrum of inhibitory activity could be extended to include gram-negative bacteria such as *Salmonella* when combined with chelating agents such as disodium ethylenediamine tetraacetate (EDTA) and citrate. Perturbation of the outer membrane of gram-negative bacteria via chelation of divalent cations located in the lipopolysaccharide layer is believed to sensitize the cells by providing access to the cytoplasmic membrane where nisin-mediated inactivation occurs.

The initial focus of this study was to optimize the inhibitory activity of nisin against a *Salmonella typhimurium* NAR test strain inoculated on broiler drumstick skin by combining nisin with various chelating agents and a surfactant.

Treatment parameters included a fixed nisin concentration (100 µg/ml), acidic solution pH, and varying EDTA, citric acid, and Tween 20 concentrations. Using a simplex search algorithm optimization program, four treatments were identified that significantly reduced the population of *S. typhimurium* NAR cells on broiler drumstick skin. *Salmonella typhimurium* NAR skin population reductions ranged from $\log_{10}$ 5.18 to 5.56 following immersion of the inoculated skin in the treatment solutions for 30 minutes (37°C). Inhibitory activity was also evident in the treatment solutions where *S. typhimurium* NAR cells washed from the inoculated skin were significantly inhibited by the treatments. In related studies, the inhibitory activity of these four optimized nisin-containing treatments were compared to either distilled, deionized water or 20 ppm chlorine against *S. typhimurium* NAR cells inoculated on the skin of broiler drumsticks. Following a 30 minute exposure to the treatment solutions at 25°C, surviving *S. typhimurium* NAR organisms were recovered from the skin and treatment solutions and enumerated. Log reductions in the skin populations were calculated relative to the control skin samples that were immersed in water under the same conditions. *Salmonella typhimurium* NAR skin populations were reduced an average of 6.67 logs following exposure to the nisin-containing treatments in comparison to 2.24 logs for the chlorine-treated broiler skin. Studies were also completed that evaluated the efficacy of the optimized nisin-containing treatments to inhibit *Salmonella*-infected broiler drumsticks. Similar reductions in the *S. typhimurium* NAR skin population, as observed with broiler drumstick skin, were detected following treatment with the four nisin-containing treatments.

Experiments were also conducted to determine the efficacy of the nisin-based treatments against *S. typhimurium* NAR-infected drumstick skin under

varying exposure times and concentrations of nisin. Exposure time significantly influenced the lethality of the treatments and depending on the treatment, nisin concentrations could be reduced from 100 μg/ml to 50 or 25 μg/ml without loss of significant biocidal activity. In other studies, the refrigerated shelf life of broiler drumsticks was extended by 1.5 to 3 days following immersion for 30 minutes in one of the optimized nisin-containing treatments in comparison to drumsticks immersed in distilled, deionized water.

These findings indicate that treatments containing nisin and varying concentrations of chelating agents and/or surfactant at an acidic pH are capable of significantly inhibiting the population of *Salmonella* microorganisms and spoilage bacteria that contaminate the surfaces of poultry carcasses. Applying the nisin-based treatment as a post chill application; either by dipping, spraying, or by incorporation into or on the surface of primary packaging materials and edible films, might be effectively used by poultry processors to reduce salmonellae populations on raw poultry products. With the poultry industry experiencing increased consumer pressure to produce *Salmonella*-free poultry products, the identification and implementation of effective preservation methods could result in several long term benefits including greater public confidence in poultry products, an increased market potential, and increased profits for the poultry industry.

DEVELOPMENT OF NISIN-BASED TREATMENTS TO CONTROL PATHOGENIC AND SPOILAGE MICROORGANISMS ASSOCIATED WITH POULTRY PRODUCTS

by

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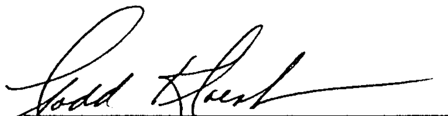

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DEDICATION

The great experience of attending this graduate school at North Carolina State University has been made possible through the encouragement and support I have received from friends on site, but mostly from my family back home in Israel.

In thanks, I would like to dedicate this thesis to my grandmother, Mrs. Fanny Bader, to my parents Mrs. Margalith and Dr. Gad Shefet and to my brother Raviv.

BIOGRAPHY

Sarid Moshe Shefet was born August 1, 1965, the son of Margalith and Dr. Gad Shefet of Givatayim, Israel. After graduating from Shimeon Ben-Zvi, where he minored in biological studies, Sarid joined the military police of the Israeli Defense Forces. He served in various positions such as commander of a police station as well as the head of the IDF's MP statistics department. After leaving the army as a lieutenant in 1987, Sarid started his undergraduate studies in Food Science and Technology at the Hebrew University of Jerusalem, Israel. During his studies he took part in the local student government organization. In 1990 he completed his Bachelor of Science degree in Food Science and Technology with success. After a year of consulting in the areas of food science and computers, Sarid initiated his graduate work in August, 1991 at North Carolina State University in the Department of Food Science under the direction of Dr. Brian W. Sheldon. He intends to pursue a Ph.D. degree in Food Science with a minor in Agricultural Engineering under the continued direction of Dr. Brian W. Sheldon.

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INTRODUCTION

A large percentage of foodborne illness in the United States is attributed to pathogenic bacteria (Cliver, 1987; Todd, 1989; Bean *et al*., 1990). The Centers for Disease Control (CDC) ascribe the majority of foodborne disease to *Salmonella* spp., *Staphylococcus aureus*, *Escherichia coli* O157:H7, *Bacillus cereus*, *Listeria monocytogenes*, *Yersinia enterocolitica*, *Vibrio* spp., *Clostridium perfringens*, and *Campylobacter jejuni* (Doyle, 1985; Todd, 1989; Bean *et al*., 1990). An estimated 24 to 81 million cases of foodborne disease occur annually in the U.S. with an average cost per case estimated to be as high as 1,000 dollars (Archer and Kvenberg, 1985; Todd, 1989). Surveillance statistics have shown a steady increase in the incidence of foodborne disease over the last forty years, and this rising trend is expected to continue (Doyle, 1985; Cliver, 1987; Todd, 1989).

These pathogenic bacteria have been isolated from a variety of foods including poultry and red meat products (Brune and Cunningham, 1971; Barnes, 1972; Goepfert *et al*., 1972; Kvenberg and Archer, 1987; Bergdoll, 1989; Mafu *et al*., 1989; Bean *et al*., 1990; Roberts, 1990). Poultry has been considered the single most important food source of *Salmonella* among food products derived from animals (Edwards, 1939; Jay, 1986). *Salmonella* contamination rates for live chickens have been reported to vary from 13 to 80% of the flock and in many cases the incidence of contamination is higher after processing (Budnick, 1976; Mead, 1976). Roberts (1988) estimated that *Salmonella*-infected poultry products cost the American population around one billion dollars in 1987 for health care-related expenses associated with salmonellosis.

In 1985, a National Academy of Sciences report called for the reduction or elimination of bacterial pathogens in fresh meat products. In recent years the poultry industry has been under increased consumer pressure to produce pathogen-free meat products. The average consumer's latent fears or suspicions about the safety of their foods are often exploited by the news media and consumer activist groups, many of whom may have less knowledge about modern animal production and processing methods than the general public. For example, several recent reports in the press such as the 1993 program broadcast by "Eye on America", the 1988 program broadcast by "60 Minutes", or the media coverage of the west coast foodborne disease outbreak involving under-cooked hamburger tainted with *Escherichia coli* O157:H7 (Jack-In-The-Box restaurant), have served to escalate consumer concerns regarding the safety of poultry and other muscle food products. In 1991, U.S. Senator H. M. Metzenbaum of Ohio introduced the "Poultry Consumer Protection Act of 1991". The bill proposed an overhaul of the poultry inspection system and a detailed study on the safety of the U.S. food supply in general. The Food Safety and Inspection Service (FSIS) of the United States Department of Agriculture is presently undergoing a major revamping of that agency with significant changes anticipated in the way poultry is inspected. This brief introduction of the current microbial problems facing the poultry industry serves to highlight the need for suitable controls to reduce or eliminate these undesirable microorganisms from our food supply. One approach to controlling these microorganisms is the use of novel biological controls such as bacteriocins to eliminate these bacteria.

Bacteriocins are proteins produced by one species of bacteria that are usually bactericidal to other closely related bacteria or in some cases bactericidal

to unrelated bacteria and bacterial spores (Tagg *et al.*, 1976; Klaenhammer, 1988). Bacteriocins produced by gram-positive bacteria are not generally inhibitory to gram-negative bacteria and likewise, bacteriocins produced by gram-negative bacteria do not inactivate gram-positive bacteria (Tagg *et al.*, 1976).

The bacteriocin nisin, produced by the dairy starter culture *Lactococcus lactis* subsp. *lactis*, was approved in 1988 by the United States Food and Drug Administration as a GRAS (general recognized as safe) substance for use in pasteurized cheese spreads to control outgrowth and toxin production by *Clostridium botulinum* (Delves-Broughton, 1990). This bacteriocin has a relatively broad spectrum of inhibitory activity against gram-positive bacteria and is commonly used as a preservative in many countries around the world in foods such as canned goods, dairy products, and beer (Daeschel, 1990; Delves-Broughton, 1990). Blackburn *et al.* (1990) and Stevens *et al.* (1991) reported that nisin's spectrum of inhibitory activity could be extended to include gram-negative bacteria such as *Salmonella* species and *Escherichia coli* O157:H7 when combined with chelating agents such as disodium ethylenediamine tetraacetate (EDTA) and citrate. Perturbation of the outer membrane of gram-negative bacteria via chelation of divalent cations located in the lipopolysaccharide layer is believed to sensitize the cells by providing access to the cytoplasmic membrane where nisin-mediated inactivation occurs.

The objective of this research was to evaluate nisin-based treatments for controlling foodborne pathogens and spoilage microorganisms associated with poultry products with the ultimate future goal of reducing the incidence of foodborne disease from consuming poultry products.

LITERATURE REVIEW

I. Poultry as a Vector of Foodborne Disease.

1. Foodborne Illnesses.

A foodborne disease outbreak is defined as an incident in which a) two or more persons experience a similar illness after ingestion of a common food, and b) epidemiological analysis implicates the food as the source of the illness (Bean *et al.*, 1990). The severity of the illness varies according to the number of microbes ingested and the efficiency of the individual's immune system to combat the infection (Roberts, 1985). FDA officials estimate an astounding 24 million cases of foodborne diarrheal illness annually in the United States (Rushing, 1990).

The factors that contribute to foodborne disease outbreaks are linked with production, processing, transportation, storage, and food service operations. Some of these factors encourage contamination of foods with pathogens or toxic agents while others permit multiplication of existing bacteria. The causes contributing to increased poultry-related foodborne disease are diverse. Some of these causes include: a) large animal population densities in grower houses which permit easier transmission of diseases or microorganisms between animals; b) cross-contamination of birds during transportation due to crowded transport cage conditions and cage to cage orientations that favor the spread of fecal contamination among birds; c) the use of improperly processed and stored animal byproducts for use in feeds that may introduce and perpetuate disease cycles; and, d) the concentration of animal slaughter into fewer and larger processing plants which may increase the probability for cross-contamination between animals and carcasses (Roberts, 1985). Although various microbial controls have

been implemented for reducing foodborne infectious diseases, foodborne illnesses appear to be increasing.

Other significant factors that contribute to the increase in foodborne diseases are failure to properly refrigerate food; failure to thoroughly heat processed foods; poor personal hygiene practices by infected employees; preparing foods a day or more in advance of serving; incorporating raw (contaminated) ingredients into foods that receive no further cooking; holding foods at improper temperatures favoring microbial growth; failure to reheat cooked foods to temperatures that kill vegetative bacteria; cross-contamination; and failure to properly clean and disinfect kitchen or processing plant equipment and utensils (Bryan, 1972). Effective reduction of foodborne disease outbreaks must be based on the elimination of these factors.

2. Salmonella.

A. General Characteristics.

Salmonella species belong to the family *Enterobacteriaceae* (D'Aoust, 1989). These organisms are classified as facultative anaerobes, gram-negative nonspore-forming rods that are ubiquitous in nature, and have as their natural habitat the intestinal tract of birds and other animals (Budnik, 1990). The conditions for growth of *Salmonella* include a temperature range between 7°C to 47°C, water activity > 0.94, and a pH range of 4 to 9 (D'Aoust, 1989; Budnik, 1990). The pH tolerance of these organisms depends on the individual strain, incubation temperature, and type of acid present (Doyle and Cliver, 1990). When citric and hydrochloric acids were used to adjust growth media pH, growth occurred at pH 4. However, use of propionic, acetic, and butyric acids inhibited

growth at this same pH. *Salmonella* species can survive for years under adverse conditions such as freezing (-23°C) and drying (Aw < 0.20) (Budnik, 1990). Under farm conditions, *Salmonella* can survive for extended time periods in animal feeds containing rendered animal byproducts, or in litter, soil, animal feces, and water (Bryan, 1972).

Salmonella contamination always relies on a transmission vehicle for spreading between humans, animals, and environmental sources. A variety of food vehicles have been associated with salmonellosis including: chicken and turkey (Brune and Cunningham, 1971; Barnes, 1972; Gallo *et al.*, 1988; Izat *et al.*, 1989; Lillard, 1989; Rampling *et al.,* 1989; Doyle and Cliver, 1990), eggs (Blaser *et al.*, 1981; Bryan, 1988; Budnik, 1990), raw beef (Walker, 1965), raw milk (Cohen, 1983; Tacket *et al.*, 1985; Bryan, 1988; Oosterom, 1991), pasteurized milk (Munce, 1980; Ryan *et al.*, 1987), meat salads (Blaser *et al.*, 1981; Beckers *et al.*, 1985; Bryan, 1988), powdered milk (Rowe *et al.*, 1987), chocolate (Craven, 1975; D'Aoust *et al.*, 1985; Kapperud *et al.*, 1989), swine (Bryan, 1988; Mafu *et al.*, 1989; Budnik, 1990), and cheese (D'Aoust *et al.*, 1985).

A direct relationship has been established between the occurrence of *Salmonella* in poultry processing plants and its presence in feed, hatcheries, and broiler flocks (Barnes, 1972). *Salmonella* contamination rates for live chickens have been reported to vary from 13 to 80% of the flock and are generally higher on processed carcasses (Budnick, 1990; Mead, 1976). The incidence of *Salmonella* in processing plants depends to a large extent on the level of contamination in the live bird (Barnes, 1972). *Salmonella* organisms are carried in the gut of a small percentage of healthy birds and are not generally considered a part of the normal intestinal microflora. One method to reduce carcass

contamination is to eliminate live birds that harbor *Salmonella* in their GI tracts (Barnes, 1972).

B. Salmonellosis.

Salmonellosis is a general term for illnesses caused by *Salmonella* species. Salmonellosis can be defined according to three syndromes: typhoid fever, enteric fever, and gastroenteritis (known also as food poisoning). The most severe syndrome, typhoid fever, is caused by *Salmonella typhi* and can lead to the following symptoms: fever, septicemia, headaches, constipation, diarrhea, and vomiting (Bryan, 1981;. Doyle and Cliver, 1990). The symptoms for enteric fever are similar to those of typhoid fever but are less severe and of a shorter duration (Doyle and Cliver, 1990). Gastroenteritis is the most common *Salmonella*-related illness found in the United States (Budnik, 1990; Doyle and Cliver, 1990). The symptoms of this disease are diarrhea, cramping, vomiting, fever, and headaches. It's incubation time ranges from 6 to 72 hours and the duration of the illness can last up to a week (Doyle and Cliver, 1990).

Salmonellosis occurs from the consumption of food contaminated by live *Salmonella* organisms (Jay, 1983; Roberts, 1988). The infective dose of *Salmonella* is dependent on individual tolerances, strain virulence, and type of food/stomach content. As few as 50 cells in a chocolate candy product have been found to cause illness (Doyle and Cliver, 1990). Outbreaks of salmonellosis are related to multiple factors: failure to properly refrigerate contaminated foods; preparing foods a day(s) in advance of serving, and holding foods at improper temperatures (4-60°C). Some factors that are involved in the initial contamination or in recontamination of food include: the presence of *Salmonella* on raw foods of animal origin; use of contaminated products (i.e. raw eggs) in

prepared foods which receive no further treatment or which are inadequately heated; cross-contamination between raw and cooked foods via workers' hands or equipment surfaces; inadequate cleaning and disinfection of kitchen or processing equipment or utensils; and to a lesser extent, contamination by human carriers (Bryan, 1972).

Todd (1989a,b) reported that salmonellosis was the most prevalent foodborne disease causing an estimated 2.9 million cases annually. Forty thousand cases of salmonellosis are actually reported annually in the United States to the Centers for Disease Control but the actual number of people believed to be infected has been estimated at 2 to 4 million (Roberts, 1988; Budnik, 1990). According to Izat (1989), approximately 1-2% of the American population suffers from salmonellosis each year. Salmonellosis cases are typically characterized by mild abdominal discomfort and diarrhea, but severe illness and even death can result. The death rate from salmonellosis is one in a thousand yielding an estimated 2,000 deaths annually (Roberts, 1985; Roberts, 1988).

All diseases are typically underreported (Cliver, 1987; Roberts, 1988; Todd, 1989a,b; Bean *et al.*, 1990). According to Roberts (1985), only one in seventy-five cases of salmonellosis are reported. Underreporting of *Salmonella*-related diseases is attributed to several factors: salmonellosis is often confused with the stomach flu and thus individuals do not associate their symptoms with foodborne causes nor seek medical attention. In addition, the lack of conclusive proof associating the disease to food consumption and lack of adequate follow-up by federal, state, city, and county government officials in reporting cases to the CDC also contributes to underreporting of foodborne disease (Cliver, 1987).

C. Economic and Public Health Impact.

Salmonella is one of the most important human pathogenic bacterial species, causing hundreds of thousands of foodborne infections each year from the consumption of contaminated meat products and other foodstuffs of animal origin. Cooked meat is not often a direct cause of infection since in most cases heating eliminates all *Salmonella* contamination present on the surface (Oosterom, 1991). Infection is more often related to contaminating cooked products prior to serving by contact with contaminated workers or food preparation utensils.

Over the last 16 years the number of reported outbreaks of salmonellosis has been increasing (Cliver, 1987; Roberts, 1988; Todd, 1989a; Bean *et al.*, 1990). However, inconsistencies in reporting of foodborne disease can be seen by examining the distribution of outbreaks by state. A few states, such as New York, California, Washington, and Hawaii, account for a disproportionately high number of outbreaks. Although these states could have a higher real incidence of salmonellosis, these figures more likely represent differences in the degree of surveillance activity (Bean, 1990). The likelihood of an outbreak coming to the attention of health authorities varies considerably depending on consumers and physicians awareness and interest, disease surveillance activities of state or local health and environmental agencies, and the degree of motivation at all levels of government to report the incident (Cliver, 1987; Bean, 1990).

Many countries in the western world annually report moderate numbers of cases of human salmonellosis, and it is generally accepted that there is considerable underreporting of salmonellosis cases (approximately 5% reported). In the United States alone, the associated costs from salmonellosis is estimated at

4 billion dollars annually (Todd, 1989a). This estimation excludes the indirect costs associated with pain, inconvenience and suffering, lost leisure time, travel expenses, etc.

The overall costs associated with all foodborne illnesses is estimated to reach 10 billion dollars annually (Rushing, 1990). This figure includes industry costs such as lost wages, reduced productivity, medical costs for treatment, loss of product via seizing of contaminated lots, loss sales, product recall and destruction, and costs associated with legal liability suits against the food distributor (e.g. restaurant, supermarket, broker, etc.) (Roberts, 1988; Rushing 1990). FDA estimates for a single outbreak of 200 cases of salmonellosis amounted to more than 62 million dollars and when these outbreaks occur in food service operations, the complete loss of a business is not unusual (Rushing, 1990).

II. Broiler Processing.

1. Basic Unit Operations in Processing Broilers.

According to federal regulations, before a poultry processing plant can begin operating, the Food Safety Inspection Service of the U.S. Department of Agriculture must approve the plant's plans for facilities, equipment, and operational procedures to ensure that the operation will be sanitary. Facilities and equipment must be cleanable and sanitized on a regularly scheduled basis (FSIS, 1987).

A processing plant is generally divided into two major areas; one in which the birds are received, killed, and defeathered, and the second area where all other unit operations are conducted (i.e. evisceration, chilling, further processing, packaging, etc.). This physical separation is required for sanitation reasons in

order to segregate the inherently dirtier phases of processing from those involved in preparation of the final product. Prior to entering the plant's kill area, USDA veterinarians examine the flock for diseases and other abnormal conditions. Dead or dying birds are culled from the flock and not allowed into the slaughtering plant (FSIS, 1987).

Broiler processing can best be described as a sequence of several separate unit operations (Bailey *et al.*, 1987; Brune and Cunningham, 1971):

- *Receiving*- Birds are transferred by conveyers to the shackling area where they are shackled by their feet. Prior to the carcass chilling operation, the shackle conveyer is the main mode of transporting carcasses.
- *Killing*- Birds may be slaughtered by two general methods. The Kosher method is performed manually by severing the trachea and jugular vein in a single knife stroke. The more traditional commercial method includes severing the jugular vein. Most plants apply an electric stunning procedure prior to killing to facilitate a more efficient slaughter, to reduce struggling, and to relax the feather papillaes, thereby promoting better feather removal and an improved processing efficiency and product quality. The electric stunning procedure will also eliminate hemorrhagic muscle sores, blood splashing, and broken bones (Sheldon *et al.*, 1993). A recent advancement in stunning technology includes the use of progressively higher levels of CO_2 in an enclosed stun tunnel. Birds lose consciousness in less than one minute. Carbon dioxide stunning is believed to cause less stress on the bird in comparison to electric stunning.
- *Bleeding*- Carcasses are bled for approximately 90 sec. Insufficient bleed time results in excess blood accumulation in the tissues which may lead to

discoloration of the carcasses and a consequent downgrading by the USDA inspector.

- *Scalding*- The most common hot water scald temperature range is 36°C to 39°C for 90 to 120 sec. This temperature range helps keep the meat tender, allows for easy feather removal, and maintains the outer layer of skin (cuticle) intact. Under-scalding may cause incomplete feather removal in the automated pickers and over-scalding results in irregular removal of the outer layer of skin. New generation scalders are now designed with countercurrent flow and air agitation for improved water penetration. In this design fresh scald water enters the scalder at the carcass exit point and circulated countercurrent to the flow of carcasses. Accordingly, carcasses pass through constantly cleaner water. A third type of scalder involves a two-stage operation. The first stage includes an initial hydrofall section where cascading water is directed over the carcass to remove the majority of the dirt and debris prior to immersion scalding. The second stage is again a countercurrent flow design where water flows opposite to the direction of carcass movement (Sheldon *et al.*, 1993). According to USDA regulations, 1 quart of water per broiler must be overflowed from the scalder during operation. It has been suggested that the elimination of immersion scalding would improve the overall microbiological quality of carcasses (Shackelford, 1988).
- *Defeathering*- The most common way to removes feathers from carcasses is by using rotating drums or wheels containing finger-like rubber projections. The design and configuration of these defeatherers have been suggested by some researchers to aid in microbial cross-contamination between carcasses.

- *Singeing*- Remaining hairlike pin feathers are removed by conveying carcasses through a gas flame.
- *Washing*- Any remaining feathers, singed pin feathers, or other foreign material are washed from the carcass surface by spray washers.
- *Pinning*- Developing feathers that have just pierced the skin are removed manually. In addition, the oil gland used for feather preening is also removed at this stage, either manually or via automation.
- *Automatic evisceration*- The evisceration operation can be thought of as the beginning of the ‘clean’ processing steps. The first cut is made from the posterior tip of the sternum to the vent, and with the vent still attached, the viscera are drawn out from the body cavity but remains attached to the carcass for the postmortem inspection. After inspection, the liver and gizzard are harvested and the remaining viscera are removed and discarded. Eviscerated carcasses are next sprayed with a 20 ppm aqueous chlorine solution that rinses the inside and outside of the carcass thereby reducing product contamination and cross-contamination by approximately tenfold (Mead, 1976). Between carcasses, each evisceration station is sprayed with high pressure water sprayers followed by a 20 ppm chlorine water sanitation rinse (Shackelford, 1988). Until the late 1970's, most evisceration procedures were accomplished manually.
- *Removal of lungs and kidneys*- A strong vacuum aspirator is inserted into the thoracic and dorsal abdominal cavities to remove the lung and kidney tissues.
- *Removal of head*- As the carcasses are conveyed by shackles, the head is drawn through a V-shaped knife which severs the head from the cervical vertebrae.

- *Removal of neck, esophagus, and crop*- Neck skin is cut along the dorsal side of the carcass to the shoulder position thereby facilitating the severing of the neck adjacent to the shoulders.
- *Inside/outside bird washer*- Prior to releasing carcasses into the chiller, the abdominable cavity and skin are sprayed with water to remove any remaining traces of blood, loosely attached tissues, or foreign substances.
- *Chilling*- The objective of this unit operation is to reduce carcass body temperature. Food Safety and Inspection Service regulations state that the carcass temperature must be below 4.4°C within 4 hours after slaughter and that 2 quarts (1.89 liters) of water must overflow from the chiller for each carcass introduced unto the chiller. Rapid cooling of the carcass prevents the buildup of microbial populations and promotes a longer product shelf life. Ice water, slush ice, and/or refrigerated chillers may be used as coolants. In some coolers, carcasses are chilled to less than 4°C in 30 min. Two types of chiller designs are commercially available including one and two-stage chillers. Most chillers employ a countercurrent flow pattern similar to scalders and may use chlorinated water (20 ppm) at the discretion of the processor. Carcasses are conveyed through the chiller using a tumbling action that acts to remove residual blood, fat, and other particulate matter from the carcasses.
- *Weighing & cutup*- Carcass weighing is generally an automated procedure while carcass cutup is accomplished by hand and/or through automated operations. A new approach in portion cutting of boneless products involves a computer-controlled portioning system that utilizes high pressure water jets for cutting. The use of water jets has the added advantage of sanitary slicing

when compared with knives, saws, blades, and human handling (Sheldon *et al.*, 1993).

- *Packing/packaging*- With the exception of those products intended for further processing, packing/packaging is the last stage of the slaughter process where carcasses or their parts are manually or automatically placed in plastic bags or bulk containers or positioned on foam trays containing absorbing drip pads and overwrapped with an oxygen permeable film.

Inspectors constantly monitor the processing facilities and equipment for maintenance of general sanitation conditions. These USDA inspectors have the authority to stop the processing line when they detect unsanitary conditions in the plant (FSIS, 1987). Furthermore, they are responsible to assure overflow volumes in the scalders and chillers. The combined water use for all processing steps, including clean-up operations, totals about 4 gallons per broiler, in comparison to an average of 10 gallons used in the late 1970's (Shackelford, 1988). Much of this water, including the mandated water overflows in the scalders and chillers, contributes to the sanitary condition of the carcasses and processing equipment. Another major role of the inspectors is to inspect live poultry and carcasses for evidence of disease, fecal and non-fecal contamination, and product wholesomeness. New technologies may someday replace the need for some inspectors and/or quality control personnel. For example, high speed video imaging cameras linked to computers can rapidly scan carcasses for quality defects, compare those images to stored standard images, and either accept or reject carcasses or assign a quality grade based on specific criteria (Sheldon *et al.*, 1993).

2. Major Sites for Microbial Cross-Contamination During Processing.

Several studies have reported an increase in bacterial contamination of carcasses as birds are processed suggesting that processing plants can aid in spreading microorganisms among carcasses (Lillard, 1971; Barnes, 1972; Dawson, 1979). Any processing step where carcasses must be handled, contact a surface, or are allowed to contact other carcasses raises the potential for cross-contamination (Shackelford, 1988). Psychrophilic bacteria, the primary food spoilage microorganisms of muscle food products, enter poultry processing plants on the feathers and feet of birds and sometimes through the water (Barnes, 1972). In general, the population of psychrophilic bacteria on carcasses is directly related to the number of birds processed per operating shift. Thus, higher carcass populations are detected at the end of the shift. These spoilage organisms may eventually contribute to the development of strong off odors and/or slime formation in products held under refrigeration or temperature abuse conditions. Fortunately, most of these organisms are not associated with human illness. A reduction in their population and/or suppression of their growth often results in increased product shelf life and consumer acceptability. Psychrophiles initially comprise about one hundred organisms per cm^2 of skin tissue following processing whereas spoiled products generally have a skin population of 10^7-10^8 cells/cm^2 (Barnes, 1972).

Salmonella contamination rates on carcasses are invariably higher after processing which reflects the ongoing problem that poultry processors face in preventing cross-contamination between infected and noninfected birds during slaughter and further processing (Brune and Cunningham 1971; Budnik, 1990).

Contamination of live animals and carcasses by *Salmonella* species can occur at several stages during production and processing.

The control of *Salmonella* in the processing plant depends largely on the control or destruction of fecal-associated bacteria (Notermans *et al.*, 1978). Studies have clearly shown that poultry may harbor *Salmonella* organisms in the intestinal tract at high incidence rates and in high populations without any symptoms of disease (Notermans *et al.*, 1978). The source of the pathogen may be related to the consumption of contaminated poultry feed containing rendered animal byproducts infected with *Salmonella* (Jones *et al.*, 1991). During slaughtering, ruptures in the digestive tract during eviceration can easily occur allowing the spread of intestinal contents within the carcass body cavity and over the skin surface. This surface contamination can be spread from carcass to carcass during subsequent chilling, cutting, packaging, and carcass distribution operations. Shackelford (1988) demonstrated that conveying shackles and other associated processing equipment surfaces that come in contact with carcasses have an increased bacterial buildup throughout the processing day which greatly contributes to carcass contamination. The incidence of *Salmonella*-contamination in poultry processing plants depends to a great extent on the population of these organisms in or on live birds arriving at the plant (Barnes, 1972).

Transportation of live animals to the processing plant contributes significantly to cross-contamination among live birds (Jones *et al.*, 1991). Due to the confined quarters encountered during transporting and the manner in which cages are stacked, broilers are typically contaminated with feces on their feet, feathers, and skin. When processed, approximately 6 to 8 hours after catching

and loading, most broilers show some evidence of fecal contamination on the feet and breast feathers (Shackelford, 1988). Kim and Doores (1993) reported that the current technologies used for scalding and picking birds have a significant impact on the attachment of bacteria to carcass skin. Examination of conventionally-scalded and picked carcasses revealed some bacterial attachment, while kosher-processed carcass skin had little bacterial attachment, and steam-sprayed skin had bacterial levels three times higher than conventionally-processed carcasses. When fecal matter on feathers and feet wash into the scalding vat, the potential for cross-contamination between carcasses increases. For this reason, the use of immersion scalders has been questioned as a possible source of carcass contamination or cross-contamination (Brune and Cunningham, 1971; Dawson *et al.*, 1979).

The low population of microorganisms commonly detected in some scald waters can be attributed to a dilution effect from adding fresh scald water; a minimum of one quart per bird as required by USDA regulations (Brune and Cunningham, 1971). Studies indicate that although the total bacterial count on broiler skin is usually less than 10,000 CFU/cm^2 immediately after scalding, this count does not differ markedly from the microbial population detected on the skin of live birds (Surkiewicz *et al.*, 1969; Bailey *et al.*, 1987). The reduction in significant numbers of psychrophilic bacteria and specifically *Pseudomonas* species on the surfaces of most carcasses during scalding indicate that scald water contamination plays a relatively minor role in contributing to the spoilage microflora on broiler carcasses (Bailey *et al.*, 1987). The potential uptake of contaminated scald water by carcasses, with subsequent contamination of air

sacs, lungs, and possibly other internal tissues by pathogenic bacteria, is a major objection to immersion scalding.

Only a few studies have been conducted to evaluate the effect of picking and pinning (feather removal) on the microbiological quality of poultry meat. May (1961) and Bailey (1987) found bacterial counts to be relatively low following the picking and subsequent spray wash operations. Surkiewicz *et al.* (1969) found total aerobic plate counts of picked broiler skin to be between 1200 CFU/cm^2 and 35,000 CFU/cm^2, although the post-pick counts reported in their studies were higher than the skin counts taken from carcasses exiting the scald tank. In another study where broiler carcass skin was artificially contaminated with a marker *E. coli* strain, cross-contamination between carcasses was confirmed following the feather removal process (Mulder, 1978). Furthermore, more frequent carcass cross-contamination occurred during scalding and picking when lower scalding temperatures (52-54°C) were used in comparison to a higher scalding temperature (60°C) (Mulder *et al.*, 1978).

The potential for additional carcass cross-contamination occurs during evisceration. As reported previously, studies by Schuler and Badenhop (1972) and Shackelford (1988) demonstrated that the shackles and associated equipment surfaces that come in contact with carcasses have an increased bacterial buildup throughout the processing day which greatly contributes to carcass cross-contamination. Following evisceration and through packaging, the number of bacteria including fecal-associated organisms on carcasses tend to decline in a modern processing plant. Effective spray washing of equipment and carcasses removes gross fecal contamination and reduces the total number of bacteria on the carcass, although a considerable number still remain on the carcass surface

and lodged beneath the feather follicles (Barnes, 1972). Brune and Cunningham (1971) reported that 20.5% of the eviscerated carcasses sampled in a poultry processing plant contained *Salmonella* species. The defeathering machines were incriminated as being among the main sources of cross-contamination in the plant.

Several important factors contribute to the potential of cross-contamination during immersion chilling and include: 1) initial bacterial populations on carcasses prior to chilling; 2) the volume of metered water overflow used per carcass; and, 3) the ratio of carcasses to chiller water volume (Peric *et al.,* 1971). Carcass chillers are designed to rapidly reduce carcass temperature thereby preventing the growth of spoilage and pathogenic microorganisms that comprise the normal flora of poultry carcasses. To achieve this temperature reduction carcasses are submerged and conveyed through vats containing chilled water. By regulations, fresh water must be metered into the chiller at a specified rate of one-half gallon per broiler carcass introduced into the chiller and one gallon per turkey. Although the intent of chilling is to prevent bacterial growth and prolong shelf life, the potential still exists for bacterial cross-contamination between carcasses while conveyed through a common chill vat. As carcasses are passed through the chiller, residual blood, fat, and other particulate matter are washed from the carcasses and become suspended in the chiller water. There is evidence to suggest that cross-contamination of carcasses occurs in the chiller yet some studies have reported no evidence of bacterial buildup during the chilling of carcasses (Lillard, 1982; Shackelford, 1988). In fact, Barnes (1972) reported a reduction in fecal bacteria, *Staphylococci*, and *Salmonella* on carcasses following immersion chilling. Conversely, carcasses

chilled by air had higher bacterial populations than water-chilled carcasses (Barnes, 1972).

May (1961) found that the bacterial population on chickens increased approximately six-fold during processing and eight-fold in retail stores and that the increased contamination was attributed to contaminated work surfaces and worker contact. In a study by Jones *et al.* (1991), *Salmonella* spp. were found half as frequently on carcasses exiting an immersion chiller (10.7%) than on carcasses following packaging (21.4%). This 21% *Salmonella* incidence rate reported by Jones *et al.* (1991) was lower than the 37% *Salmonella*-positive rate reported by Green *et al*. (1982). These studies suggest that greater manual handling of carcasses results in higher carcass bacterial populations.

3. Disinfectants Used by the Poultry Industry.

A. Non Chlorine-Based Disinfectants.

Various disinfectants have been developed for use by poultry producers and processors to inhibit poultry-borne microbial pathogens and spoilage microorganisms. These disinfectants protect both the bird and the consumer, as well as extend product shelf life. Disinfectants can be separated into two groups: those used by poultry producers and those used by poultry processors. Fate *et al.* (1985) evaluated the efficacy of four commercial disinfectants for sanitizing poultry houses. Rodac plates were used to sample representative surface materials commonly found in poultry rearing facilities. Pre-disinfection samples were taken following the removal of chickens and litter, while post-disinfection samples were taken four hours after room treatment. The disinfectants were diluted in water and applied evenly to all surfaces of the rooms with a high-

pressure spray unit. The rooms were allowed to dry for about 4 hours prior to sampling. The most effective disinfectant was a product that contained 0.78% glutaraldehyde. The other products, ranking in order of greater to lesser bacterial inhibitory activity, contained 0.5% cresylic acid, 0.63% iodophores, and 0.78% of a quaternary ammonium compound combined with formaldehyde. The most effective fungicide was the product containing cresylic acid.

In other studies, the efficacy of glutaraldehyde (1,5 pentanedial) as a disinfectant was evaluated under simulated commercial carcass chilling conditions where filtered air was bubbled through a continuous chiller (Mast and MacNeil, 1978). In these studies, broiler carcasses were held in chillers (3°C) containing either 50 ppm glutaraldehyde, 50 ppm chlorine, or water (control) for 45 minutes. Chilling reduced the number of mesophiles on all carcasses; those chilled in glutaraldehyde had the lowest mesophilic count throughout 10 days of refrigerated storage at 3°C. Glutaraldehyde-chilled carcasses had a shelf life two days longer than chlorine-chilled carcasses and three days longer than carcasses chilled in water. In a second chiller study that tested the efficacy of glutaraldehyde (Bailey *et al.*, 1987), a population of 2.5×10^2 *Salmonella typhimurium* cells were inoculated onto the skin of broiler carcasses prior to chilling. No detectable *S. typhimurium* cells were recovered from the skin following immersion for 30 minutes in a chill water bath containing 0.5% glutaraldehyde (pH 8.6). However, the *S. typhimurium* population was not eliminated when the inoculum load was increased to 3.6×10^5 cells per carcass. Passage of *S. typhimurium* cells from inoculated to uninoculated carcasses (*i.e.* cross-contamination) was prevented by adding at least 0.01% glutaraldehyde to the prechill water when *S. typhimurium* inoculum levels were 2.5×10^2 cells per

carcass (Mast and MacNeil, 1978). In comparison to control carcasses that were prechilled in water, the shelf life of broiler carcasses held at 2°C was extended by 6 days following the prechilling of carcasses for 10 minutes in a chill bath containing 0.5% glutaraldehyde (adjusted to pH 8.6). This extension in shelf life appeared to result from a reduction in the initial total mesophilic counts on glutaraldehyde-treated carcasses, and not from a reduced rate of bacterial growth following an initial lag phase.

Ansari (1984) compared the germicidal properties of 11 disinfectants (Table 1.1). These disinfectants were comprised of the most commonly used active ingredients: quaternary ammonium, cresylic acid, synthetic phenol, formaldehyde, or combinations of the above. The use-dilution methods recommended by the American Association of Analytical Chemists (AOAC) were used to test the germicidal activity of each disinfectant against 6 specific bacterial strains. To simulate field conditions, horse serum (16.4%) was added to the cultures as a substitute for the normal organic matter found in grower houses. At a 1:256 dilution, disinfectants C and J were the most effective against all 6 bacterial strains (Table 1.2). Disinfectants F, I, H, and K were effective at the recommended dilutions as broad spectrum germicidal agents. All disinfectants tested, with the exception of disinfectants G and H, inhibited *S. pullorum* and *S. gallinarum*. Disinfectants D, H, J, and K were bactericidal against *A. faecalis*, while C, D (phenolics), and J (cresylic acid) were effective against *P. aeruginosa*. *S. aureus* was sensitive to all the disinfectants tested, except formulations containing quaternary ammonium compounds (A, B) and propionic acid (G) which were applied as single active ingredient component systems. The *E. coli*

Table 1.1. Disinfectants description.

Source*	Active Ingredients	Intended use**	Dilution Used
A	Quat. Amm. (4.9%), Isopropanol (1.6%)	Det.-Dis.,G.D.	1:64
B	Quat. Amm. (9.7%), Glycol (4%)	H.D.,A.S.	1:128
C	Phenol (9.6%), Glycol (12.6%)	G.D.,A.S.	1:256
D	Phenol (22.9%)	G.D.	1:256
E	Propanediol-HNP (19.2%), Formaldehyde (2.02%), Quat. Amm. (2.3%)	G.D.	1:128
F	Quat. Amm. (12.5%) + T-EDTA (1.69%)	G.D.	1:256
G	Propionic acid (20%)	W.S.,fungicidal	1:64
H	Phenol (17.72%)	G.D.,H.D.,A.S.	1:256
I	Quat. Amm. (2.11%) + Formaldehyde (27.78%) +Thymol (2.58%), Alcohol (24.9%)	G.D.	1:100
J	Cresylic acid (50%)	G.D.	1:256
K	Quat. Amm. (20%) + EDTA (1.75%)	Det.-Dis., G.D.	1:400

* Source of Disinfectants:
A,B: Bio-lab
C: Ceva Laboratories
D: Chris-Tex Chemicals, Inc.
E: Hess and Clark, Inc.
F: Huntington Laboratories
G: Kemin Industries, Inc.
H: Oxford
I: Vineland Laboratories
J: Whitmoyer Laboratories, Inc.
K: Winthrop Sterling Drugs, Inc.

** Intended use:
G.D.: General disinfectant for housing and equipment
H.D.: Hatchery disinfectant.
A.S.: Air sanitizer.
Det.-Dis.: Detergent and disinfectant.
W.S.: Water sanitizer.

Ansari, 1984. Poultry Digest **43**: 230-232.

Table 1.2. Efficacy of several disinfectants[*] against specified microorganisms[1].

I. Disinfectant killed microorganisms in at least 18 of 20 cultures tested (90% confidence).
II. Disinfectant killed microorganisms in 14-17 cultures of 20 cultures tested.

Microorganism	Category	Disinfectants
Salmonella pullorum	I.	A[*], B, C, D, E, F, I, J, K
	II.	E
Salmonella gallinarum	I.	B, C, E, F, I, J
	II.	A, D
Alcaligenes faecalis	I.	D, H, J, K
	II.	C, E
Pseudomonas aeruginosa	I.	C, D, J
	II.	A, H
Staphylococcus aureus	I.	C, I, J
	II.	D, E, F, H, K
Escherichia coli	I.	All disinfectants except G
	II.	-

[1] No additional organic load present.
[*] See Table 1.1 for description of disinfectant source abbreviations.

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culture was inhibited by all disinfectants except the propionic acid-containing formulation (G). Although quaternary ammonium compounds are nontoxic, they do have a bitter taste, and as such they are likely to alter the flavor of food. In addition, quaternary ammonium compounds are not too effective against many common gram-negative bacteria (Guthrie, 1992).

Table 1.3 shows the inhibitory efficacy of these disinfectants when an organic load was introduced to simulate conditions found in poultry hatcheries and houses. Considering the general environmental conditions present where disinfectants are used to combat disease agents, these results are clearly more relevant to the issues faced by the poultry industry. In general, disinfectants are more effective in inhibiting or controlling microbial pathogens when used on relatively clean surfaces (Ansari, 1984). For some of the tested microorganisms, the presence of horse serum had little or no adverse effect on the inhibitory activity of the disinfectant (*e.g. P. aeruginosa*); whereas for other organisms (*e.g. S. pullorum*), the addition of an organic load reduced the effectiveness of some disinfectants (*e.g.* Quats- A,B,F). These disinfectants are contact surface inhibitors and must be in contact with pathogenic organisms at the correct concentrations to inhibit poultry contaminants.

Hydrogen peroxide (H_2O_2) has been used successfully for many years as a disinfectant, particularly as a surface decontaminant and sterilant in industrial and commercial sanitation programs (Spaulding *et al.*, 1977). Sheldon and Brake (1991) evaluated H_2O_2 as an alternative hatching egg disinfectant to replace formaldehyde. Sheldon and Brake (1991) reported that 0.5% H_2O_2 yielded greater than a 6-log kill in three potential eggshell bacterial contaminants in 30

Table 1.3. Efficacy of disinfectants* in the presence of 16% horse serum.

I. Disinfectant killed microorganisms in at least 18 of 20 cultures tested.
II. Disinfectant killed microorganisms in 14-17 out of 20 cultures tested.

Organism	Class	Disinfectants
Salmonella pullorum	I.	C*, D, J
	II.	E, K
Salmonella gallinarum	I.	All disinfectants except G
	II.	G
Alcaligenes faecalis	I.	D, J, K
	II.	C, F
Pseudomonas aeruginosa	I.	C, F, J
	II.	A, I, K
Staphylococcus aureus	I.	F, I, J, K
	II.	B, C, E
Escherichia coli	I.	A, B, C, D, E, G, H, I
	II.	J, K

* See Table 1.1 for description of disinfectant source abbreviations.

Ansari, 1984. Poultry Digest **43**: 230-232.

seconds. In comparison with formaldehyde fumigation, no significant difference in hatchability was detected for 0.5% H_2O_2-treated eggs from 30- or 56-wk-old broiler flocks. Under circumstances of higher H_2O_2 demand, such as occurs on eggshell surfaces, H_2O_2 concentrations of 5% were required to disinfect the shell surfaces (~5-log reduction). Hatchability of fertile broiler eggs from a 44-wk-old flock was significantly increased following treatment with a 5% H_2O_2 spray in comparison to the untreated control eggs. These results demonstrate that H_2O_2 compared favorably to formaldehyde as a hatching egg disinfectant without adversely affecting hatching potential. Unlike some disinfectants, H_2O_2 readily decomposes to environmentally safe and nontoxic end products (*i.e.* water and oxygen) and can easily be destroyed after use. However, as with any strong oxidizing agent, H_2O_2 should be handled with care because it can irritate the skin, eyes, and mucous membranes.

Ozone has also been considered to be an efficacious hatchery disinfectant (Sheldon and Ball, 1986). Masaoka *et al.* (1982) documented ozone's bactericidal and fungicidal properties in the decontamination of hospital rooms. In their study, formaldehyde and ozone were compared, and found to have similar bactericidal properties. However, ozone was superior to formaldehyde with regard to convenience, ease of expulsion after use, and degree of respiratory hazard threat to the hospital staff. Whistler and Sheldon (1989) reported that ozone (1.41 to 1.68% by weight) killed greater than 99.9% of three selected bacteria [*E. coli* ATCC MM294, *P. aeruginosa* B-14, and *S. typhimurium* B-13] representative of poultry pathogens that routinely contaminate the surfaces of eggshells, setters, and hatchers. In another study, broiler carcasses chilled in 3.0-4.5 ppm ozonated water (1-2°C) for 45 minutes had lower whole carcass rinse

mesophilic and psychrotrophic bacterial counts compared to carcasses chilled under non-ozonated conditions (Sheldon and Brown, 1986).

Following a spray application of either an invert soap, iodophor, dichloroisocyanate, or orthodichlorobenzene onto the surface of a test plate previously inoculated with 1.0 x 10^7 cells of *S. aureus* or with chicken droppings, Kitani *et al.* (1983) reported only a 10% reduction in the respective bacterial populations. No significant difference in the degree of disinfection was observed between the four disinfectants. Furuta (1982) obtained similar results when dipping contaminated transport containers used in the delivery of broilers for 2, 5, or 10 seconds in three types of disinfectant solutions: orthoderivative (orthodichlorobenzene); invert (methyl dodecyl benzyl trimethyl ammonium chloride); and amphoteric soaps (methyl dodecyl xylilena bis trimethyl ammonium chloride).

B. Chlorine-Based Disinfectants.

In 1881, the German bacteriologist Koch demonstrated that bacteria could be destroyed by hypochlorite. Five years later, the American Public Health Association issued a favorable report on the use of hypochlorites as disinfectants (Hadfield, 1957). In 1908, Johnson introduced to North America the use of chloride of lime for water purification (Race, 1918). Since then, the use of chlorine treatment for water purification has increased rapidly in the United States. Today, it is rare to find municipal water supplies that are not chlorinated (Dychdala, 1977). Following its introduction to the water treatment industry, the use of chlorine as a disinfectant gained widespread acceptance by food industries. In the United States, chlorine and hypochlorites are accepted for use in food processing and bottled water as prescribed by the 1958 amendment to the

Federal Food, Drug and Cosmetic Act (FD&C Act) of 1938. This amendment allowed for the continued use of "generally recognized as safe" (GRAS) substances that were commonly used in the United States before 1958. In certain instances, chlorine compounds are considered as GRAS substances under the FD&C Act, the Poultry Products Inspection Act, and the Meat Inspection Act. Aqueous chlorine is used extensively in the food industry to sanitize food processing equipment and food containers (100-200 ppm), to rinse and convey raw fruits and vegetables (1-5 ppm), and to cool heat-sterilized canned foods (1-2 ppm) (Foegeding, 1983). Chlorine is also widely used by the fishing industry (Lane, 1974), in washing nutmeats (Smith and Arends, 1976), and in processing seafood (Moody, 1976) and red meats (Kotula *et al.*, 1974).

The use of chlorine as an effective disinfectant in the poultry industry is well documented (Barnes and Mead, 1971; Lillard, 1979; Wei *et al.*, 1985; Fukayama *et al.*, 1986; Tsai *et al.*, 1992). Low levels of chlorination have been effective in reducing the number of microorganisms present in chiller water and on poultry equipment surfaces. Goresline *et al.* (1951) reported that 10 ppm chlorine added to the final wash of poultry carcasses reduced the bacterial counts by 78% and that 20 ppm yielded a 1-log (90%) reduction in carcass bacterial populations. Several studies have also reported increases in poultry carcass shelf life following chilling in solutions containing 20 to 109 ppm chlorine (Ziegler and Stadelman, 1955; Mead, 1976). Barnes (1972) reported that the maintenance of at least a 50 ppm available chlorine concentration in poultry chillers can destroy almost all the bacteria present in the water, thereby reducing the possibility of bacterial cross-contamination between carcasses. Similar results were obtained when using 100 ppm or 200 ppm chlorine in poultry chillers. Furthermore, the

production of objectionable carcass off odors was not detectable at these chlorine levels. Mallman *et al.* (1959) reported that 400 ppm chlorine in the chiller water yielded broiler carcasses with lower mesophilic bacterial counts after 17 days of refrigerated storage than control carcasses chilled without chlorine, with no objectionable off flavors detected. Nilsson and Regner (1963) found that 20 ppm chlorine added to the chiller water completely inhibited the *Salmonella typhimurium* population on artificially contaminated chicken legs (10 or 100 CFU/cm^2) following a 10 minute contact time. The chlorine concentration in the chiller tank was maintained at 20 ppm ± 2-3 ppm. Following spraying poultry carcasses with a solution containing 20 ppm chlorine, *S. typhimurium* in the chiller water was reduced from $\log_{10}$ 1.67 to 1.53 after a 5 minute holding period and from $\log_{10}$ 1.06 to 1.03 after a 30 minute holding period (Thomson *et al.,* 1967). The laboratory trials conducted by Tsai *et al.* (1992) indicated that because of a high chlorine demand in poultry chiller water, dosages of 100 to 150 ppm chlorine were required to achieve a 99% reduction in the number of bacteria following a 3 to 5 minute exposure. When the exposure time was increased, the bacterial population was further reduced, although at a slower rate of inactivation than seen with shorter exposure times. Chlorine's bactericidal properties may be severely reduced or completely destroyed due to reactivity with organic materials (Barnes, 1972; Mead, 1976; Williams *et al.*, 1990). Chlorine also reacts with some nitrogenous compounds to form chloramines which are less effective than equivalent concentrations of chlorine in the form of hypochlorite (Allen, 1961).

Lillard (1979, 1980) compared the bactericidal effect of chlorine dioxide (ClO_2) and chlorine (Cl_2) added to chiller water for the decontamination of broiler carcasses. When metered directly into the chiller water where carcasses entered

the chiller, 5 ppm ClO_2 and 34 ppm Cl_2 were equally bactericidal. When introduced into the chiller as a component of the fresh-water input, 3 ppm ClO_2 and 20 ppm Cl_2 were equally effective. The use of about 86% less ClO_2 than Cl_2 would result in less potential toxicity and corrosion of processing equipment, and would provide the poultry industry with an alternative to chlorine.

Wabeck *et al.* (1968) measured the effect of 20, 40, and 60 ppm sodium hypochlorite in aqueous solutions on the development of off odors and flavors in turkey drumsticks. In addition, they determined chlorine depletion and the reduction of *Salmonella montevideo*, *Salmonella heidelberg*, *Salmonella typhimurium*, *Salmonella thompson*, and total bacterial mesophilic populations. Organoleptic changes were evaluated using turkey meat thawed four hours in 20, 40, and 60 ppm hypochlorite solutions. Odors and off-flavors were detected at the highest hypochlorite concentration. Most of the available hypochlorite was depleted within 30 minutes after initiation of the experiments. When the four strains of *Salmonella* were suspended for 30 minutes in 20 and 40 ppm hypochlorite, all strains were completely inhibited. However, the same treatments yielded only a 0.39 and 0.22 log reduction when the four *Salmonella* strains were inoculated onto the drumsticks at 10^3 CFU/cm^2. Numerous other studies have concluded that sodium hypochlorite is much more bactericidal in the presence of very low organic loads (*e.g.* purified water) compared to high organic loads (*e.g.* poultry carcasses) (Barnes and Mead, 1971; Dzialoszynska and Wojton, 1978; Guthrie, 1992; Mullerat *et al.*,1992; Tsai *et al.*, 1992).

Williams *et al.* (1990) evaluated the inhibitory activities of free chlorine (as calcium hypochlorite) and a combination of several halogens against *S. typhimurium* and an unidentified normal bacterial strain isolated from broiler

carcasses. They reported that under controlled conditions of pH (pH 7) and temperature (4° and 48°C) similar to those encountered in poultry processing, two of the compounds (3-chloro-4,4-dimethyl-2-oxazolidinone and 1,3-dichloro-4,4,5,5-tetramethyl-2-imidazolidinone) at a concentration of 50 mg/L produced a 6-log reduction in viable organisms in less than 1 minute of exposure at 48°C, whereas a third compound (1-bromo-3-chloro-4,4,5,5-tetramethyl-2-imidazolidinone) was less effective (5.6 minutes to produce a 6-log reduction).

When chlorine and chlorine-containing compounds are used properly, they are among the most useful and least damaging disinfectants available. However, chlorine compounds are corrosive and have a bleaching action. Therefore, they must be used carefully around metals and materials that may be bleached or faded, and thereby damaged. Perhaps the greatest drawback or deficiency to the use of chlorine compounds as disinfectants is that they very readily react with, and are inactivated by, any organic compounds including the target microorganisms.

III. Bacteriocins.

1. Description.

The term bacteriocin was originally applied to proteins of the colicin type produced by *Escherichia coli* and characterized by a lethal biosynthesis based on adsorption onto a specific receptor located on the cell surface. Bacteriocins are produced by many strains of gram-positive and gram-negative bacteria. Tagg *et al.* (1976) described bacteriocins produced by gram-positive bacteria as antimicrobial compounds having biologically active protein moieties, a narrow spectrum of inhibitory activity, and bactericidal to sensitive strains. However,

some well defined bacteriocins produced by gram-positive bacteria have wide spectrums of activity. Antagonistic substances of a similar nature to bacteriocins that not have been completely characterized are called 'bacteriocin-like compounds.' Bacteriocins produced by lactic acid bacteria, such as nisin, have often been associated with the term 'antibiotic.' The term antibiotic actually defines a culture-induced antagonism which cannot be attributed to organic acids, hydrogen peroxide, or bacteriocins (Klaenhammer, 1990).

Bacteriocins are ribosomally synthesized as primary or secondary metabolites and may undergo post-translational modifications. Their genetic determinants are routinely encoded by either plasmids or transposons. They vary in molecular weight from 100 Da to 40,000 Da and are classified into two molecular weight group designations, as low- and high molecular weight bacteriocins. Low molecular weight forms are generally more susceptible to trypsin digestion but less sensitive to heat inactivation (Tagg *et al.*, 1976). Some bacteriocins may be simple proteins; however, many others seem to be quite complex molecules that contain lipid and carbohydrate components in addition to protein (Tagg *et al.*, 1976; Hardy, 1982). These proteins are composed of hydrophobic cationic peptides and often contain at least one lantibiotic, a name coined to describe unusual amino acids having antimicrobial properties, such as lanthionine (alanine-S-alanine).

Bacteriocins produced by gram-positive bacteria vary greatly in their spectrum of activity. While some bacteriocins inhibit only a single or a few bacterial strains of the same species or a few species from the same genus, others have activities targeted against many strains of organisms from different genera of gram-positive bacteria (Ray, 1992a). With the addition of some cell wall-

modifying agents such as chelators, the spectrum of inhibitory activity of bacteriocins produced by gram-positive bacteria can be extended to include some gram-negative bacteria (Tagg *et al.*, 1976; Blackburn *et al.*, 1989; Stevens, 1991).

The stability of bacteriocin preparations has often been shown to decrease significantly with increased purification (Tagg *et al.*, 1976). Many peptides, however, are stable over several months of refrigeration, freezing, and freeze drying and remain biologically active under acidic pH conditions (Ray, 1992a,b). Bacteriocins are susceptible to proteolytic enzyme digestion including gastric proteinases resulting in a loss of biological activity. Except for a few bacteriocins, most are heat stable and thus suitable for inclusion into food products requiring a thermal process. The heat stability of these peptides is associated with their relative small size and hydrophobic nature.

2. Modes of Action.

Bacteriocins are generally bactericidal rather than bacteriostatic under most treatment conditions. Bacterial cells are killed in a variety of ways although most bacteriocins target the cytoplasmic membrane. Some can also damage RNA or DNA in sensitive cells (Ray, 1992a, Hurst and Hoover, 1993). In general, bacteriocins kill sensitive cells in a two stage process. The first stage corresponds to the physical adsorption of bacteriocin molecules to exposed cell envelope receptors, probably a reversible phase. No permanent physiological damage is produced during this stage and removal of the bacteriocin during this stage apparently leaves the cell intact. Within a measurable time, the second stage develops in which irreversible pathological changes are effected via specific biochemical lesions (Tagg *et al.*, 1976).

Bacteriocins in general bind to specific protein receptor(s) on the cell wall in step one followed in step two by a destabilization of the cell membrane function and an increase in the permeability of the cytoplasmic membrane to ions, especially cations. Producer strains and many resistant gram-positive bacteria adsorb bacteriocins to the cell wall with no consequences (Ray, 1992a). These protein receptors may also function as receptors for other metabolites which are transported into the cell. The release of important cations, such as K+, from bacteriocin-treated cells results in a dissipation of the proton motive force potential. This force serves to control many membrane functions such as the active transport of amino acids and sugars across the cellular membrane. These and other cell membrane functions that depend on the existence of the proton motive force are lost following bacteriocin treatment. A further consequence of the loss of membrane potential is that cellular ATP reserves are expended in an attempt to maintain the membrane potential. The decrease in the intracellular concentration of ATP inhibits many-energy dependent reactions in the cells (Hurst and Hoover, 1993). Tagg *et al*. (1976) suggested that attachment of a lethal unit of bacteriocin to its specific outer cell membrane receptor causes a reversible change that is then transmitted and amplified via the cell envelop to membrane-bound biochemical targets. If the bacterial cell membrane is understood as consisting of a complex arrangement of repeating units, then conformational changes in these units, induced by attachment of the bacteriocin, may be sequentially propagated throughout the membrane (Tagg *et al.*, 1976).

3. Current Food Applications for Bacteriocins.

One of the major reasons for the current search and interest in biopreservatives is the consumer perception that many currently used food preservatives are unsafe and unnatural (Ray, 1992a). Except for a few, most bacteriocins are heat stable, a fact that makes them of use when applied to food products prior to heat processing (Hurst and Hoover, 1993).

The antibacterial properties of bacteriocins have mostly been studied with pure cultures in bacteriological media. Only nisin produced by *L. lactis* subsp. *lactis*, pediocin AcH from *P. acidilactici* H, and pediocin PA1 from *P. acidilactici* PAC 1.0 have been extensively studied in many different food systems that include dairy, meat, fish, vegetables, wine, and beer. They have proven to be effective in preserving these foods against gram-positive spoilage and pathogenic bacteria. Many countries have approved the use of bacteriocins for the preservation of specific foods that include dairy products, meat products, bakery products and fillings, canned vegetables and soups, and baby foods. In the United States, nisin was approved as a GRAS substance for use in selected pasteurized cheese spreads to prevent the outgrowth and toxin production of *Clostridium botulinum* (Federal Register, 1988).

4. Use of Bacteriocins in Meat.

The use of controlled starter cultures or isolated bacteriocins for the extension of shelf life of fresh and vacuum packed meats has received limited attention over the last 40 years. In a study by Saleh and Ordal (1955), botulinum toxin formation in chicken a'la king (30°C, 5 days) was prevented when inoculated with a nisin-producing strain of *Streptococcus* (*Lactococcus*) *lactis*. Nisin alone failed to prevent toxin formation or growth of Clostridium, indicating

the presence of another antimicrobial substance(s) (e.g. lactic acid, hydrogen peroxide).

Much of the current literature describing the use of bacteriocins as preservatives in meat and meat products involves the fermented foods industry. Hanna *et al*. (1980) observed that the use of lactic acid bacteria in vacuum packaged beef steaks replaced the aerobic spoilage microflora and promoted an increase in shelf life. The preservative effect was attributed to: (a) lowering of pH, (b) production of lactate ions, (c) generation of hydrogen peroxide, and (d) production of antibiotic-like substances (bacteriocins).

In a similar study, Schoebitz *et al*. (1988) evaluated the influence of two lactic acid bacterial strains, added to vacuum packaged ground beef, on the survival of several contaminating bacteria including *S. aureus*, *E. coli*, and *P. fluorescens*. An inhibitory action was observed which could not be explained by an acidity effect alone but also to the production of a bacteriocin by the inoculated *Lactobacillus*.

Rayman *et al.* (1981) used nisin as a possible alternative or adjunct to nitrite in simulated cooked ham. In their study, a slurry of cooked ham was inoculated with spores of *C. sporogenes* and heated to 70°C over 2.5-3 hr. At pH values ranging from 5.7 to 6.5, 75 ppm of nisin completely prevented spoilage after incubation at 37°C for 56 days. Nisin and nitrite were reported to have an additive inhibitory effect against *C. sporogenes*. The preservative effect was also obtained at 40 ppm nisin. However, these researchers reported that nisin was unstable when stored at 4°C, and refrigerated packs stored for 56 days, and then temperature abused at 37°C, could not be relied upon to remain unspoiled.

Past research efforts have also focused on the use of starter cultures to reduce foodborne pathogens and spoilage microorganisms in fermented meats. Goepfert and Chung (1970) demonstrated a reduction in *Salmonella* during fermentation of baker sausage. Similar results have been reported for turkey sausage (Baran and Stevenson, 1975) and lebanon bologna (Smith *et al.*, 1975)

Several workers have reported on the inhibition of meat spoilage microorganisms by lactic acid bacteria. Reddy *et al.* (1970, 1975) extended the shelf life of refrigerated (7°C) ground beef and steaks by inoculating strains of *Streptococcus* (*Lactococcus*) *lactis* and *Leuconostoc citrovorum.* Roth and Clark (1975) inhibited the growth of *Microbacterium thermosphactum* in vacuum-packed meat by introducing lactic acid bacteria. Furthermore, the shelf life of poultry meat was extended by inoculation of a commercial starter culture containing *Pediococcus cerevisiae* and *Lactobacillus plantarum*. This same starter culture was also capable of inhibiting the growth of *Salmonella typhimurium* and *Staphylococcus aureus* in culture medium (Raccach *et al.* 1979).

More recently, Chung *et al*. (1989) examined the effect of nisin on attachment and growth of *Listeria monocytogenes* on fresh lean beef muscle. Nisin addition caused a delay in growth of *L. monocytogenes* and *S. aureus* of at least 1 day when held at room temperature and for more than 2 weeks when stored at 5°C. These researchers observed that nisin activity, as measured by a standard bioassay procedure, decreased very rapidly on meat held at room temperature. By 4 days the activity of nisin decreased to 4.7% of it's original value. Richter *et al*. (1989) examined the behavior of *L. monocytogenes* in summer sausage fermented with a bacteriocin produced by *Pediococcus*

acidilactici that had been previously shown to inhibit this pathogen under culture conditions. A 2 $\log_{10}$ reduction in population of *L. monocytogenes* was observed during sausage fermentation with the bacteriocin-producing strain, as compared to less than 1 $\log_{10}$ reduction in sausage fermented with a non-inhibitory strain. In related studies, Berry *et al.* (1990, 1991) conducted two studies involving the use of a bacteriocin-producing *P. acidilactici* culture to inhibit *L. monocytogenes* during the manufacture of fermented semidry sausage and to control postprocessing *L. monocytogenes* contamination of frankfurters. Both studies reported a bacteriocin-related inhibitory effect on *Listeria* growth although complete inactivation was not achieved under their experimental conditions. Foegeding *et al.* (1992) conducted a similar study to determine whether a pediocin-producing strain of *P. acidilactici* PAC 1.0 could effectively control *L. monocytogenes* in a dry fermented sausage product. Their study showed that meat fermentation in and of itself was effective in controlling *L. monocytogenes*, especially if the pH at the end of fermentation was less than 4.9. Pediocin was responsible for part of the antilisterial activity during the fermentation. Furthermore, inhibition of *L. monocytogenes* during drying was enhanced in the presence in the presence of pediocin.

Nielsen *et al*. (1990) examined the effect of a bacteriocin produced by *P. acidilactici* on the inhibition of *L. monocytogenes* associated with fresh beef. Depending upon the *Pediococcus* population, the bacteriocin reduced the levels of attached *L. monocytogenes* from 0.5 to 2.2 orders of magnitude within 2 min. Fewer *L. monocytogenes* cells were able to attach to the meat when treated with the bacteriocin prior in inoculation.

Lücke and Schillinger (1990) described the use of a bacteriocin-producing strain of *Lactobacillus sake* to control *L. monocytogenes* in refrigerated meat products. No bactericidal effect was observed in pasteurized minced meat held at 8°C although growth of *L. monocytogenes* was initially inhibited by the producer strain. Conversely, microbial inhibition attributed to a bacteriocin was observed in comminuted cured pork (pH 5.7) stored at 15°C. *Listeria* populations were reduced approximately 1 log cycle. This effect, however, was not observed in pork at pH 6.3 to 6.4. Nettles and Siragusa (1993) evaluated the use of nisin combined with chelating agents to inhibit gram-negative pathogens attached to lean beef tissue. Less than 1 log reduction was detected against a *Salmonella typhimurium* test strain following a 15 min (25°C) dip in a treatment composed of nisin and EDTA.

Rozbeh et al. (1993) tested the efficacy of several biopreservatives including pediocin Ach (1,400 AU/g), nisin (1,400 AU/g), and Nisaplin (500 IU/g) on refrigerated vacuum-packaged beef. All three biopreservatives exerted an immediate bactericidal effect on *Leuconostoc*-inoculates. Furthermore, all biopreservatives kept bacterial populations in vacuum-packaged beef within $\log_{10}$ 6 CFU/g for up to 8 weeks at 3°C. These researchers demonstrated that spoilage of red meat products due to growth of bacteria can be reduced by treating meat with bacteriocins. However, they reported that proper techniques must be developed to uniformly apply small volumes of antibacterial metabolites (i.e. bacteriocins) to fresh meat.

IV. Nisin.

1. Production.

Nisin is a bacteriocin produced by *Lactococcus lactis* subsp. *lactis* during the exponential phase of growth (Hurst and Dring, 1968; Buchman *et al.*, 1988). The release of nisin from the cell into the propagation medium is dependent upon the pH of this environment (Hurst and Kruse, 1972). At pH < 6, more then 80% of the nisin produced is released into the growth media. While at pH > 6, the majority of nisin remains associated within the cell. In addition, an increased lag growth phase yields an increase in nisin production (Hurst and Dring, 1968).

2. Structure.

Nisin is a pentacyclic protein composed of 34 amino acids. The molecular weight of nisin is 3,354 daltons, although it usually occurs in dimers or tetramers. Due to its characteristic lanthionine bridges and thioether amino acids (lanthionine and ß-methyllanthionine), nisin is classified as a lantibiotic. The nisin molecule has a net positive charge due to a high proportion of basic amino acids. Nisin's complete structure was elucidated by Gross and Morell (1971) and was later confirmed by chemical synthesis (Fukase *et al.*, 1988) and DNA sequencing (Hansen, 1993).

The solubility of nisin is 57 mg/ml at pH 2 and 0.25 mg/ml at pH 8 (Liu and Hansen, 1990). At higher pH, nisin undergoes a chemical modification and loss of activity that is reversible when pH is decreased (Hurst, 1981; Liu and Hansen, 1990). At pH 2, nisin can be autoclaved without inactivation (Hurst, 1981). However, as pH increases there is a corresponding decrease in stability and solubility (Hurst, 1981; Liu and Hansen, 1990). Purified nisin contains

approximately 4×10^7 IU/g (Frazer *et al.*, 1962). Forty international units (IU) are equivalent to 1 μg/ml of pure nisin (Hurst, 1981), however, specific activity is dependent on the purity of the preparation.

3. Mode of Action.

In general, nisin does not inhibit molds, yeasts, or gram-negative bacteria, but has a wide spectrum of inhibitory activity against spores during outgrowth and gram-positive vegetative cells including lactococci, staphylococci, clostridia, and *Listeria monocytogenes* (Mattick and Hirsh, 1944; Lipinska, 1977; Hurst, 1981; Klaenhammer, 1988). From the lack of cited literature, it is obvious that more research is needed to elucidate the mechanisms of action of nisin towards bacterial spores and vegetative cells.

A. Spore Inactivation.

For some spores, nisin exerts a sporicidal effect while in others nisin acts as a sporostat. The sporocidal activity of nisin depends on environmental conditions, nisin concentration, spore loads, population dynamics, and molecular kinetic factors. The number of nisin molecules required to inhibit individual spores is unknown. Furthermore, the number of nisin receptor sites associated with the spore coat is unknown.

Spores undergo three developmental stages during germination: germination swelling; pre-emergence swelling; and emergence and elongation (Gould, 1964). Nisin's sporicidal activity is generally confined to the clostridia, whose spores are killed during the late stage of emergence and elongation (Ramseier, 1960). The inhibitory activity observed for clostridia spores is inversely related to the concentration of spores (Hirsh and Grinsted, 1954). The

exact mechanism of inactivation of these spores is not yet understood although nisin is believed to target membrane sulfhydryl groups in newly-germinated spores (Morris et al., 1984). Presumably, the sporicidial efficacy of nisin is related to population dynamics and molecular kinetics. In theory, nisin is randomly distributed throughout the medium and thus has an equal probability for distribution and binding to the receptors. The higher the concentration of nisin, the greater the probability for attachment to a receptor. Moreover, binding of nisin molecules to spore receptor sites in excess of what is needed for inhibition would result in a reduction in the killing efficiency. Nisin molecules also bind to other nonmicrobial materials (i.e. lipids, proteins) rendering the molecule biologically inactive (Ray, 1992b).

As a sporostat, nisin prevents the germination of spores (e.g. *Bacillu s* species). It does not inhibit germination swelling but instead inhibits pre-emergence swelling. Pre-emergence swelling precedes the emergence of vegetative cells from the spore envelope, which is then followed by cell growth. This sporostatic phenomenon is especially evident in spore types that emerge from their spore coats by the exertion of internal mechanical pressures (e.g. *Bacillus subtilis*, Gould and Hurst, 1962). Bacteria that produce small-sized spores (e.g. *Bacillus subtilis*) are more sensitive to nisin than large-sized spores (i.e. *Bacillus cereus*, Gould and Hurst, 1962). Presumably the greater sensitivity of small-sized spores is related to the ratio of nisin concentration to spore surface area, to possibly more nisin binding sites, or to anatomical differences in spore structure (e.g. spore coat thickness variations).

Studies have shown that exposure of spores to heat enhances nisin's sporocidal properties. For example, addition of nisin to milk prior to heating

reduced the D-value for *Clostridium sporogenes* by 55% (Lewis *et al.* 1954). The reduction in D-values may be related to better adsorptivity of nisin onto the heat treated spores. Moreover, the heating process may injure the spores, making them more sensitive to nisin attack. Addition of trypsin to nisin reduces the lethal effect of nisin on heat-treated spores, yet trypsin by itself does not inactivate nisin (Hurst, 1981). This loss of sporicidial activity may be due to a greater binding affinity of trypsin to spore coat receptor sites than nisin or trypsin-induced degradation of proteins used as nisin receptors.

B. Vegetative Cell Inactivation.

The site of nisin's inhibitory activity against vegetative cells is the cytoplasmic membrane. Damage can range from the dissipation of the membrane potential and pH gradient, which causes a rapid efflux of amino acids, ATP, and ions from the cellular membrane, the disruption of the proton motive force, and loss of cellular biosynthesis (Ray, 1992b).

Nisin-mediated cell lysis is associated with it's ability to change the surface free energy on the cytoplasmic membrane which leads to a disturbance in cell permeability, and ultimately to cell lysis. Moreover, nisin also interacts with phospholipid components of the cytoplasmic membrane leading to an interference of membrane function (Lipinska, 1977). Since nisin is positively charged, it behaves as a surface active cationic detergent with the capacity to destabilize the cytoplasmic membrane by binding membrane ions. In order to interact with the membrane nisin must first be adsorbed on the cell surface. This adsorbance is reversible for the first 15 minutes but then it becomes irreversible (Hirsh and Grinsted, 1954). Another factor that effects nisin's activity is the pH of the medium. The optimum pH for maximum activity is pH 6.5 (Hurst and Kruse,

1972). This nearly neutral charge environment increases the chances of interaction between the membrane ions and nisin. The death of the cell following the above stages is marked by cell lysis and/or release of intracellular components through the cytoplasmic membrane. As evidence of cell lysis some studies have documented a high rate of loss of amino acids from the cytoplasm following nisin treatment (Ruhr and Shal, 1985).

Another aspect of nisin's mode of inhibition is it's ability to interrupt the proton motive force and cellular biosynthesis (Sahl *et al.* 1987). This interruption of the proton motive force may be related to the interaction between the cationic tail of the nisin molecule and cytoplasmic membrane anions in such a way that the membrane potential is dissipated resulting in the formation of discrete size pores which leads to cell lysis and death. This hypothesis is supported by studies which documented that when an anionic detergent is added to a medium containing nisin, the bactericidal activity of nisin is lost (Ramseier, 1960). Nisin's strong affinity to anions would result in contact between nisin and the detergent's ions instead of membrane ions. The steps that occur between the adsorption of nisin onto the cell wall and it's contact with the cytoplasmic membrane have yet to be elucidated.

C. Membrane Vesicle and Liposomal Disruption.

Inhibition studies conducted using membrane vesicles have shown that nisin blocks the transport of L-proline into cytoplasmic membranes and causes a release of agglomerated amino acids from bacterial cytoplasmic membranes (Ruhr and Shal, 1985). In cytoplasmic membranes of non-bacterial sources (e.g. asolectin liposomes from soybean phospholipids), captured amino acids were not released from the membrane upon treatment with nisin (Ruhr and Shal, 1985).

However, liposomes treated with nisin showed a loss of membrane potential as well as pH gradient (Kordel *et al.* 1989). Further studies conducted with proteolipids indicated that nisin inhibits the binding of oxygen by cytochrome C (Gao *et al.* 1991). In bacterial cells, nisin may be incorporated within the membrane forming an ionic channel which leads to a lost of ions and a deterioration of the membrane potential and pH gradient.

From the above documented studies it can be concluded that the active site for nisin is on the cytoplasmic membrane. This assumption is supported by observations that undamaged gram-negative bacteria, which have an external cell membrane, are not effected by nisin. However, damage to the outer membrane of gram-negative bacteria (i.e. mechanical, chemical, physical, heat) results in sensitivity to nisin (Morris *et al.* 1984; Kordel and Sahl, 1986; Blackburn et al. 1989; Stevens *et al.* 1991).

4. Nisin + EDTA Inhibition of Gram-Negative Microorganisms.

The resistance of gram-negative bacteria to nisin is attributed to the presence of the outer membrane which acts as a permeability barrier excluding many compounds from reaching the cytoplasmic membrane and the cell interior. Gram-negative bacteria with defective outer membranes (via mutation or chemically-induced) exhibit increased permeability and sensitivity to antibiotics. The increase in outer membrane permeability to nisin is proposed to facilitate inactivation of the cell via bactericidal action at the cytoplasmic membrane (Nikaido and Vaara., 1987; Stevens, 1991).

EDTA is a strong divalent ion chelator. When gram-negative bacteria are treated with EDTA, the cell walls become more permeable to many antibiotics and dyes (Nikaido and Vaara, 1979; Hancock, 1984). Leive (1974) suggested that the

entry of antibiotics into EDTA-treated cells is due to passive diffusion. EDTA disrupts the barrier function of the outer membrane by chelating magnesium and calcium ions which serve to stabilize the lipopolysaccharide (LPS) layer. The LPS carries a large negative charge due to the presence of phosphate and carboxyl groups. Exposure to EDTA results in a loss of up to 50% of the lipopolysacharides and lipids from the outer membrane (Leive, 1974; Nikaido and Vaara, 1987; Marvin, 1989). The loss of LPS from the outer membrane leaves gaps which are believed to be filled by phospholipids, resulting in regions of phospholipid bilayer in the outer membrane (Nikaido and Vaara, 1987; Marvin *et al.*, 1989). The filling of gaps with phospholipids is believed to reestablish the permeability barrier properties of the outer membrane; however, the membrane functions with less efficiency compared to unaltered outer membranes.

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Optimization of Nisin-Based Disinfectant Formulations for Maximum Bactericidal Activity†

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ABSTRACT

The objective of this study was to maximize the bactericidal activity of nisin-based formulations against *Salmonella typhimurium*-contaminated broiler drumstick skin by varying the pH and the components of the formulation. A systematic statistical approach based on a simplex algorithm program was followed for optimizing the formulations. Treatment conditions included a constant nisin concentration (100 µg/ml) with varying solution pH (3.5-4.5), citric acid (3.0-3.45%), disodium ethylenediamine tetraacetate (EDTA) (4.52-7.45 mM), and Tween 20 (0.5-0.68%) concentrations.

After completion of optimization testing, the most efficacious bactericidal formulations were identified and further compared in broiler skin decontamination studies. Of nine optimized formulations compared, four formulations yielded significantly greater bactericidal activity (3.12-4.86 log reduction) and were composed of 100 µg/ml nisin and: 3.1% citric acid, 7.45 mM EDTA (pH 3.8); 3.1% citric acid, 5.4 mM EDTA, 0.68% Tween 20 (pH 3.7); 3.27% citric acid, 0.61% Tween 20 (pH 3.5); 3% citric acid, 5 mM EDTA, and 0.5% Tween 20 (pH 3.5). These results demonstrate the utility of applying a systematic approach to maximize the biocidal activity of a multiple component nisin-based formulation.

INTRODUCTION

The statistical design of experiments to optimize the inhibitory activity of a complex multiple ingredient disinfectant can be approached in several ways. Most experimental approaches systematically adjust the concentration of the ingredients in question in order to study their effect on the inhibitory response. Traditional optimization techniques that require considerable experimentation generally optimize a response by adjusting the concentration of only one variable at a time while all other variables are held constant. These one-variable-at-a-time strategies generally make progress towards reaching an optimum although the most inefficient paths may be followed (4, 5). Alternatively, designed experimentation is a systematic approach whereby several ingredients and their interactions are examined simultaneously, allowing the researcher to derive the greatest amount of meaningful data from the fewest number of experiments; thus saving time, resources, and expense.

A very common experimental design method is the response surface method (RSM) which use simultaneously-derived quantitative data to build an empirical model that describes the relationships between each factor or variable (independent variable) investigated and the response (dependent variable). These RSM models take into consideration the effects of each variable, interactions between and among variables, and curvature effects (e.g. two different levels of the same variable producing similar response values and higher or lower responses at intermediate variable levels). Response surface methods help explain how a system (e.g. disinfectant) will behave (bactericidal activity) when variable (disinfectant ingredients) concentrations are altered. Secondly, as a byproduct, these methods suggest the optimum concentrations of ingredients

needed for achieving specific responses. In general, RSM models require testing of many ingredient levels to increase its accuracy. Following simultaneous testing of all formulations, a prediction equation, usually in the form of a quadratic equation, is determined by multiple linear regression to describe the mathematical relationships between each ingredient. These relationships are then used to calculate optimum ingredient levels to achieve a maximum inhibitory response (4,14).

The second of the optimization categories is the EVOP (Evolutionary operations) methods. In the EVOP method a starting point (e.g. initial ingredient concentrations) is chosen and then one proceeds in steps of a predetermined size to sequentially advance toward the maximum (optimum) response point. Several EVOP methods exist including one-factor-at-a-time, steepest ascent strategy, conjugate gradient search, and simplex search algorithm. The EVOP methods are less precise in locating the exact optimum levels of the ingredients but reach the vicinity of the optimum response faster (fewer experiments) than RSMs. The EVOP methods do not generate a prediction equation. Furthermore, EVOP-based experiments are in general not replicated within each experimental step because it lowers the efficiency of the method (e.g. more time consuming, increased expense) (4, 5).

The EVOP method evaluated in this study was the simplex search algorithm. A simplex is a geometrical figure which has n+1 vertices when a response is being optimized with respect to n factors (for two factors the simplex will be a triangle). This procedure begins with the assignment of specific values to several terms. Reasonable starting concentrations or values (base points) for each ingredient in the disinfectant formulation are chosen based on knowledge or

past experience of what their optimums may be. Step sizes (ingredient concentration increments) for each ingredient are established that allow for reasonable progress towards reaching the optimum. Very small step sizes lead to slow progress while large step sizes may result in overshooting the optimum concentrations for achieving maximum inhibition. Ingredient range limits for testing (concentration or pH limitations) are assigned based on such factors as ingredient cost, solubility, legal limitations, impact on product quality, preliminary findings, etc. Finally, an end-point value is established which defines when the optimization search concludes (5,10).

With these terms defined, n+1 formulations are prepared for each experimental step, where n is equal to the number of ingredients evaluated in the disinfectant formula. These formulations constitute a simplex in space. The n+1 formulations are then simultaneously tested in standard broiler skin decontamination assays where the formulation yielding the lowest microbial kill is identified, eliminated, and a new mirror image disinfectant formulation is calculated, formulated, and tested against the retained formulas. These experimental steps are continued until the pre-established end-point criteria is satisfied. At this stage the final step is replicated three times to confirm which formulation yields the maximum inhibition (10, 14).

The objective of this study was to maximize the bactericidal activity of nisin-based formulations against *Salmonella typhimurium*-contaminated broiler drumstick skin by varying the pH and composition of the formulation. Although nisin exhibits a relatively broad spectrum of inhibitory activity against gram-positive microorganisms, it is not generally inhibitory towards gram-negative organisms. Recent studies have shown that the spectrum of nisin activity can be

extended to gram-negative bacteria (2, 17). Application of nisin in combination with the chelating agent EDTA resulted in inhibition of *Salmonella* species and other gram-negative bacteria (17). Magnesium and calcium ions present in the lipopolysaccharide layer (LPS) of the outer membrane serve to stabilize the LPS layer rendering the outer membrane resistant to the penetration of such molecules as antibiotics, detergents, dyes, and nisin (8). Chelating agents, such as EDTA, bind magnesium ions in the LPS layer and produce cells with a loss of lipopolysaccharides and lipids from the outer membrane and an increased susceptibility to these molecules by permeabilizing the outer membrane and allowing their passage to the cytoplasmic membrane, the site where nisin activity is believed to occur (11). Stevens *et al*. (17) reported that in addition to EDTA, other chelators including citric acid monohydrate, sodium phosphate dibasic, and ethylenebis (oxyethylene-nitrilo) tetraacetic acid were also inhibitory against *Salmonella* species and other gram-negative bacteria when combined with nisin. A systematic approach was followed for optimizing the formulations using a simplex algorithm optimization procedure.

MATERIALS AND METHODS

Test organism. A nalidixic acid-resistant strain of *Salmonella typhimurium* (*S. typhimurium* NAR) was obtained from Frank T. Jones, North Carolina State University, Raleigh, NC. This organism was resistant to >1000 ppm of nalidixic acid. The bacterial culture was maintained at 4°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) and transferred to fresh BHI broth 24 h prior to conducting an experiment.

Reagents. Purified nisin (Applied Microbiology Inc., New York, NY) was stored at -20°C. The nisin solutions were prepared in 0.02 N hydrochloric acid

(Fisher Scientific Co., Dallas, TX). Nisin activity was verified using *Lactococcus lactis* subsp. *cremoris* ATCC 14365 and *Listeria monocytogenes* Scott A (North Carolina State University Culture Collection, Raleigh, NC) in an inhibition assay based on that of Barefoot and Klaenhammer (1). Two chelating agents and one surfactant were tested in these studies and included disodium ethylenediamine tetraacetate (EDTA) (Fisher Scientific Co., Dallas, TX), citric acid anhydrous (citrate) (Sigma Chemical Co., St. Louis, MO), and Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma Chemical Co.).

Standard broiler skin decontamination protocol. A standard broiler skin decontamination protocol was used throughout these studies for testing each disinfectant formulation. The *S. typhimurium* NAR test strain was grown to a population density of approximately 10^{8-9} CFU/ml (OD_{600} ~0.8, mid-log phase) and a 0.1 ml volume spread evenly with a sterile L-shaped glass rod across the surface of a 5.1 cm x 5.1 cm skin sample aseptically excised from fresh, nonfrozen broiler drumsticks obtained from a local supermarket. Following inoculation the skin samples were held under a laminar flow hood for 15-minutes to allow for adsorption and attachment of the test strain followed by immersion of the inoculated skin for 30 min in 20 ml of a defined treatment solution maintained at 37°C. During exposure the treatment solutions were agitated at 150 rpm on a rotating incubator. Viable *S. typhimurium* NAR cells were enumerated from the initial culture, treatment solutions following skin immersion, and treated skin using duplicate pour plates of BHI agar supplemented with 800 ppm nalidixic acid (37°C for 48 h). Surviving cells were recovered from the skin using a rinse procedure in 20 ml of 0.1% peptone water vortexed for 60 sec. All solutions were serially diluted in 0.1% peptone water. Three skin samples were tested per

formulation with three untreated (immersed in distilled, deionized water) and inoculated skin samples serving as the controls. Three uninoculated control skin samples were tested for the presence of nalidixic acid resistant bacteria to account for any background populations in calculating log survivors of *S. typhimurium* NAR. Recovered *Salmonella typhimurium* NAR populations were reported as $\log_{10}$ survivors per ml of either the initial inoculum, treatment solution (referred to as Treatment), or skin rinse (referred to as Rinse).

Simplex search algorithm. The nisin-containing formulations contained 100 µg/ml nisin and varied solution pH, citric acid, EDTA, and Tween 20 concentrations. Since the pH variable cannot be omitted from the test formula, it was not taken into consideration in determining the total number of possible treatment combinations. Furthermore, because the biocidal activity of nisin against *Salmonella* species is dependent on the presence of chelating agents such as EDTA or citric acid (16, 17), formulations not containing EDTA or citric acid were omitted from the experimental design. The total number of possible treatment combinations that were tested are summarized in Table 1. Table 2 summarizes the concentration and pH values assigned for base point, step size, and range limits for the four variables (ingredients and pH) tested in the nisin-containing formulations. As previously indicated, n+1 test formulas were evaluated per experimental step, where n equals the total number of disinfectant components excluding nisin. For example, a two component formulation (e.g. citric acid and EDTA + nisin) would test three formulations per experimental step.

Initial formulation compositions for the first step of each search were calculated according to the equations presented in Table 3a. Following the testing of these step 1 formulations using the standard decontamination protocol,

the test formulation that yielded the least microbial inhibition on the skin (Rinse recovery) was eliminated, a new test formula was generated using equation b (Table 3), and subsequently tested in step 2 against the formulations retained from step 1. This same procedure was followed until the end-point was reached. The end-point was defined when two or more treatments within a step yielded $\log_{10}$ reductions in skin rinse populations that were 0.5 log of each other following three replications. End-point criteria was not established for treatment solution population reductions. If during any step of the search the algorithm replaced a formulation with a new formulation that was also the least inhibitory, and therefore scheduled for removal in the next step, equation c (Table 3) was used to calculate a new formula; essentially reducing the simplex in half. Following the randomized completion of the optimization search for 3, 4, and 5 component formulations (including nisin), the nine most effective nisin-containing formulations were selected from among all of the optimization searches and further compared in separate broiler skin decontamination studies. These studies were replicated three times and the average log survivors calculated on a rinse-, treatment solution-, and total treatment-effect basis were statistically compared. The total treatment effect was calculated as the sum of log CFU/ml survivors recovered from both the skin rinse and treatment solution.

General statistics. Bacterial populations were converted to $\log_{10}$ prior to statistical analysis. Formulation differences were determined by comparing each mean log survivor pair using the Student's t-test at P 0.05 (LSD) (13, 14, 15).

RESULTS AND DISCUSSION

The inhibitory activity of treatments combining 100 µg/ml nisin and varying solution pH, citric acid, EDTA, and Tween 20 concentrations on *S. typhimurium* NAR-contaminated broiler drumstick skin was optimized using the simplex algorithm design as described by Spendley et al. (14). A summary of the optimization searches for three ingredient formulations (nisin + pH and citric acid, nisin + pH and EDTA) is presented in Table 4. The search for the optimum nisin, pH, and citric acid concentration was suspended following the completion of one experimental step due to the detection of an unacceptable rubbery skin texture following the 30 min treatment. Treatment C was the most inhibitory of the three yielding a mean log reduction in *S. typhimurium* NAR skin rinse population of 5.20. Treatment B was the least inhibitory yielding a skin rinse population reduction of less than 3.83 logs. This loss of activity at higher treatment pH (pH 4.4 - treatment B) may be associated with a loss of nisin stability and solubility as treatment solution pH increased (6, 9).

The search for the optimum inhibitory treatment combining treatment pH and EDTA with nisin was completed after four experimental steps (Table 4). In step 1 three treatments (A, B, C) were formulated according to the general simplex algorithm equations (Table 3) and tested. Treatment B was the least inhibitory (3.23 log reduction) and therefore was omitted from the second step. Although treatments A and C yielded log reductions of within 0.5 log of each other, the end-point criteria was not satisfied over three replications necessitating further testing in subsequent steps. A new treatment (B-1) was formulated according to equation b in Table 3 and tested against treatments A and C in step 2. Treatment B-1, the least effective, was replaced with formula B-2 in step 3 using equation c

to calculate the formula composition (Table 3). The end-point criteria for completing this search was met after 4 steps. Treatment B-3 which consisted of 100 μg/ml nisin, 5.65 mM EDTA and a solution pH of 3.6 was the most lethal of the final three treatments evaluated (4.13 log reduction). A graphical illustration of these four steps is presented in Figure 1.

A summary of the three component treatments (nisin + pH, EDTA, citric acid; pH, EDTA, Tween 20; pH, citric acid, Tween 20) is presented in Table 5. The search for the most inhibitory treatment containing EDTA and citric acid was completed after two steps indicating that the base points and step sizes chosen for these treatment parameters were initially set near the optimum and thus the search required minimal experimentation. Treatment D yielded the largest reduction in the *S. typhimurium* NAR skin population of 4.93 logs. In previous studies Stevens *et al.* (17) demonstrated that the degree of nisin inactivation of gram-negative bacterial species was dependent on chelator type and bacterial strain. Although EDTA was consistently the most effective chelator tested, citric acid was not significantly different from EDTA for 3 of the 6 organisms tested. Both chelators were combined in this study to capitalize on their chelation properties and the acidulant properties of citric acid.

Four steps were required to optimize the treatments composed of EDTA, Tween 20, and solution pH (Table 5). Treatment A (pH 3.5, 5.0 mM EDTA, 0.50% Tween 20) was the most inhibitory of the four treatments tested in step 4. The search for the most effective treatment containing citric acid and Tween 20 ended after two experimental steps with treatment B-1 yielding a 4.66 log reduction in *S. typhimurium* NAR skin population. The surfactant Tween 20 was added to the treatment solution to reduce the surface or interfacial energies between the

treatment components (i.e. nisin, EDTA, citric acid) and *S. typhimurium* NAR cells, thereby facilitating better distribution of nisin and/or other treatment components and potentially maximizing their interaction with the bacterial cell (12). In a related study (7), the nonionic emulsifier Tween 80 was shown to increase the biocidal activity of nisin against *Listeria monocytogenes* in a high fat whole milk system. These authors reported that Tween 80 has the ability to displace proteins such as nisin from the milk fat globule leading to the restoration or retention of nisin bioactivity.

In the final search, three steps were required to optimize the five component formulations (nisin + pH, EDTA, citric acid, Tween 20, Table 6). Treatment D yielded a 5.45 log reduction in population followed by treatments E (5.14), A (4.77), C-1 (4.60), and B-1 (4.40).

The final experiment was designed to compare the efficacy of the most inhibitory formulations identified in the 3, 4, and 5 variable optimization searches. Nine formulations were selected for further testing (Table 7). When expressed as log survivors on a skin rinse basis, formulations 1, 3, 5, 6, 7, and 9 yielded significantly more inhibition than formulations 2, 4, and 8 (Figure 2a). Log survivors ranged from 0.83 (formulation 9) to 2.63 (formulation 2) log CFU/ml of skin rinse. The average number of survivors recovered from the nine treatment solutions following the 30 min exposure ranged from 1.49 (formulation 9) to 3.69 log CFU/ml (Figure 2b). Formulations 4, 5, 8, and 9 yielded significantly fewer survivors than the other five formulations. When expressed as the total effect (skin rinse plus treatment solution survivors, log CFU/ml), formulations 9 and 5 yielded significantly fewer *S. typhimurium* NAR organisms than the other formulations (Figure 2c). Based on the average initial inoculum loads,

formulations 5 and 9 resulted in a 3.89 and 4.86 log reduction in the target population, respectively. The fact that formulations containing both citric acid and EDTA (#3, #5, #9) yielded significantly fewer skin rinse survivors than those containing just EDTA (#2, #4 and #8) suggests an enhancement effect between citric acid and EDTA. Both EDTA and citric acid are strong divalent ion chelators (3).

The usefulness of the simplex algorithm procedure for optimizing the biocidal activity of nisin-based formulations was assessed by comparing the log reductions in *S. typhimurium* NAR populations on broiler drumstick skin and in treatment solutions. In general, formulations containing both chelating agents and the surfactant were more inhibitory than formulations containing only one chelating agent and/or surfactant. Moreover, lower treatment pH enhanced the biocidal activity of the nisin-based formulations. Overall, the simplex algorithm procedure provided a rapid means of optimizing the lethality of a multi-component disinfectant in response to the presence and interaction of 3, 4, and 5 formulation variables.

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Table 1. Treatment combinations evaluated in the simplex algorithm optimization search[a].

Formulation ingredients		
Citric Acid	EDTA	Tween 20
-	+	-
-	+	+
+	-	-
+	-	+
+	+	-
+	+	+

[a] +, present, -, absent in the formulation.

Table 2. Base points, step sizes, and maximum and minimum variable limits used in the simplex algorithm optimization search.

Variables	Base point	Step size	Minimum limit	Maximum limit
pH	3.5	1.0	3.5	-
Citric acid (%)	3.0	0.5	-	5.0
EDTA (mM)	5.0	2.0	-	35.0
Tween 20 (%)	0.5	0.2	-	1.5

Table 3. General equations used in the simplex algorithm optimization search.

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a) General equations used in calculating the ingredient concentration and pH values tested in step 1 of the optimization search.

Formula 1:	$(Z_1$	$; Z_2$	;... ;	$Z_n)$
Formula 2:	$(Z_1 + 0.9 \times y_1$	$; Z_2 + 0.2 \times y_2$	;... ;	$Z_n + 0.2 \times y_n)$
Formula 3:	$(Z_1 + 0.2 \times y_1$	$; Z_2 + 0.9 \times y_2$	;... ;	$Z_n + 0.2 \times y_n)$
"	"	"	"	"
Formula n+1:	$(Z_1 + 0.2 \times y_1$	$; Z_2 + 0.2 \times y_2$	;... ;	$Z_n + 0.9 \times y_n)$

Where Z = base points (concentration or pH) for each formulation component and y= step size for each formulation ingredient.

b) General equation used in calculating new ingredient concentrations or pH values for all remaining search steps.

new ingredient value = (2 × average of each ingredient value excluding the omitted value[a]) - (omitted value[a])

c) General equation used in calculating new ingredient concentrations or pH values where the simplex is reduced in size.

new ingredient value = (0.5 × Average of each ingredient value excluding the omitted value[a]) + (0.5 × omitted value[a])

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[a] The least inhibitory ingredient value. This equation is applied to each formulation ingredient.

Table 4. Experimental steps completed in the optimization of three variable formulations[a].

Step	Treatment designation	pH	EDTA mM	Citric acid %	Tween 20 %	Treatment solution[b]	Skin rinse[b]
						Log reduction	
1	A	3.5		3.00		3.18[c]	4.53[c]
1	B	4.4		3.10		< 2.83	< 3.83
1	C	3.7		3.45		3.04	5.20
1	A	3.5	5.00			5.46	3.48
1	B	4.4	5.40			4.80	3.23
1	C	3.7	6.80			4.84	4.13
2	A	3.5	5.00			5.36	4.32
2	B-1	3.5	6.40			5.36	3.52
2	C	3.7	6.80			6.33	4.38
3	A	3.5	5.00			5.43	3.55
3	B-2	3.7	5.40			5.35	3.24
3	C	3.7	6.80			5.36	3.87
4	A	3.5	5.00			3.79[c]	3.89[c]
4	B-3	3.6	5.65			5.28	4.13
4	C	3.7	6.80			3.70	3.73

[a] All formulations contained 100 µg/ml nisin.

[b] Log reduction represents the log reduction in *S. typhimurium* NAR population exposed to the specific treatment at 37°C for 30 min. in comparison to the untreated control in distilled, deionized water, n=3, 5.1cm x 5.1cm inoculated broiler drumstick skin samples per formulation.

[c] Final experimental steps were replicated three times to satisfy the end-point criteria (n=9).

Table 5. Experimental steps completed in the optimization of four variable formulations[a].

Step	Treatment designation	pH	EDTA mM	Citric acid %	Tween 20 %	Treatment solution[b]	Skin rinse[b]
						Log reduction	
1	A	3.6	5.65	3.00		2.45	2.30
1	B	4.5	6.05	3.10		3.75	5.11
1	C	3.8	7.45	3.10		3.46	4.90
1	D	3.8	6.05	3.45		3.70	4.27
2	A-1	4.5	7.38	3.45		3.15[c]	3.83[c]
2	B	4.5	6.05	3.10		5.01	4.88
2	C	3.8	7.45	3.10		6.06	4.90
2	D	3.8	6.05	3.45		5.65	4.93
1	A	3.5	5.00		0.50	4.84	4.09
1	B	4.4	5.40		0.54	4.77	4.14
1	C	3.7	6.80		0.54	4.86	3.71
1	D	3.7	5.40		0.68	4.57	4.65
2	A	3.5	5.00		0.50	4.45	3.92
2	B	4.4	5.40		0.54	3.89	3.47
2	C-1	4	3.73		0.61	4.07	2.75
2	D	3.7	5.40		0.68	4.50	3.27
3	A	3.5	5.00		0.50	4.90	5.23
3	B	4.4	5.40		0.54	5.11	4.88
3	C-2	3.9	4.48		0.59	5.06	4.56
3	D	3.7	5.40		0.68	5.17	4.21
4	A	3.5	5.00		0.50	5.38[c]	4.49[c]
4	B	4.4	5.40		0.54	5.73	3.46
4	C-2	3.9	4.48		0.59	6.06	3.16
4	D-1	4.2	4.52		0.4	5.86	3.15
1	A	3.5		3.00	0.50	2.16	3.60
1	B	4.4		3.10	0.54	1.12	2.66
1	C	3.7		3.45	0.54	2.05	3.66
1	D	3.7		3.10	0.68	2.13	3.38
2	A	3.5		3.00	0.50	2.84[c]	4.18[c]
2	B-1	3.5		3.27	0.61	3.24	4.66
2	C	3.7		3.45	0.54	2.80	3.60
2	D	3.7		3.10	0.68	2.60	3.64

[a] All formulations contained 100 μg/ml nisin.

[b] Log reduction represents the log reduction in *S. typhimurium* NAR population exposed to the specific treatment at 37°C for 30 min. in comparison to the untreated control in distilled, deionized water, n=3, 5.1cm x 5.1cm inoculated broiler drumstick skin samples per formulation.

[c] Final experimental steps were replicated three times to satisfy the end-point criteria (n=9).

Table 6. Experimental steps completed in the optimization of five variable formulations[a].

Step	Treatment designation	pH	EDTA mM	Citric acid %	Tween 20 %	Treatment solution[b]	Skin rinse[b]
						Log reduction	
1	A	3.5	5.00	3.00	0.50	3.81	5.25
1	B	4.4	5.40	3.10	0.54	1.65	3.81
1	C	3.7	6.80	3.10	0.54	4.72	4.74
1	D	3.7	5.40	3.10	0.68	3.80	4.79
1	E	3.7	5.40	3.45	0.54	3.45	4.87
2	A	3.5	5.00	3.00	0.50	6.38	3.86
2	B-1	3.5	5.90	3.23	0.59	3.88	4.22
2	C	3.7	6.80	3.10	0.54	3.07	3.35
2	D	3.7	5.40	3.10	0.68	3.50	5.30
2	E	3.7	5.40	3.45	0.54	3.43	4.48
3	A	3.5	5.00	3.00	0.50	6.25[c]	4.77[c]
3	B-1	3.5	5.90	3.23	0.59	6.55	4.40
3	C-1	3.5	4.05	3.29	0.62	4.31	4.60
3	D	3.7	5.40	3.10	0.68	4.07	5.45
3	E	3.7	5.40	3.45	0.54	4.09	5.14

[a] All formulations contained 100 µg/ml nisin.

[b] Log reduction represents the log reduction in *S. typhimurium* NAR population exposed to the specific treatment at 37°C for 30 min. in comparison to the untreated control in distilled, deionized water, n=3, 5.1cm x 5.1cm inoculated broiler drumstick skin samples per formulation.

[c] Final experimental steps were replicated three times to satisfy the end-point criteria (n=9).

Table 7. Composition of the nine optimized nisin-based formulations evaluated in the final broiler skin decontamination study.

Formulation no.	Solution pH	EDTA (mM)	Citric acid (%)	Tween 20 (%)
1	3.7		3.45	
2	3.6	5.85		
3	3.8	7.45	3.1	
4	3.5	5		0.5
5	3.7	5.4	3.1	0.68
6	3.5		3.27	0.61
7	3.5		3.00	
8	3.5	5.00		
9	3.5	5.00	3.00	0.5

Figure 1. Graphic illustration of the simplex search algorithm for optimizing a three variable formulation (nisin + EDTA and treatment solution pH) (see Table 4 for corresponding inhibitory responses for each formulation within each search step).

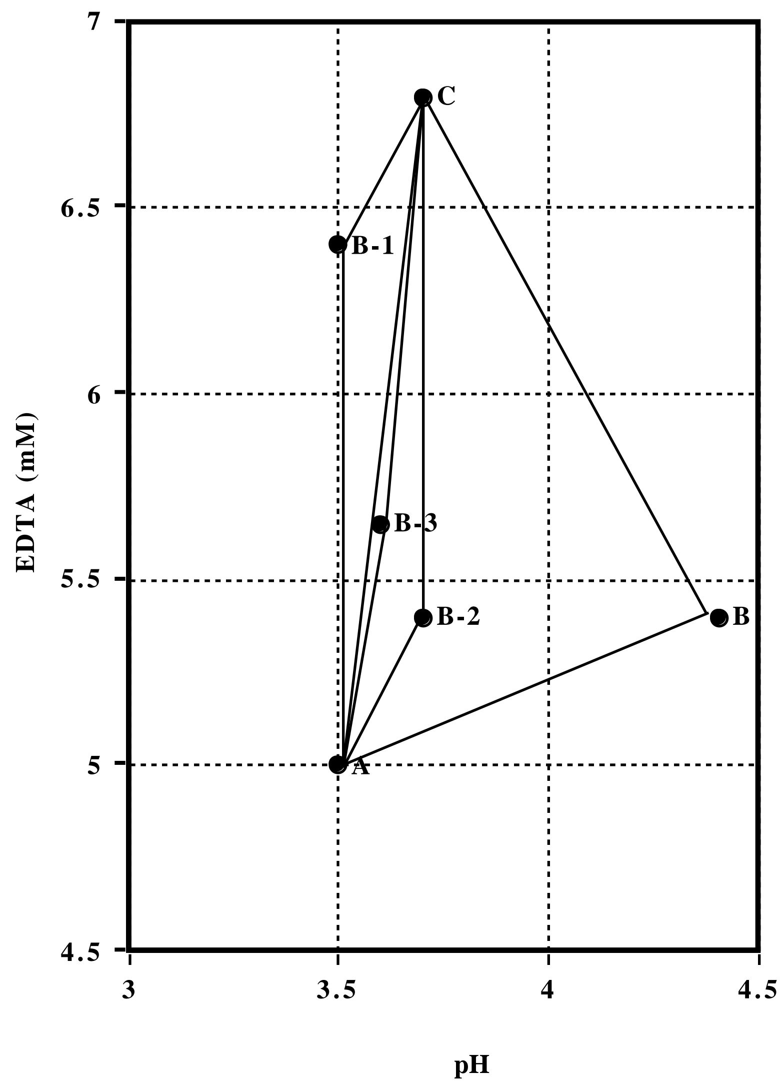
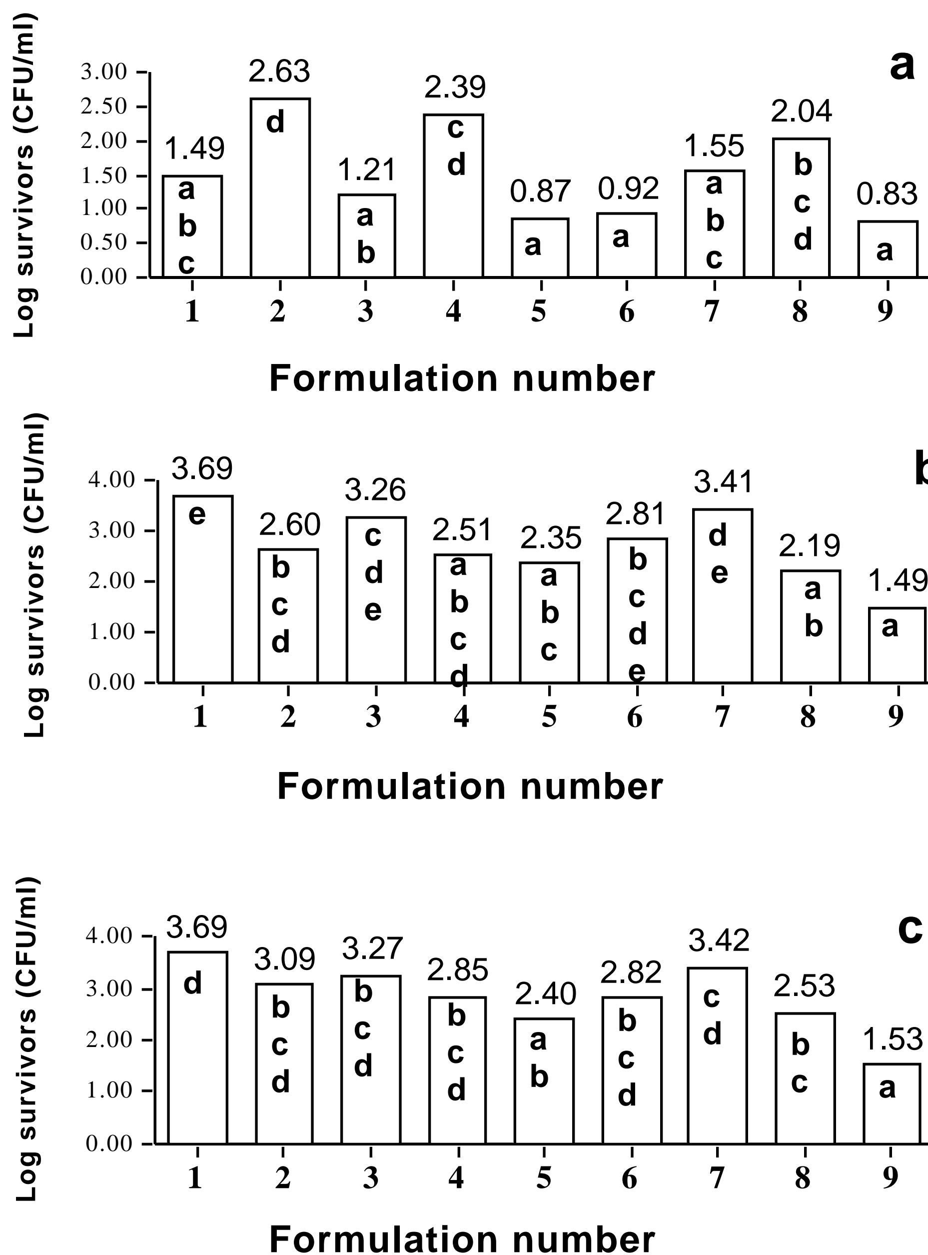


Figure 2. Log_{10} survivors following treatment (37°C, 30 min.) with nine optimized nisin-based formulations against *Salmonella typhimurium* NAR-contaminated broiler drumstick skin; a. treatment solution, b. skin rinse solution, c. total effect. Means (n=6) with different letters are significantly different (p 0.05). See Table 7 for composition of the nine formulations (initial inoculum load, log_{10} 6.39 cells/skin sample).



Efficacy of Optimized Nisin-Based Treatments to Inhibit *Salmonella typhimurium* and Extend Shelf Life of Broiler Carcasses†

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ABSTRACT

Nisin is an antimicrobial peptide produced by *Lactococcus lactis* subsp. *lactis* and exhibits a broad spectrum of inhibitory activity against gram-positive microorganisms such as *Listeria monocytogenes* and *Clostridium botulinum*. In previous studies, a method was developed using nisin in combination with food-grade chelating agents to inactivate *Salmonella* species and other gram-negative bacteria. The objectives of this study were twofold. First, determine the efficacy of several optimized nisin-containing preparations on reducing the population of *Salmonella typhimurium* NAR on broiler drumstick skin and whole drumsticks; and second, to determine if these treatments also extended the refrigerated shelf life of broiler drumsticks.

In previous studies, a simplex algorithm was used to optimize the biocidal activity of nisin towards *Salmonella* by adjusting formula pH and combining it with varying concentrations of chelating agents (EDTA, citric acid) and a surfactant (Tween 20). From these studies, four optimal treatments were identified and are further tested in the present study. Significant reductions in the viable *S. typhimurium* NAR populations on broiler drumstick skin ranged from 3.12 to 4.86 $\log_{10}$ cycles following immersion for 30 min at 25°C. The inhibitory activity of these four treatments against *S. typhimurium* NAR-contaminated drumsticks were also compared to treatments of 20 ppm chlorine. Number of survivors following a 30 minute drumstick dip ranged from 1.5 to 794 organisms per ml of skin rinse for the nisin formulations versus 131,826 organisms per ml on the chlorine-treated drumsticks. The lethality of the four nisin-containing treatments against *S. typhimurium* NAR-infected drumstick skin was also compared under varying exposure time and nisin concentrations. Depending on

the formulation, nisin concentrations could be reduced from 100 µg/ml to 50 or 25 µg/ml without loss of biocidal activity. In other studies, the refrigerated shelf life of broiler drumsticks was extended by 1.5 to 3 days following immersion for 30 minutes in one of the optimized nisin-containing treatments in comparison to drumsticks dipped in sterile distilled, deionized water. In conclusion, nisin-containing formulations effectively reduced the population of *S. typhimurium* NAR and psychrotrophic microorganisms on broiler drumsticks and compared with current chemical treatment.

INTRODUCTION

In 1992 the U.S. poultry industry produced 12 billion dollars worth of birds at the farm level, which were processed into products valued at 32 billion dollars (8). In 1993 over 27.6 billion pounds of ready-to-eat poultry products were produced (1). Although the United States has one of the world's best poultry production, processing, and inspection programs, an estimated 4,000 deaths a year are still associated with consumption of *Salmonella* and *Campylobacter*-infected poultry products (10).

Nisin is an antimicrobial peptide produced by the dairy fermentation bacterium *Lactococcus lactis* subsp. *lactis*. It exhibits a broad spectrum of inhibitory activity against gram-positive microorganisms such as *Listeria monocytogenes* and *Clostridium botulinum*, but it is not generally inhibitory towards gram-negative organisms. Recent studies have shown that the spectrum of nisin activity can be extended to gram-negative bacteria (5, 18). Application of nisin in combination with the chelating agent EDTA (disodium ethylenediamine tetraacetate) resulted in inhibition of *Salmonella* species and other gram-negative bacteria (19). Magnesium and calcium ions present in the lipopolysaccharide layer (LPS) of the outer membrane of gram-negative bacteria serve to stabilize the LPS layer rendering the outer membrane resistant to the penetration of such molecules as antibiotics, detergents, dyes, and nisin (11). Chelating agents, such as EDTA, bind magnesium ions in the LPS layer and result in a loss of lipopolysaccharides and lipids from the outer membrane and increased susceptibility of the cells to nisin. Permeabilizing the outer membrane allows passage of membrane active agents to their primary target (13). Stevens *et al*. (18) reported that in addition to EDTA, other chelators including citric acid

monohydrate, sodium phosphate dibasic, and ethylenebis (oxyethylene-nitrilo) tetraacetic acid were also inhibitory against *Salmonella* species and other gram-negative bacteria when combined with nisin.

In related studies, the bactericidal activity was maximized against *Salmonella typhimurium* NAR-contaminated broiler drumstick skin by varying the pH and composition of nisin-based formulations (16). A systematic statistical approach based on a simplex algorithm program was employed to optimize formulations. Treatment conditions included a constant nisin concentration (100 μg/ml) with varied solution pH (3.5-4.5), citric acid (3.0-3.45%), EDTA (4.52-7.45 mM), and Tween 20 (0.5-0.68%) concentrations.

This study evaluated the bactericidal activity of several optimized nisin-containing formulations against an antibiotic resistant *Salmonella typhimurium* NAR strain inoculated onto fresh broiler drumstick skin or whole drumsticks. The effects of nisin concentration and exposure time on contaminated broiler drumstick skin were determined. In addition, the effect of these treatments on the refrigerated shelf life of broiler drumsticks was evaluated.

MATERIALS AND METHODS

Test organism. A nalidixic acid-resistant strain of *Salmonella typhimurium* (*S. typhimurium* NAR) was obtained from Frank T. Jones, North Carolina State University, Raleigh, NC. This organism was resistant to >1000 ppm of nalidixic acid. The bacterial culture was maintained at 4°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) and transferred to fresh BHI broth 24 h prior to conducting an experiment.

Reagents. Purified nisin (Applied Microbiology, Inc., New York, NY) was stored at -20°C. The nisin solutions were prepared in 0.02 N hydrochloric acid

(Fisher Scientific Co., Dallas, TX). Nisin inhibitory activity was verified using *Lactococcus lactis* subsp. *cremoris* ATCC 14365 and *Listeria monocytogenes* Scott A (North Carolina State University Culture Collection, Raleigh, NC) in an inhibition assay based on that of Barefoot and Klaenhammer (3). Two chelating agents and one surfactant were used in these studies and included disodium ethylenediamine tetraacetate (EDTA) (Fisher Scientific Co., Dallas, TX), citric acid anhydrous (citrate) (Sigma Chemical Co., St. Louis, MO), Tween 20 (polyoxyethylenesorbitan monolaurate, Sigma Chemical Co.), and 20 ppm chlorine in distilled, deionized water (Fisher Scientific Co., Dallas, TX).

Standard inactivation protocol. A standard inactivation protocol was used throughout these studies for testing each disinfectant formulation. The *S. typhimurium* NAR test strain was grown to a population density of approximately 10^{8-9} CFU/ml (OD_{600} ~0.8, mid-log phase) and a 0.1 ml volume spread evenly with a sterile L-shaped glass rod across the surface of a 5.1 cm x 5.1 cm skin sample aseptically excised from fresh, nonfrozen broiler drumsticks obtained from a local supermarket. Following inoculation, the skin samples were held under a laminar flow hood for 15-minutes to allow for adsorption and attachment of the test strain followed by immersion of the inoculated skin for 30 min in 20 ml of a defined treatment solution maintained at 25°C. Four nisin-containing treatments, previously formulated (16) to optimize bactericidal activity against *S. typhimurium* NAR-infected broiler skin, were evaluated in the present study (Table 1). During exposure the treatment solutions were agitated at 150 rpm in a rotating incubator. Viable *S. typhimurium* NAR cells were enumerated from the initial culture, treatment solutions following skin immersion, and treated skin using duplicate pour plates of BHI agar supplemented with 800 ppm

nalidixic acid (37°C for 48 h). Surviving cells were recovered from the skin using a rinse procedure in 20 ml of 0.1% peptone water vortexed for 60 sec. All solutions were serially diluted in 0.1% peptone water. Three skin samples were tested per formulation with three untreated (immersed in distilled, deionized water) and inoculated skin samples serving as the controls. Recovered *Salmonella typhimurium* NAR populations were reported as $\log_{10}$ survivors per ml of either the initial inoculum, treatment solution (referred to as Treatment), or skin rinse (referred to as Rinse). Unless otherwise stated, experiments were replicated twice.

Decontamination of *S. typhimurium*-contaminated broiler drumstick skin and whole drumsticks. Several experiments were conducted to compare the bactericidal efficacy of four optimized nisin-containing formulations (Table 1) against a *Salmonella typhimurium* NAR test strain inoculated onto either broiler drumstick skin or whole drumsticks. Three 5.1 cm x 5.1 cm inoculated skin samples per treatment were each submerged for 30 min at 25°C in 20 ml of one of the following treatment solutions: (i) nisin treatment no. 1, 2, 3, or 4 (Table 1) and (ii) 20 ppm chlorine in distilled, deionized water. The standard inactivation protocol including skin inoculation procedures were followed as previously described. In similar studies involving whole broiler drumsticks, three inoculated drumsticks per treatment were submerged in 150 ml of one of the following treatment solutions maintained at 25°C: (i) nisin treatment no. 1, 2, 3, or 4 (Table 1), (ii) 20 ppm chlorine in distilled, deionized water, and (iii) distilled, deionized water. The standard inactivation protocol was followed as described earlier with the following exceptions. The whole drumsticks were artificially contaminated by suspending three drumsticks for 1 min into a 300 ml volume of a mid-log phase

culture of *S. typhimurium* NAR. Following inoculation the drumsticks were blotted with sterile absorbing paper towels to remove excess inoculum and the total number of viable *S. typhimurium* NAR organisms recovered immediately from three untreated drumsticks using a whole drumstick rinse procedure in 20 ml of 0.1% peptone water (6). The drumstick and peptone water were shaken in a sterile Whirl-Pak bag (NASCO, Fort Atkinson, WI) for 1 min to facilitate removal of the surviving microorganisms. The recovered organisms were enumerated on duplicate BHI agar pour plates supplemented with 800 ppm nalidixic acid. Following treatment, the remaining drumsticks were individually placed on standard commercial foam traypacks containing an absorbent drip pad, covered with plastic overwrap, and stored at 4°C for 24 hours. Following storage, surviving *S. typhimurium* NAR organisms were recovered from the drumsticks using the same procedures as described for the untreated control drumsticks.

Shelf life studies. The objective of this experiment was to determine the efficacy of nisin treatment no. 4 (Table 1) in extending the refrigerated shelf life of whole broiler drumsticks. Treatment no. 4 was chosen since it consistently yielded the greatest lethality towards the *Salmonella* test strain. One hundred and twenty fresh non-chilled broiler drumsticks were obtained from a local processor prior to chilling, bagged, and transported on ice (no physical contact with ice) to the laboratory (~45 min). Sixty air chilled drumsticks per treatment were submerged for 30 min in 7.2 liters (1.05 liters per kg tissue) of one of the following treatment solutions (25°C): (i) treatment no. 4 (Table 1) and (ii) sterile distilled, deionized water (pH 3.5). Following treatment and a 1 minute drip time, the drumsticks were packaged in groups of two in commercial foam traypacks, covered with plastic overwrap, and stored in a refrigerated incubator maintained

at 4°C. At specified sampling times (0, 2, 4, 5, 6, 7, 8, 9, 10, 11 days), two traypacks per treatment (n = 4) were sampled and the population of mesophilic and psychrotrophic organisms enumerated per drumstick using the same rinse procedures as described earlier for whole drumsticks. The rinse solution was pour-plated on duplicate BHI agar plates and incubated at 37°C for 48 hours for the enumeration of total mesophiles and 4°C for 10 days to estimate the psychrotrophic population. This experiment was replicated twice and the data analyzed separately.

Varying nisin concentration and exposure time. Two experiments were conducted to compare the lethality of the four optimized nisin-containing formulations, under varying nisin concentration and exposure time, in decontaminating broiler drumstick skin infected with *Salmonella typhimurium* NAR. Since the cost of nisin may be somewhat limiting at 100 µg/ml for decontaminating poultry carcasses, the efficacy of these four optimized nisin-containing treatments was evaluated at reduced nisin concentrations. Furthermore, a reduction in treatment time to less than 30 min may be more desirable for processors to reduce the overall process time. This experiment was divided into two trials.

Trial I. Three broiler drumstick skin samples per treatment were each submerged for either 1, 3, 10, or 30 min in 20 ml of nisin treatment no. 4 maintained at room temperature (25°C) (Table 1). The standard inactivation protocol was followed as described previously.

Trial II. Three skin samples per treatment were each submerged for 30 min in 20 ml of one of the following treatment solutions (25°C): (i) nisin treatment no. 1, 2, 3, or 4 at nisin concentrations of 0, 25, 50, 75, and 100 µg/ml, and (ii)

sterile distilled, deionized water (pH 3.5) (Table 1). The standard inactivation protocol was followed as described previously.

General statistics. Bacterial populations were converted to $\log_{10}$ prior to statistical analysis. Significant differences between treatment mean populations were determined by comparing each mean pair using the Student's t-test at P 0.05 (LSD) (15, 17).

RESULTS AND DISCUSSION

Decontamination of *S. typhimurium* NAR-contaminated broiler drumstick skin and whole drumsticks. The lethality of four optimized nisin-containing formulations (no. 1, 2, 3, and 4) in comparison to 20 ppm chlorine against *S. typhimurium*-contaminated broiler drumstick skin was investigated. The average inoculum load per skin sample was 1.05 x 10^7 cells. Treatments 1, 2, 3, and 4 yielded similar reductions in *S. typhimurium* NAR skin rinse populations ranging from no detectable organisms for treatment 4 to 0.51 log-cycles (3.2 CFU/ml) for treatment 1 (Figure 1a). Chlorine had only a limited effect on reducing the *Salmonella typhimurium* NAR skin rinse population. When expressed as the surviving population of *S. typhimurium* NAR recovered from the treatment solution (Figure 1b) or total effect that represents a summation of the survivors recovered from both the skin rinse and treatment solution (Figure 1c), treatment 4 was significantly more lethal than the other treatments. Less than 2 CFU/ml were recovered from the treatment 4 drumstick solution. This same pattern of microbial inactivation was also detected in the total effect category (Figure 1c). These findings indicate that the widely used disinfectant, chlorine, is considerably less effective in decontaminating poultry drumstick skin than the nisin-containing formulations. Furthermore, treatment No. 4 was significantly

more effective in decontaminating poultry skin (total effect) than the other three nisin-containing formulations. Chlorine was tested at 20 ppm since this is the USDA-accepted concentration generally used by poultry processors in whole carcass chiller tanks. Our results indicate that Cl-treatment, under these conditions, was relatively ineffective.

The next trial sought to evaluate the biocidal activity of the four nisin-containing treatments (1, 2, 3, 4), 20 ppm chlorine, and distilled, deionized water in reducing the population of *S. typhimurium* NAR on whole broiler drumsticks. The average *S. typhimurium* NAR population recovered from the untreated drumsticks was log 2.26 (182 CFU/ml) per ml of drumstick rinse. No significant differences in the drumstick rinse populations were observed between the untreated drumsticks (log 2.26) and the 20 ppm chlorine and distilled, deionized water treatments which averaged log 2.12 and 2.30 CFU/ml, respectively (Figure 2a). Significant reductions in the drumstick rinse populations were achieved with the four nisin-containing treatments which averaged 16.8 CFU/ml of rinse. These results clearly show that the nisin-containing treatments resulted in a significant reduction of the target organism drumstick population of around 1 log/ml of rinse.

Treatments 2 and 4 contained the same components at different concentrations and pH values yet treatment 4 was consistently more inhibitory toward *S. typhimurium* NAR than treatment 2. Variation in the lethality of treatments 2 and 4 may be associated with differences in the formulation pH. In an accompanying study, higher pH resulted in a loss of nisin bioactivity (12). Treatments 1 and 3 were deficient in Tween 20 and EDTA, respectively. The surfactant Tween 20 was added to the treatment formulation to reduce the surface or interfacial energies between the treatment components (e.g. nisin) and

S. typhimurium NAR cells, thereby facilitating better distribution of nisin and/or other treatment components and potentially maximizing their interaction with the bacterial cell (14). In a related study (9), the nonionic emulsifier Tween 80 was shown to increase the biocidal activity of nisin against *Listeria monocytogenes* in a high fat whole milk system. It was proposed that Tween 80 displaced nisin from milk fat globules which led to a restoration or retention of nisin inhibitory activity. Stevens *et al.* (19) suggested that the presence and concentration of fat in foods (e.g. sub-cutaneous fat associated with broiler drumstick skin), can decrease nisin activity by partitioning the molecule in hydrophobic regions where it is unavailable for action against bacteria.

Stevens *et al*. (19) reported that in addition to EDTA, other chelators including citric acid monohydrate, sodium phosphate dibasic, and ethylenebis (oxyethylene-nitrilo) tetraacetic acid were also inhibitory against *Salmonella* species and other gram-negative bacteria when combined with nisin. Although EDTA was consistently the most effective chelator tested by these authors, citric acid was not significantly different from EDTA for 3 of the organisms tested. Both chelators were combined in treatments 2 and 4 to capitalize on their chelation properties and the acidulant properties of citric acid.

Shelf life studies. The results of the replicated shelf life studies are summarized in Figures 3a and 4a for Trial 1 and Figures 3b and 4b for Trial 2. Figure 3 reports the mesophilic and psychrotrophic drumstick populations as CFU/ml of rinse, whereas Figure 4 reports data on a CFU/cm^2 basis. To estimate the total number of microorganisms per drumstick, the CFU/ml of rinse were multiplied by 20, the drumstick rinse volume, and converted to $\log_{10}$ values (20). Counts expressed on a per cm^2 of skin surface basis were derived by dividing the

total number of microorganisms per drumstick by the average surface area of a 114 g drumstick (191.0 cm^2) and converting to log_{10}. Following the 30-minute treatment (Trial 1), drumsticks submerged in treatment 4 had lower initial (day 0) mesophilic and psychrotrophic populations than drumsticks submerged in pH 3.5 distilled, deionized water (2.58 and 1.17 log reduction, respectively) (Figure 3a). The initial mesophilic bacterial population averaged 1.6×10^5 CFU/ml of rinse for the control drumsticks and 4.2×10^2 CFU/ml for the treated drumsticks. Gradual increases in the mesophilic and psychrotrophic counts were detected throughout the refrigerated storage period for both treatments. The range of mesophilic population differences between the treated and control drumsticks was 2.08 logs at day 6 and 4.31 logs at day 7. For psychrotrophs, the range of population differences between treatments was 1.17 logs at day 0 and 2.87 logs at day 7. As expected, the psychrotrophic populations were initially lower than the mesophilic populations. However, as storage time progressed, psychrotrophic populations surpassed the mesophilic counts due to the use of selective refrigeration storage temperatures.

When drumstick mesophilic and psychrotrophic bacterial populations were expressed on a per cm^2 basis (Figure 4a), the log survivor counts are about 1 log lower than the CFU/ml of rinse counts. Off odors and slime formation on broiler carcasses held at 4.4°C are first detectable when aerobic plate counts reach a population of log 8.0 CFU/cm^2 (2). However, shelf life is dependent on the initial population of microorganisms present on carcasses with lower populations yielding a longer shelf life. Other studies designed to test the efficacy of disinfectants on broiler carcasses (16) revealed that a small decrease in the initial carcass bacterial populations resulting from chlorine dioxide treatment (~ 0.5 log

reduction) lengthened shelf life by around 2 days when compared to control carcasses. In the present study, there was a 2.58 and 1.17 log reduction in the initial mesophilic and psychrotrophic count, respectively, following treatment with the nisin formulation (day 0). Based on the amount of storage time required to reach 1 x 10^8 CFU/cm^2 (2), the nisin-treated drumsticks had an estimated shelf life of around 2 (based on a psychrotrophic count comparison of treatments) to 3 days (based on a mesophilic count comparison of treatments) longer than the control drumsticks. Other studies have detected these same spoilage conditions at $\log_{10}$ aerobic counts ranging from 7.0 to 9.0 CFU/cm^2 (4, 7).

The results of the second shelf life study are summarized in Figures 3b and 4b. In this trial the initial mean control mesophilic population was about 1-log lower than observed in trial 1 (4.24 versus 5.20, respectively). Similar treatment patterns in the mesophilic and psychrotrophic counts as seen in trial 1 were detected in trial 2 (Figure 3b). Nisin-treated drumsticks had significantly lower counts throughout refrigerated storage than the water-treated drumsticks. Initially, the mesophilic and psychrotrophic counts of the nisin-treated drumsticks were reduced by 1.54 and 0.99 logs, respectively, in comparison to the control drumsticks. The estimated refrigerated storage time to reach a log 8.0 CFU/cm^2 psychrotrophic population was approximately 6.75 days for the control drumsticks and 8.5 days for the nisin-treated drumsticks (Figure 4b). Based on mesophilic counts, the projected number of days of refrigeration required to reach log 8.0 CFU/cm^2 was 8 for the controls and 9.5 days for the treated drumsticks. Thus, shelf life of the nisin-treated drumsticks was extended by 1.5 to 1.75 days in comparison to the control drumsticks. These finding clearly establish that treating drumsticks with treatment No. 4 results in an extension of product shelf life.

Varying nisin concentration and exposure time. The objective of this trial was to compare the efficacy of treatment 4 under varying exposure times (Trial I: 1, 3, 10, and 30 minutes) and nisin concentrations (Trial II: 0, 25, 50, 75, and 100 µg/ml) in decontaminating *S. typhimurium* NAR-infected broiler drumstick skin. The average inoculum load spread on each skin sample in trial I was log 6.6. Exposure time significantly influenced the lethality of treatment 4. As expected, longer exposure times yielded greater kill (Figure 5). Thirty minutes of exposure resulted in a log survivor population of 1.59. No significant difference in log survivors was detected between 3 and 10 minutes of exposure (3.01 and 2.64, respectively). One minute of exposure resulted in a 2.02 log reduction in the *S. typhimurium* NAR skin rinse population in comparison to a 5.01 log reduction after 30 minutes of exposure to the nisin formulation. Thus, exposure time may be reduced to between 10 and 3 minutes without a significant decline in lethality. This finding is important to consider when evaluating how these formulations may be applied to poultry products. Furthermore, variation in exposure times would also impact overall processing time and processing costs. In trial II, the average inoculum load spread over each skin sample was log 7.89. The results of this study indicate that the concentration of nisin can be reduced from 100 µg/ml to at least 50 µg/ml (treatments 1, 2, 3, 4, Figure 6a) without significantly affecting the lethality of the treatments against the target organism skin population. In some cases, nisin concentrations can be reduced to 25 µg/ml without loss of biocidal activity (treatment 4 - Figure 6a, treatments 2, 3, 4 - Figure 6c). These findings also indicate that the pH 3.5 water control treatment also inhibited the target organism (~ 3-log reduction) (Figure 6c). Moreover, the four test treatments deficient in nisin resulted in a 4.5 to 5 log reduction in the *S.*

typhimurium NAR population. The findings of this trial demonstrate that nisin concentrations can be significantly reduced or possibly eliminated in these four optimized treatments without loss of significant biocidal activity. Thus, a reduction in application costs would be realized through concentration reductions of nisin, the most expensive component of this formulation.

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Table 1. Composition of the four optimized nisin-based (100 µg nisin/ml) formulations.

Formulation no.	Solution pH	EDTA (mM)	Citric acid (%)	Tween 20 (%)
1	3.8	7.45	3.1	
2	3.7	5.4	3.1	0.68
3	3.5		3.27	0.61
4	3.5	5.00	3.00	0.5

Figure 1. Bactericidal effect of four optimized nisin-containing formulations in comparison to 20 ppm chlorine against *Salmonella typhimurium* NAR-contaminated broiler drumstick skin (a. Rinse solution. b. Treatment solution. c. Total effect) (initial inoculum load, $\log_{10}$ 7.02 cells/skin sample) n=6 .

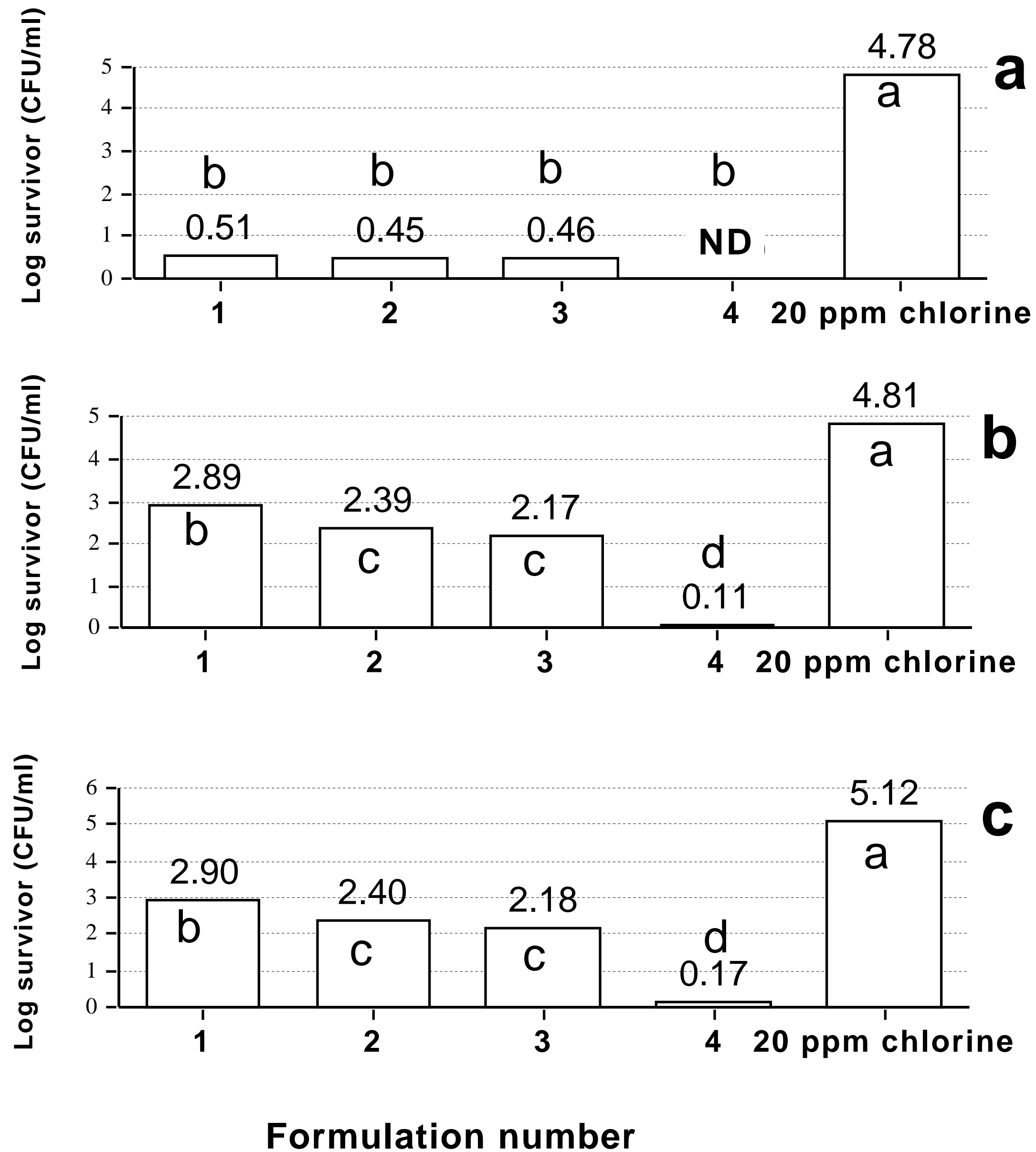


Figure 2. Bactericidal effect of four optimized nisin-containing formulations in comparison to 20 ppm chlorine and distilled deionized water against *Salmonella typhimurium* NAR-contaminated broiler drumsticks (a. Rinse solution b. Treatment solution) (initial inoculum load, $\log_{10}$ 2.26 cells/skin sample) n=6.

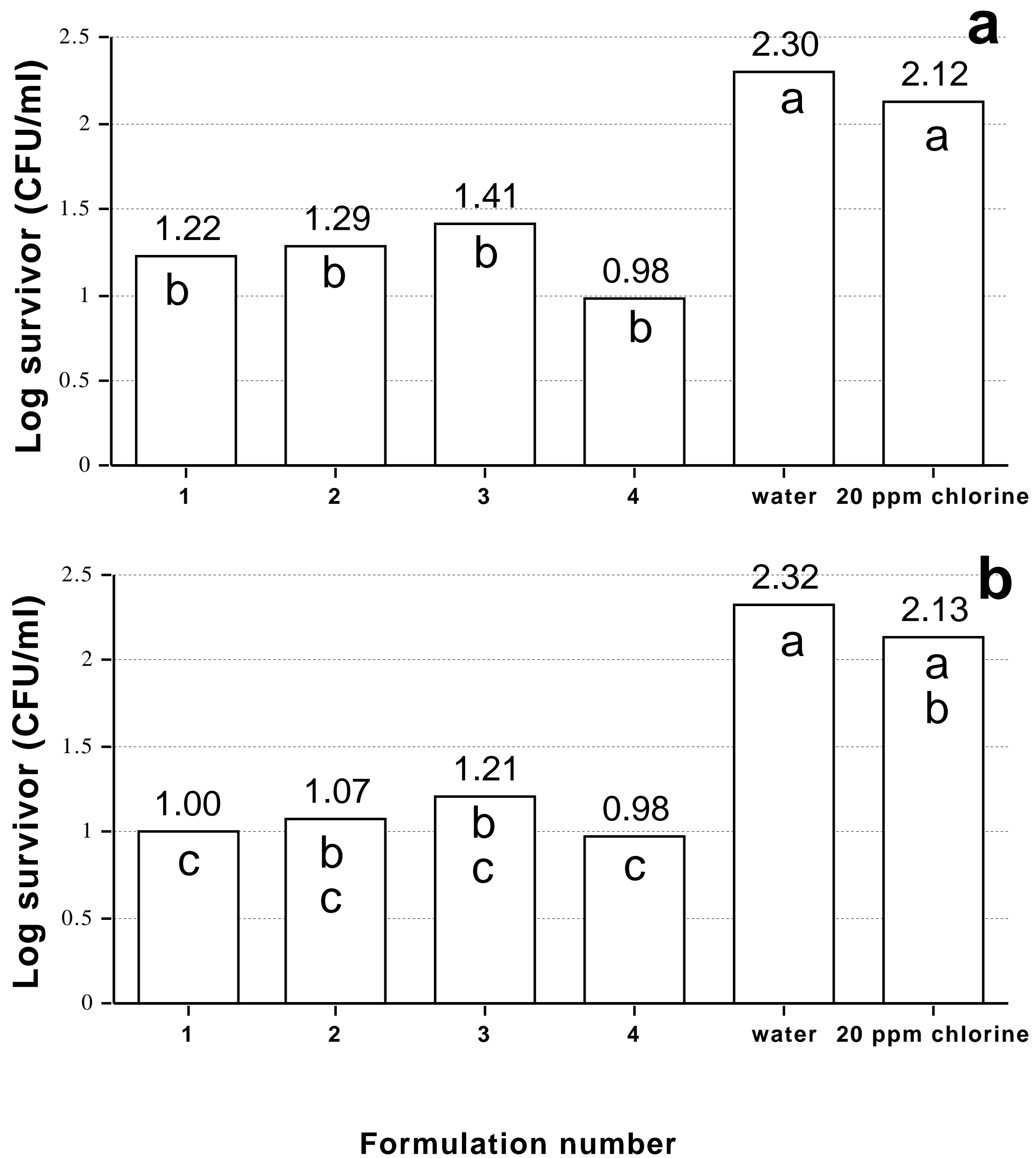


Figure 3. Effect of an optimized nisin-containing formulation (no. 4) on refrigerated shelf life of broiler drumsticks expressed as CFU/ml of drumstick rinse (a. Trial 1. b. Trial 2) n=4.

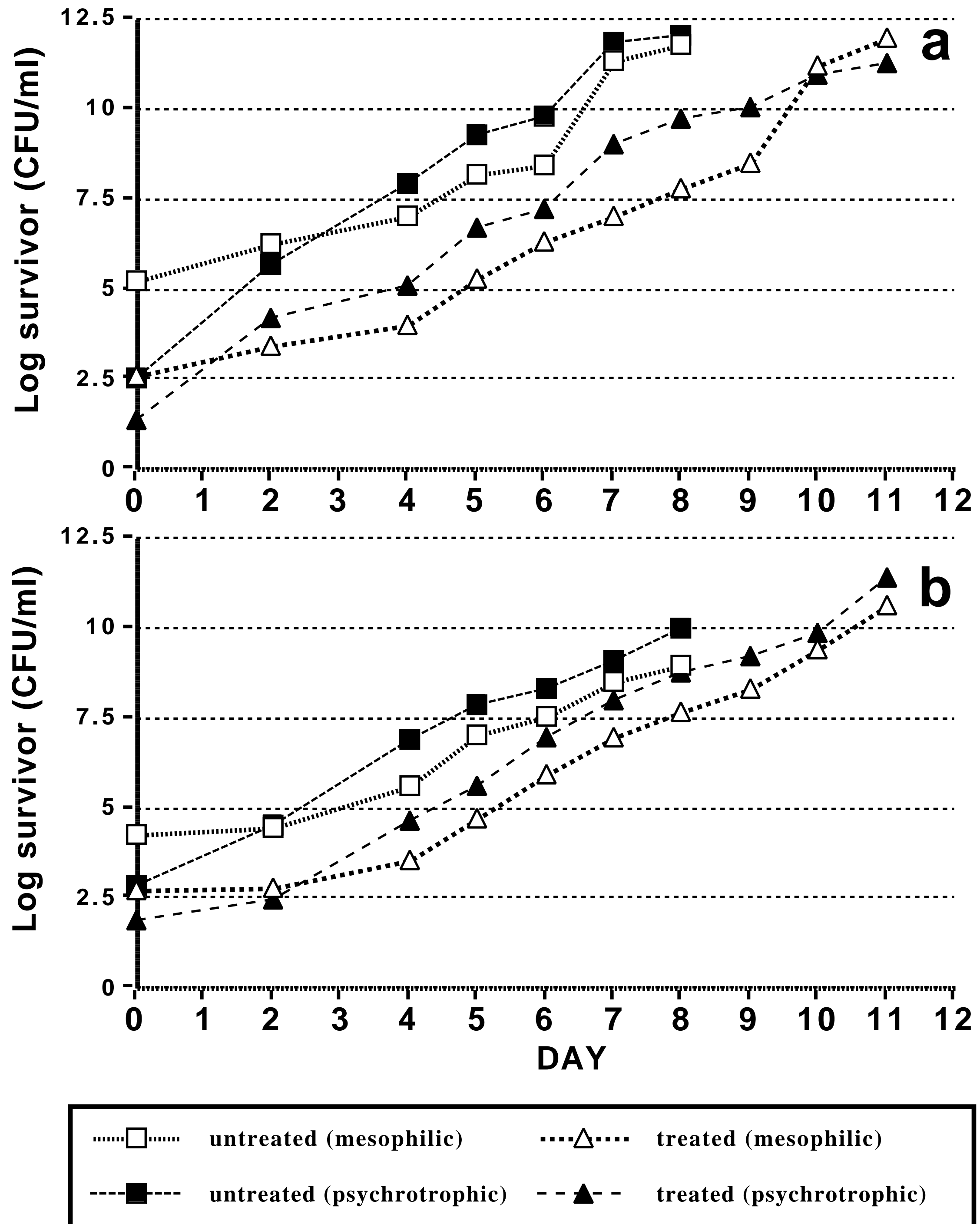


Figure 4. Effect of an optimized nisin-containing formulation (no. 4) on refrigerated shelf life of broiler drumsticks expressed as CFU/cm^2 of drumstick rinse (a. Trial 1. b. Trial 2) n=4.

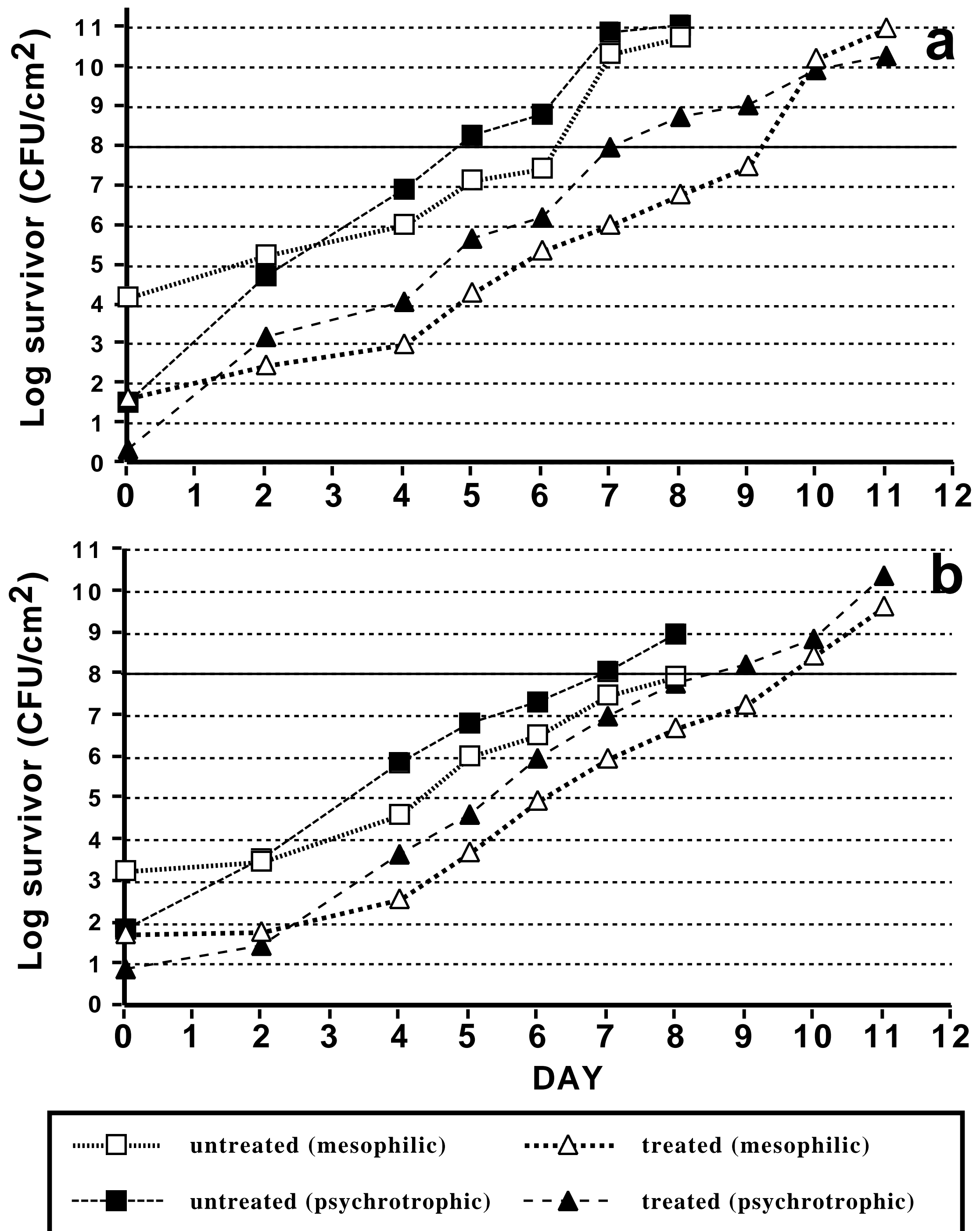


Figure 5. Bactericidal effect of an optimized nisin-containing formulation (no. 4), under varying exposure times, against *Salmonella typhimurium* NAR-contaminated broiler drumstick skin (skin rinse basis) (initial inoculum load, log_{10} 6.6 cells/skin sample) n=6.

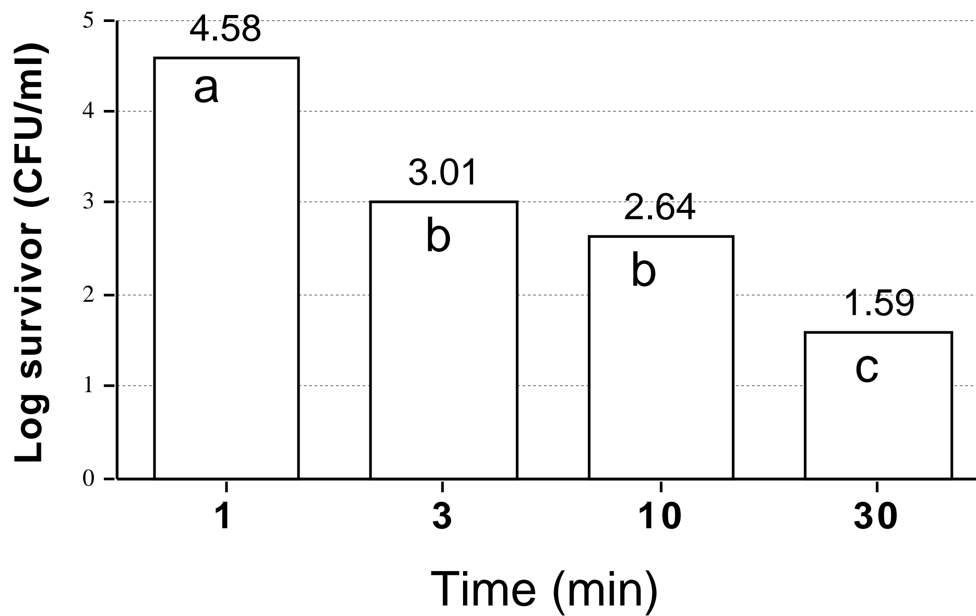
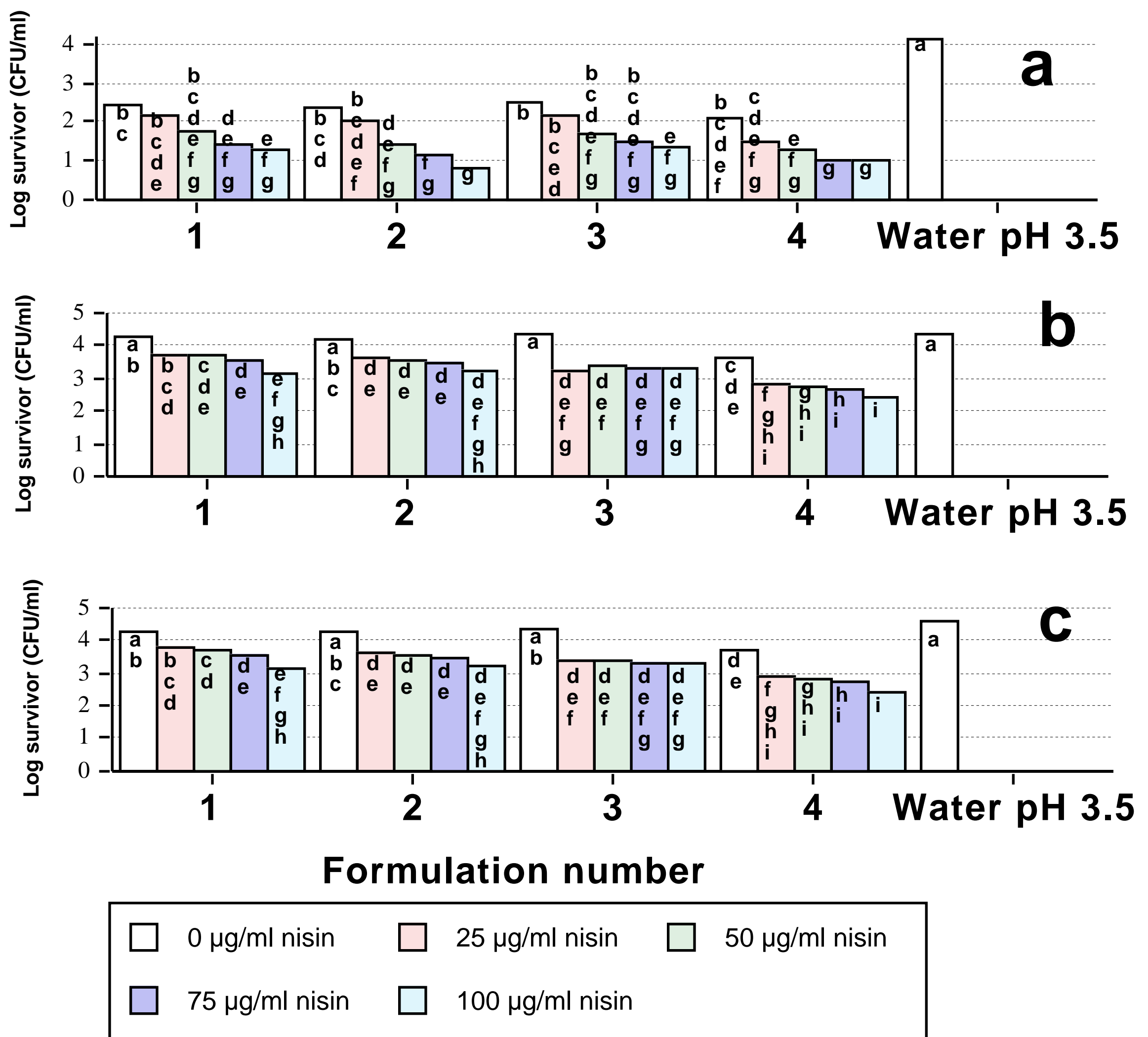


Figure 6. Bactericidal effect of four optimized nisin-containing formulations, under varying nisin concentrations, on *Salmonella typhimurium* NAR-contaminated broiler drumstick skin (a. Skin rinse population b. Treatment solution population, c. Total effect - treatment solution population + skin rinse population, CFU/ml) (initial inoculum load, $\log_{10}$ 7.89 cells/skin sample) n=6.



SUMMARY

Nisin is an antimicrobial peptide produced by the dairy fermentation bacterium *Lactococcus lactis* subsp. *lactis*. It exhibits a broad spectrum of inhibitory activity against gram-positive microorganisms such as *Listeria monocytogenes* and *Clostridium botulinum*. In previous studies from our laboratory and others, a method was developed using nisin in combination with food-grade chelating agents to inactivate *Salmonella* species and other gram-negative bacteria.

At a time of increased public interest in "natural foods" and chemical "additives", it is prudent to consider natural antimicrobials such as nisin as food preservatives. In the United States, nisin has received GRAS (generally recognized as safe) status and is approved for use in some processed cheese spreads to prevent the outgrowth of clostridial spores and toxin production. The fact that nisin has already received regulatory approval for use in some food applications should support its application for approval and use in other foods such as poultry products. Recently, the M. G. Waldbaum Co. filed a petition with the FDA seeking to affirm GRAS status for nisin as an antimicrobial agent in reduced-cholesterol liquid whole eggs (Feedstuffs staff editor, 1994).

The objectives of this study were to optimize and determine the efficacy of nisin-based treatments in reducing the population of *Salmonella typhimurium* organisms on infected broiler drumsticks, to extend the refrigerated shelf life of broiler drumsticks, and to determine the efficacy of varying exposure time and nisin concentration on inactivating *S. typhimurium* populations associated with infected broiler drumstick skin.

This study initially sought to optimize the biocidal activity of nisin towards *Salmonella* by altering formula pH and combining it with various chelating agents (EDTA, citric acid) and a surfactant (Tween 20). Using an improved simplex algorithm optimization program, four treatments were identified that significantly reduced the viable *Salmonella typhimurium* population on broiler drumstick skin by 3.12 to 4.86 logs (99.92 to 99.9998% reduction). Furthermore, a reduction in the *Salmonella* carcass-to-carcass cross contamination potential was observed following the simultaneous immersion of *S. typhimurium* free and contaminated drumsticks in nisin-containing formulations. In other experiments, the biocidal activity of these optimized nisin treatments against *S. typhimurium*-contaminated drumsticks were compared to 20 ppm chlorine, the industry standard. Log_{10} survivors of the *Salmonella* test strain following a 30 minute drumstick dip ranged from 1.5 to 794 organisms per ml of skin rinse for the nisin treatments versus 131,826 organisms per ml of the chlorine-treated drumsticks. The lethality of four nisin formulations against *S. typhimurium*-infected drumstick skin was also evaluated under varying concentrations of nisin. Nisin concentrations could be reduced depending on the formulation, from 100 μg/ml to 50 or 25 μg/ml without loss of significant biocidal activity. In other studies, the refrigerated shelf life of broiler drumsticks was extended by 1.5 to 3 days following immersion for 30 minutes in a treatment solution containing the most efficacious nisin-containing formulation in comparison to the drumsticks immersed in sterile distilled, deionized water.

These studies have demonstrated that several acidified nisin-containing formulations composed of chelating agent(s) and a surfactant are very effective in reducing the population of *Salmonella* and spoilage organisms on broiler

drumsticks and extending product shelf life. Furthermore, the absence of nisin in these treatments resulted in significant reductions of the contaminating *Salmonella* populations. Applying these nisin-based treatments as a carcass post chill application; either by dipping, spraying, or possibly by incorporation into or on the surface of primary packaging materials or edible films, might be an effective microbial control strategy for poultry processors.

As previously discussed, bacteriocin-based treatments have been successfully evaluated by other investigators in other muscle food systems. In view of these previous reports and the successful findings reported in this study, the application of bacteriocins and bacteriocin-containing treatments to food systems should be seriously considered by food processors as a means of controlling undesirable microorganisms in food products.

Although nisin was approved as GRAS nearly six years ago, it is commercially available from only one source at a cost of 300 dollars per gram of purified nisin. As techniques for the production and purification of nisin are further developed and refined and more regulatory approvals of product applications sought, the cost of nisin should decrease as market competition is encouraged, making it more economically competitive with other less effective disinfectants. In response to these cost reductions, the poultry industry may be more willing to use nisin-based treatments as part of their microbial reduction program, to assure consumer safety, enhance public confidence in poultry products, improve the overall product quality, and extend its shelf life.

Appendix 1.

A Typical Growth Curve of *Salmonella typhimurium* NAR Incubated at 37°C in BHI Broth.

Objective

To construct a typical growth curve of *Salmonella typhimurium* NAR for use in future experiments.

Materials and Methods

A nalidixic acid-resistant (>1,000 ppm) strain of *Salmonella typhimurium* (*S. typhimurium* NAR) was obtained from Frank T. Jones, North Carolina State University, Raleigh, NC. The bacterial culture was maintained at 4°C in brain heart infusion (BHI) broth (Difco Laboratories, Detroit, MI) and transferred to fresh BHI broth 24 h prior to conducting each experiment. One hundred microliters of the 24 h culture were transferred into 100 ml of BHI broth and incubated at 37°C for 390 min. The cultured was sampled at 10 min increments for determining optical density (at OD_{600}) and viable cell population using duplicate pour plates of BHI agar supplemented with 800 ppm nalidixic acid (37°C for 48 h).

Results and Discussion

The results are based on the average of 6 runs (3 replications per two experiments). The data are presented in Table A1-1 and graphically displayed in Figure A1-1. Regression equations (linear and polynomial) were derived separately for the lag (0 to 150 min), log (160 to 290 min), and stationary (300 to 390 min) phases of growth and are presented in Table A1-2.

Table A1-1. Typical growth data for *Salmonella typhimurium* NAR incubated at 37°C in BHI broth (n=6).

Incubation time (min)	Log CFU/ml	O.D.$_{600}$
0	5.86	0.0037
10	5.97	0.0064
20	6.08	0.0079
30	6.19	0.0085
40	6.30	0.0088
50	6.41	0.0090
60	6.52	0.0096
70	6.63	0.0110
80	6.74	0.0136
90	6.85	0.0177
100	6.96	0.0238
110	7.07	0.0323
120	7.18	0.0435
130	7.29	0.0578
140	7.40	0.0758
150	7.51	0.0976
160	7.68	0.1197
170	7.81	0.1668
180	7.94	0.2341
190	8.07	0.3198
200	8.20	0.4221
210	8.33	0.5389
220	8.45	0.6683
230	8.58	0.8080
240	8.71	0.9559
250	8.84	1.1096
260	8.97	1.2667
270	9.10	1.4248
280	9.22	1.5811
290	9.35	1.7330
300	9.48	1.7832
310	9.42	1.8067
320	9.42	1.8273
330	9.43	1.8448
340	9.43	1.8593
350	9.44	1.8708
360	9.44	1.8793
370	9.46	1.8849
380	9.47	1.8874
390	9.48	1.8869

Figure A1-1. Typical growth curve of *Salmonella typhimurium* NAR incubated at 37°C in BHI broth.

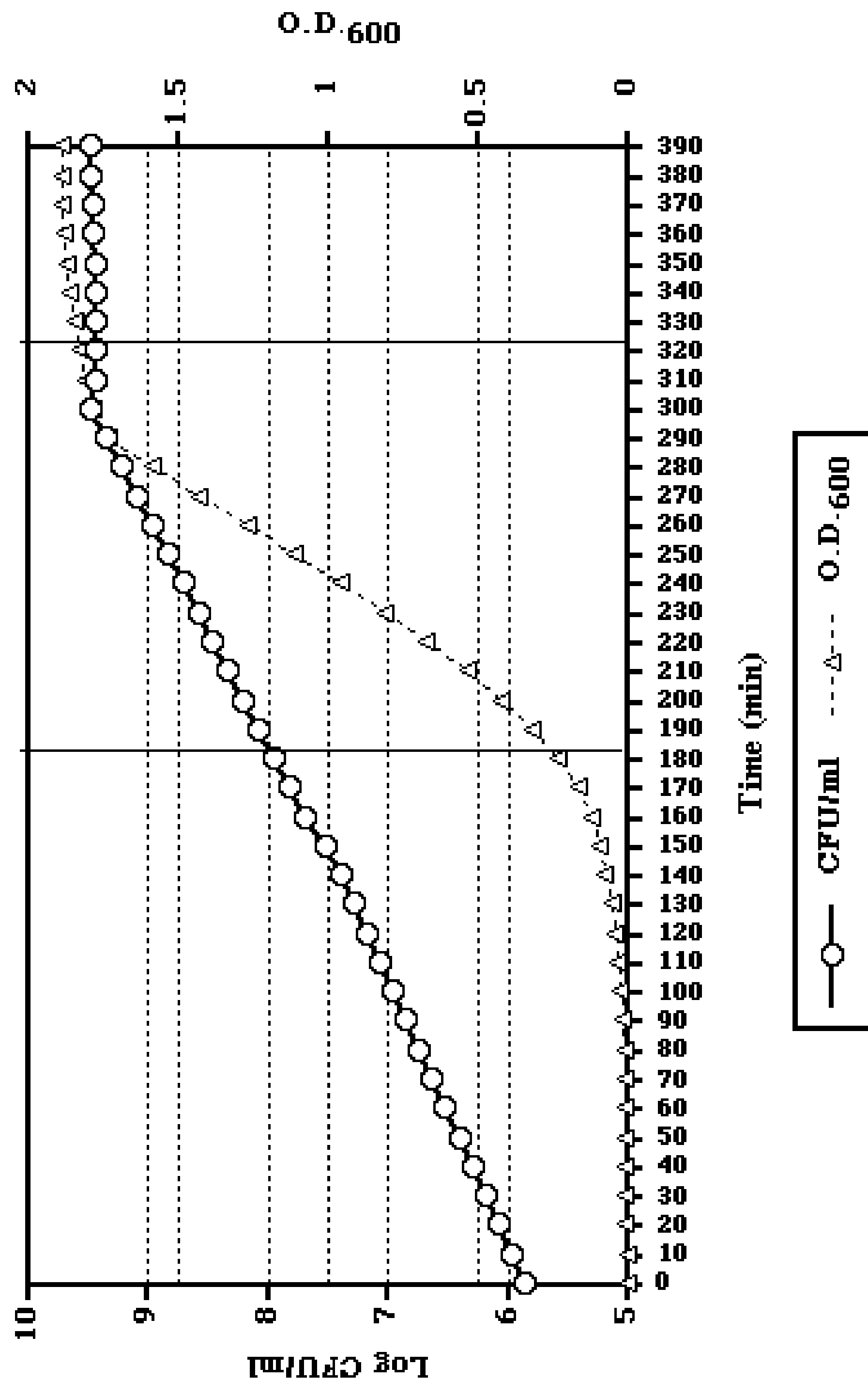


Table A1-2. Regression equations derived from growth data of *Salmonella typhimurium* NAR incubated at 37°C in BHI broth.

Growth phase	Equations
CFU/ml by time	
lag	$= 5.8606413 + 0.0110006 \times time$ (r^2=0.998)
log	$= 5.6265527 + 0.0128514 \times time$ (r^2=0.995)
stationary	$= 10.352157 - 0.005976 \times time + 0.0000096 \times time^2$ (r^2=1.0)
Optical density (OD_{600}) by time	
lag	$= 0.0037295 + 0.0003409 \times time - 0.000008 \times time^2 + 0.000000066 \times time^3$ (r^2=1.0)
log	$= 0.0129835 + 0.025418 \times time - 0.000075 \times time^2 + 5E^{-7} \times time^3 - 8.7E^{-10} \times time^4$ (r^2=0.992)
stationary	$= -0.317382 - 0.011502 \times time - 0.000015 \times time^2$ (r^2=1.0)

Regression equations (linear and polynomial) were derived separately for the lag (0 to 150 min), log (160 to 290 min), and stationary (300 to 390 min) phases of growth.

Appendix 2.

Bactericidal Effect of Varying Concentrations of Citric Acid in Combination with 100 μg/ml Nisin on *S. typhimurium* NAR-Contaminated Broiler Drumstick Skin.

Objective

The objective of this experiment was to test the bactericidal efficacy of varying concentrations of citric acid (CA) in combination with 100 µg/ml nisin against a *Salmonella typhimurium* NAR test strain inoculated on broiler drumstick skin. This preliminary study was conducted to identify the optimum inhibitory concentration range of citric acid to be used in future studies to optimize nisin-containing formulations.

Materials and Methods

Three *S. typhimurium*-inoculated broiler drumstick skin samples per treatment were each submerged for 30 min in 20 ml of one of the following treatment solutions (37°C): (i) distilled, deionized water (pH 3.5), (ii) 0.3% CA (pH 3.5), (iii) 3% CA (pH 3.5), (iv) 100 µg/ml nisin in distilled water (pH 3.5), (v) 0.3% CA and 100 µg/ml nisin (pH 3.5), and (vi) 3% CA and 100 µg/ml nisin (pH 3.5). Adjustment of pH was performed using 3 M NaOH or 12 N HCl (all chemicals supplied by Fisher Scientific Co., Dallas, TX). The standard broiler skin decontamination protocol was used throughout this study (see description on page 65).

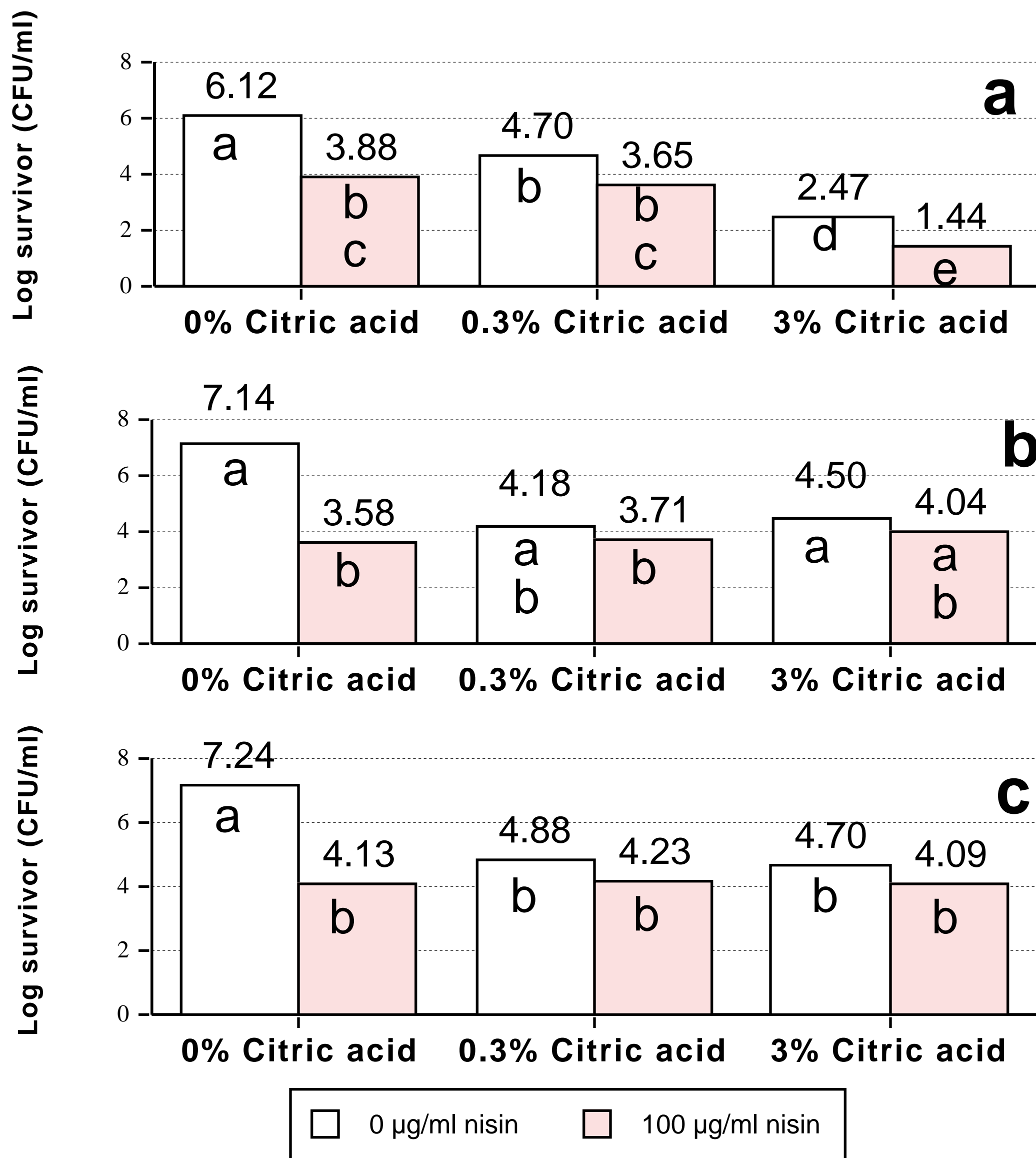
Results and Discussion

Skin samples were inoculated with approximately 4 x 10^6 cells of the test strain. The average skin mesophilic population was log 3.43. The combination of nisin and acidic pH yielded a significant reduction in the test strain population when compared to the acidic pH alone (0% CA/0 µg/ml nisin, Figure A2-1a, b, c). The addition of nisin to the pH 3.5 solutions significantly reduced the number of survivors detected in the skin rinse (Figure A2-1a, 6.12 vs. 3.88), treatment

solution (Figure A2-1b, 7.14 vs. 3.58), and total effect (Figure A2-1c, 7.24 vs. 4.13).

The presence of citric acid appeared to alter the physical characteristics of the skin as noted by elevation of feather follicles and a rubbery skin texture. The treatment containing 0 or 100 µg/ml nisin and 3% CA yielded significantly fewer survivors in the skin rinse samples (Figure A2-1a, log 2.47 and 1.44). These results indicate that both acidic pH and the presence of CA significantly influenced the inhibitory activity of nisin.

Figure A2-1. Bactericidal effect of varying citric acid and nisin concentrations against *Salmonella typhimurium* NAR-contaminated broiler drumstick skin (a. Rinse solution. b. Treatment solution. c. Total effect[1]).



1 Total effect = recovered skin rinse population + treatment solution population.

a-e Mean log reductions within a, b, or c without common superscripts are significantly different (P < .05); n=6; all replicates were within 1-log-cycle of each other.

Appendix 3.

Bactericidal Effect of Varying Nisin Concentrations in Combination with 20 mM EDTA on *Salmonella typhimurium* NAR-Contaminated Broiler Drumstick Skin.

Objective

The objective of this experiment was to determine the bactericidal effect of varying nisin concentrations in combination with 20 mM EDTA on *Salmonella typhimurium* NAR-contaminated broiler drumstick skin.

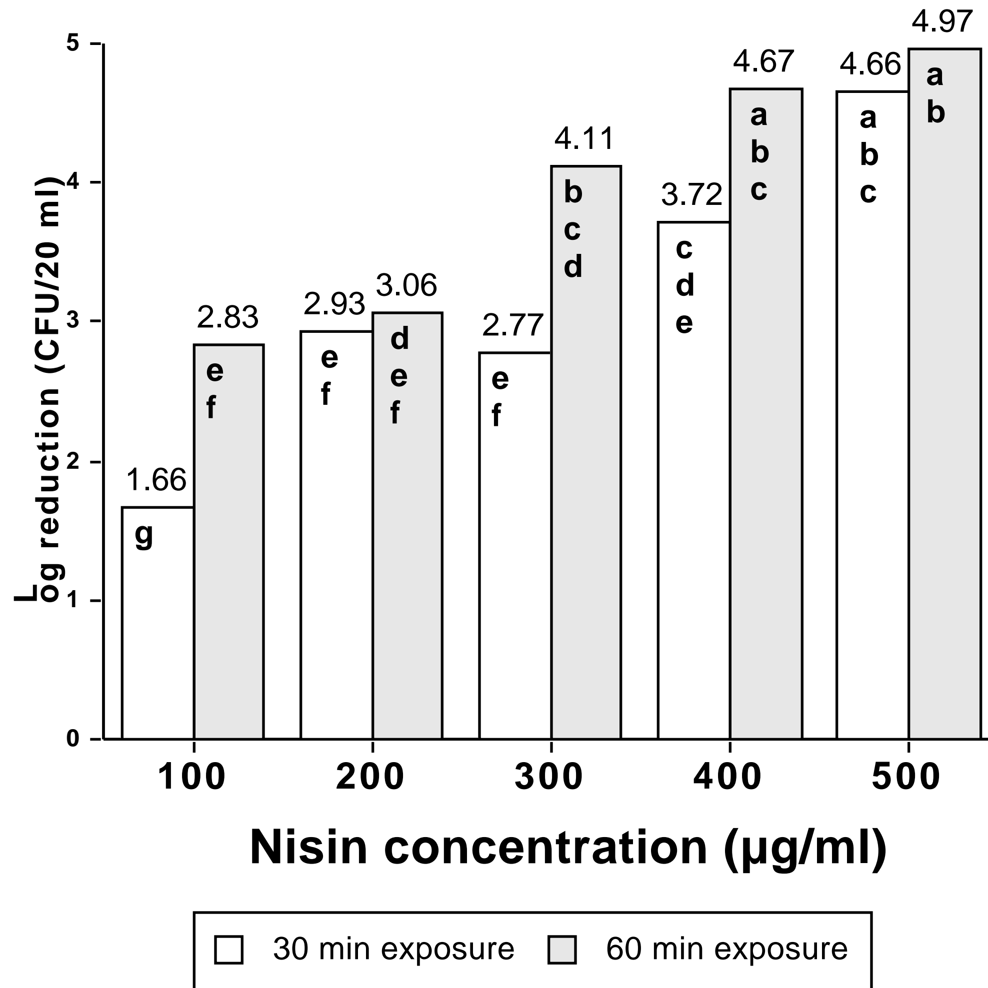
Materials and Methods

The standard broiler skin decontamination protocol was used (see description on page 65). Three *S. typhimurium* NAR-inoculated broiler drumstick skin samples per treatment were submerged for 30 or 60 min in 20 ml of one of the following treatments: (1) 20 mM EDTA in cell buffer, pH 6.5, (2) 20 mM EDTA and 100 µg/ml nisin in cell buffer, pH 6.5, (3) 20 mM EDTA and 200 µg/ml nisin in cell buffer, pH 6.5, (4) 20 mM EDTA and 300 µg/ml nisin in cell buffer, pH 6.5, (5) 20 mM EDTA and 400 µg/ml nisin in cell buffer, pH 6.5, and (6) 20 mM EDTA and 500 µg/ml nisin in cell buffer, pH 6.5. The cell buffer was composed of 50 mM Tris-HCl (pH 7.2), 1 mM $MgSO_4$, 4 mM $CaCl_2$, 0.1 M NaCl, and 0.1% gelatin. Adjustment of pH was performed using 3 M NaOH or 12 N HCl (all chemicals supplied by Fisher Scientific, Co., Dallas, TX).

Results and Discussion

In general, increasing nisin concentration resulted in increasing inhibition of the *Salmonella typhimurium* NAR test strain. This trend was detected for both exposure times. No significant difference in inhibition was observed due to exposure time at the higher nisin concentrations (200, 400, 500 µg/ml).

Figure A3-1. Bactericidal effect of varying nisin concentrations in combination with 20 mM EDTA on *Salmonella typhimurium* NAR-contaminated broiler drumstick skin[1].



[1] Mean log reduction in population was calculated relative to the control treatment (cell buffer).

[a-g] Mean log reductions without common superscripts are significantly different ($P < .05$); n=6; all replicates were within 1-log-cycle of each other.

Appendix 4.

Bactericidal Effect of 200 μg/ml Nisin Under Varying Exposure Times on *Salmonella typhimurium* NAR-Contaminated Broiler Breast and Drumstick Skin.

Objective

The objective of this experiment was to determine the influence of skin type and exposure time on the bactericidal effect of nisin in combination with EDTA on *Salmonella typhimurium* NAR-contaminated skin.

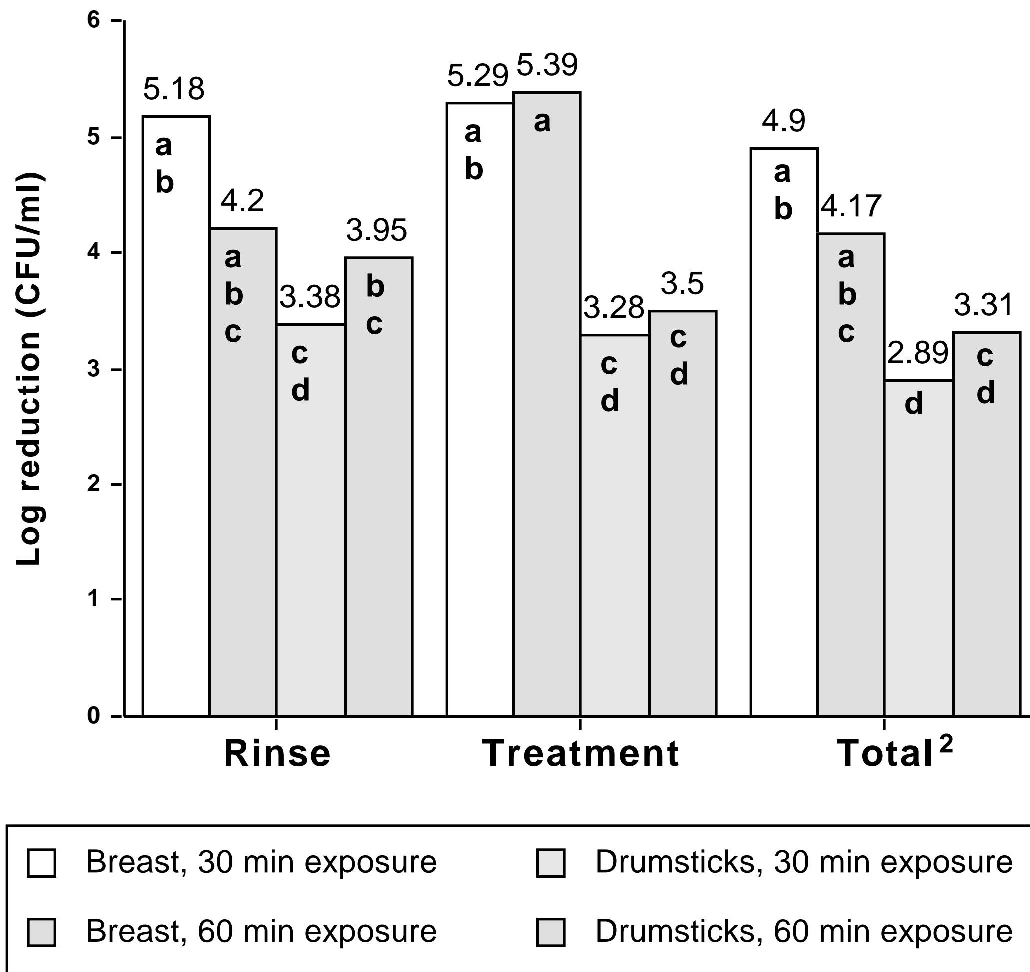
Materials and Methods

The standard broiler skin decontamination protocol was used throughout this experiment (see description on page 65). Two broiler skin types (breast, drumstick) and two exposure times (30, 60 min) were evaluated in conjunction with the following treatments (at 37°C): (1) 20 ml cell buffer, pH 6.5 (2) 200 µg/ml nisin and 20 mM EDTA in cell buffer (20 ml total), pH 6.5.

Results and Discussion

The results are based on n=6 breast skin samples (3 per rep, 2 reps) and n=33 drumstick skin samples (3 per rep, 11 reps). Exposure time within skin type did not significantly affect the inhibitory activity of nisin and EDTA. However, after 30 min of exposure variations in inhibitory activity were noted between skin types with breast skin tissues having higher microbial reductions than drumstick skin. These results indicate that exposure time can be limited to 30 min without a significant loss of nisin/EDTA activity. Furthermore, skin type will influence the degree of inhibition by nisin/EDTA. Based on these findings, all remaining studies were conducted using skin from drumsticks or whole drumsticks and unless otherwise noted, an exposure time of 30 min was employed.

Figure A4-1. Inactivation of *Salmonella typhimurium* NAR populations inoculated on broiler breast and drumstick skin following treatment with 200 µg/ml nisin in combination with 20 mM EDTA at 37°C[1].



[1] Mean log reduction in population was calculated relative to the control treatment (cell buffer).

[2] Total effect = skin rinse population + treatment solution population.

[a-d] Mean log reductions within the same category heading (rinse, treatment, total) without common superscripts are significantly different ($P < .05$); n=6 replications per breast skin and 33 per drumstick skin; all replicates were within 1-log-cycle of each other.

Appendix 5.

Bactericidal Efficacy of Four Optimized Nisin-Containing Treatments in Comparison to Chlorination in Preventing Cross-Contamination Between *Salmonella typhimurium* NAR-Contaminated and Noncontaminated Broiler Drumsticks.

Objective

The objective of this experiment was to compare the bactericidal efficacy of four optimized nisin-containing formulations identified on page 104 against a chlorine control in reducing the *Salmonella typhimurium* NAR-cross contamination potential between artificially infected and noninfected broiler drumsticks.

Materials and Methods

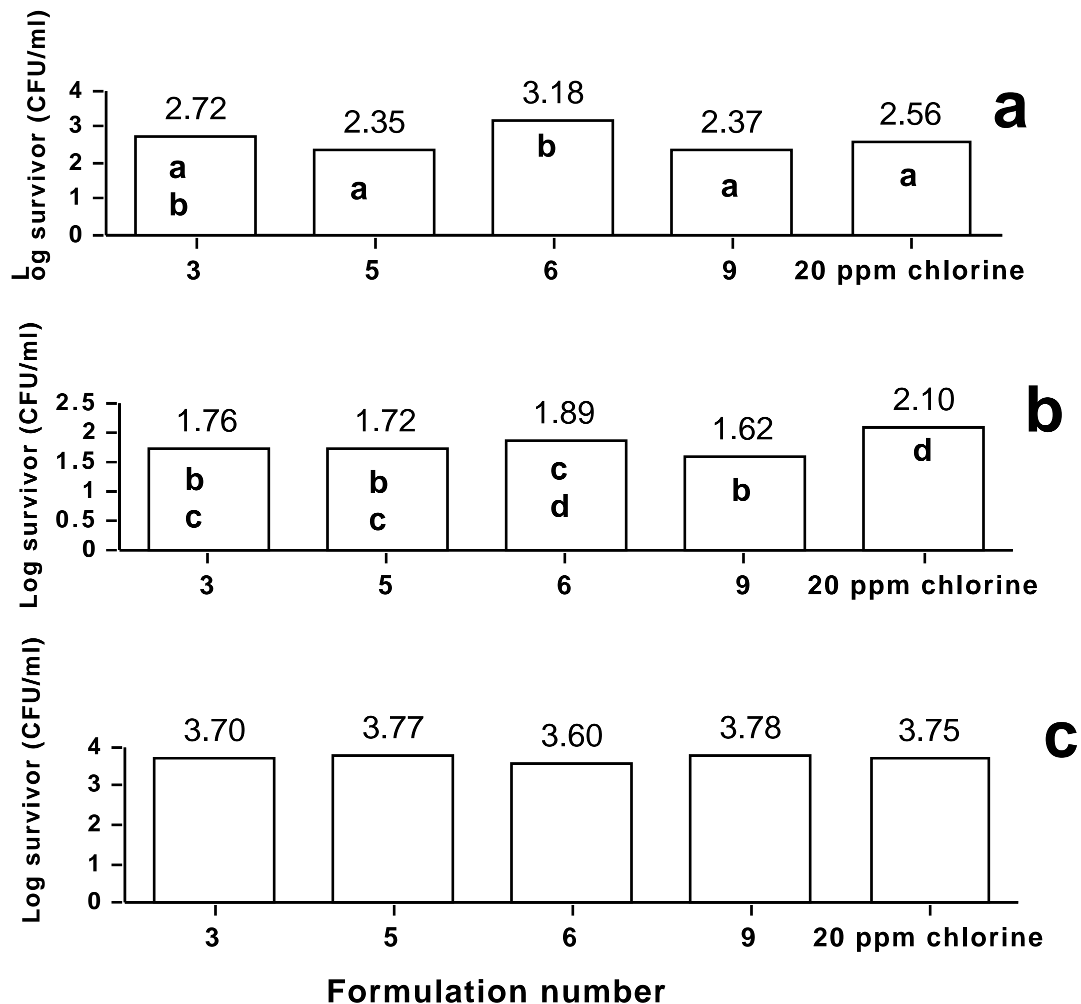
The standard broiler skin decontamination protocol was followed with exceptions (see description on page 65). Six broiler drumsticks per treatment (three *S. typhimurium* NAR-contaminated and three noncontaminated) were submerged for 1 min in 360 ml of one of the following treatment solutions (25°C): (i) Treatments 3, 5, 6, and 9 nisin-containing treatments (100 µg/ml nisin) (see page 104 for a description of each individual treatment composition), and (ii) 20 ppm chlorine. Following treatment, the pre-inoculated drumsticks were separated from the noninfected drumsticks and individually placed on standard commercial foam traypacks with absorbent pads, covered with a commercial plastic overwrap, and stored at 4°C for 24 hours. Following storage, surviving *S. typhimurium* NAR organisms were recovered from both sets of drumsticks using the same procedures as described in the standard inactivation protocol. This experiment was replicated twice.

Results and Discussion

The average *S. typhimurium* NAR population of the untreated drumsticks was log 5.47 (295,120 CFU) per ml of rinse. Although the treatments did not prevent cross-contamination between contaminated and uncontaminated

drumsticks (Figures A5-1a, A5-1b), there was a significantly lower population of *S. typhimurium* NAR organisms residing on the uncontaminated drumsticks than the contaminated drumsticks. Furthermore, in comparison to the population of target organisms recovered from the untreated, inoculated drumsticks (log 5.47), all treatments resulted in a significant reduction of the *S. typhimurium* NAR drumstick population (range of log 2.35 to 3.18). These results indicate that a 1-minute dip time is probably too short to ensure adequate bacterial inactivation and prevention of cross-contamination between drumsticks. It is also apparent that cross-contamination between carcasses can occur very rapidly. No significant differences between treatments were noted in the number of organisms recovered from the treatment solutions (Figure A5-1c).

Figure A5-1. Bactericidal effect of four optimized nisin-containing formulations in comparison to 20 ppm chlorine in preventing cross-contamination between noncontaminated broiler drumsticks and *Salmonella typhimurium* NAR-contaminated broiler drumsticks (a. Contaminated skin rinse solution. b. Noncontaminated skin rinse solution. c. Treatment solution)



[a-d] Mean log survivors within the same category (a, b, c) without common superscripts are significantly different (P < .05); n=6; all replicates were within 1-log-cycle of each other; mean log reductions in the treatment solution (c) were not significantly different.

Appendix 6.

Bactericidal Efficacy of Four Optimized Nisin-Containing Treatments in Reducing the Mesophilic Bacterial Flora of Broiler Drumstick Skin.

Objective

The objective of this experiment was to evaluate the bactericidal efficacy of four optimized nisin-containing treatments in reducing the mesophilic bacterial flora of broiler drumstick skin.

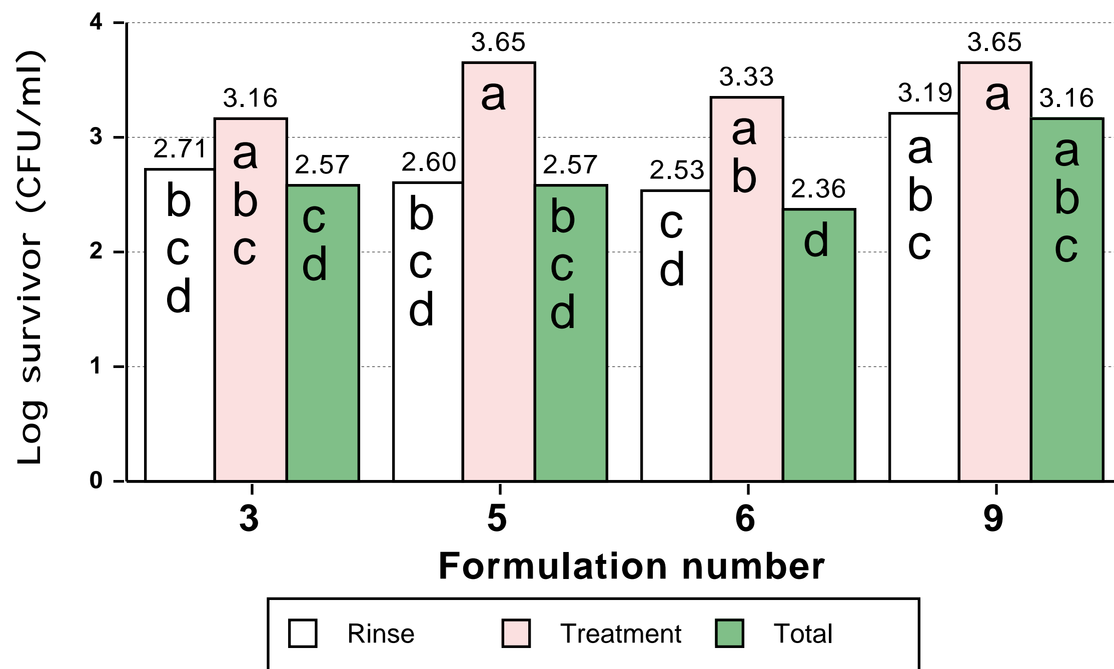
Materials and Methods

Three broiler drumstick skin samples per treatment were each submerged for 30 min in 20 ml of one of the following treatment solutions (25°C): (i) nisin treatment No. 3, (ii) nisin treatment No. 5, (iii) nisin treatment No. 6, (iv) nisin treatment No. 9, and (v) untreated skin (see page 104 for a description of each treatment composition). The standard broiler skin decontamination protocol was followed as described earlier with the following exceptions (see description on page 65). Surviving mesophilic bacteria were recovered using BHI agar pour plates without nalidixic acid. This experiment was replicated once.

Results and Discussion

The untreated drumstick mesophilic population averaged log 3.65 (4,467 CFU) per ml of skin rinse. No significant treatment differences were detected in the population of organisms recovered from the treatment solutions or from the skin rinse procedures (Figure A6-1). When expressed as the total number of surviving organisms, treatment 6 was significantly more lethal than treatment 9. No total treatment differences were noted between treatments 3, 5, and 9. These findings clearly indicate a significant reduction (2.4 to 3.2 logs) in the broiler drumstick skin heterotrophic population following treatment with the optimized nisin-containing treatments.

Figure A6-1. Bactericidal effect of four optimized nisin-containing formulations on the mesophilic flora of broiler drumstick skin.



[a-d] Mean log reductions within the same category heading (treatment, rinse, total) without common superscripts are significantly different (P < .05); n=3; all replicates were within 1-log-cycle of each other.