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Methanobactin: a potential novel biopreservative for use against the foodborne pathogen Listeria monocytogenes

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Methanobactin: A potential novel biopreservative for use against the foodborne pathogen *Listeria monocytogenes*

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ABSTRACT

Listeria monocytogenes is a ubiquitous, pathogenic foodborne bacterium, typically associated with post-processing contamination of ready-to-eat meats. Control of this pathogen has been a research topic for years, with more recent work investigating the use of novel naturally-produced antimicrobials. Methanobactin is a novel, chromopeptide recently isolated from the methanotroph, *Methylosinus trichosporium* OB3b. Its recent characterization has placed it in a new class of compounds now known as chalkophores, which are analogous to iron-binding siderophores. While it is thought to possibly serve multiple physiological roles involving copper metabolism in this bacterium, nothing is known regarding its potential as an antimicrobial. Our research focused on the use of copper-bound methanobactin (Mb-Cu) to control *L. monocytogenes*, thus the objectives of our studies were: (1) Determine the minimum inhibitory concentration (MIC) of Mb-Cu, at different pH values, and its effect on cell viability. (2) Optimize Mb-Cu activity by combining it with various surfactants. (3) Evaluate the anti-listerial efficacy of Mb-Cu, sodium lauryl sulfate (SLS), and their combination as surface treatments on frankfurters formulated with and without sodium lactate (NaL). (4) Gather preliminary evidence regarding the potential mode of action of Mb-Cu against *L. monocytogenes*. At pH 5.5 to 7.3, the MIC (4.11 mM) of Mb-Cu was lowest at pH 6.0, while the bactericidal action at the MICs ranged from 3.34- to 4.87-log reductions of the pathogen. Combination with Tween 20 or 80 lowered Mb-Cu activity while SLS enhanced it, which lowered the MIC from 2.06 to 1.03 mM. At pH 5.75, SLS (0.25%) + 1.03 mM Mb-Cu reduced *L. monocytogenes* populations by 5.33-log cycles, whereas 2-fold higher concentrations of Mb-Cu alone were

required to achieve this same reduction. On frankfurters after 24-h storage, 1% SLS + 10 mM Mb-Cu reduced *L. monocytogenes* by 1.91-2.66 log-cycles, extended the lag phase in the presence of NaL, and prevented counts from exceeding initial populations during storage at 4°C. In buffer, Mb-Cu reduced *L. monocytogenes* and inhibited respiration in a dose-dependent manner. Some leakage of UV-absorbing material was detected without cell lysis. Evidence points to the cell membrane as the potential biological target of Mb-Cu against *L. monocytogenes*.

CHAPTER 1. GENERAL INTRODUCTION

INTRODUCTION

The prevention of contamination and growth of pathogenic foodborne microorganisms is a top priority among food processors. Justifiably, relentless efforts are dedicated to prevent an outbreak of foodborne-related illness, because the cost of medical aid, decreased consumer confidence, and product recalls, will threaten a company's existence. *Listeria monocytogenes* is a well-documented, deadly (20-30% mortality rate) foodborne bacterium that poses a particular problem to the food industry and consumers, due to the high mortality rate of listeriosis, its ubiquity in nature, and ability to grow at refrigeration temperatures. It is estimated that there are ~2500 cases of foodborne listeriosis in the United States each year, and immuno-compromised persons are very susceptible to listerial infections, i.e. young, elderly, and ill populations. Unfortunately the pathogen can also infect prenatal children and result in abortion or stillbirth.

L. monocytogenes is considered a hardy organism because it can tolerate high salt concentrations, and display growth in the absence of oxygen and at refrigeration temperatures. Consumption of ready-to-eat (RTE) meats, including poultry deli meats and frankfurters has been implicated in major multi-state listeriosis outbreaks, resulting in a significant number of deaths. In addition to this organism's ability to proliferate on a number of foods, its persistence in the food processing environment makes it a potential post-processing contaminant. This, along with the uncertain infective dose and high fatality rate associated with listeriosis, led the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) to establish a "zero-tolerance" policy for its presence in

RTE meat products. In 2003, the USDA-FSIS tightened regulations even further by establishing an interim final rule, mandating food processors to choose from three alternatives. This has encouraged food processors to implement a postlethality treatment(s) to reduce or eliminate *L. monocytogenes*, and/or antimicrobial agents or processes to limit or suppress the growth of the pathogen throughout the shelf life of their products. While strict regulations are in place to control *L. monocytogenes* on RTE meat products, the consuming public currently shows a demand favoring foods that are minimally processed or that are produced to include more natural food safety measures. This is yet another major challenge for the food industry. There has been increased research interest in a variety of natural compounds, to include in hurdle technology interventions that can be used in product formulation or as surface treatments for meats by way of package incorporation, films, dips, and sprays, to control the growth of *L. monocytogenes*. The advantage of hurdle technology is that while presenting several barriers to microbial growth, lower amounts of individual antimicrobials can be used to enhance antimicrobial activity.

Organic acids and their salts have been used for years, while other biopreservative candidates for food use include plant-derived compounds, i.e. spices, essential oils, peptides, and other extracts. Also, a variety of bacterial metabolites have been evaluated for their antimicrobial properties including proteinaceous compounds such as various bacteriocins and siderophores. Siderophores are iron-chelating agents, and most of the work done on these compounds has focused on potential medical applications. Bacteriocins, however, have received a great deal of attention for their capacity to destroy certain pathogenic microorganisms in foods. Both of these classes of compounds are found

widespread in nature, however, nisin is the only bacteriocin currently allowed in foods, in its purified form. Research efforts are uncovering new and uncharacterized proteinaceous compounds that may have potential applications to control foodborne pathogenic microorganisms.

Methanobactin is a novel extracellular chromopeptide, produced by the methane-oxidizing bacterium, *Methylosinus trichosporium* OB3b, an important organism involved in global carbon cycling. This compound appears to fulfill multiple physiological roles for the producer-organism including copper sequestration and/or detoxification, as well as delivery. Its recent characterization has placed it in a new class of compounds now known as chalkophores, for fulfilling the analogous role of iron-binding siderophores. It is very difficult to compare methanobactin with other biopreservatives because it only shares similarities with groups of compounds, i.e. bacteriocins and siderophores. Methanotrophs have been examined for use in single-cell protein (SCP) production due to their ease of cultivation. Thus it seems feasible to explore the potential of this compound from an organism such as this for use as a biopreservative. There is no published research on the potential antimicrobial application of methanobactin, thus the work within this dissertation describes the antimicrobial efficacy of this naturally-produced compound against *L. monocytogenes*.

In 2004, the USDA-FSIS reported that the *L. monocytogenes* Interim Final rule had improved the safety of RTE meats and poultry, where recalls dropped one-third from 2002 to 2004. However, earlier this year, the United States Center for Disease Control and Prevention (CDC) reported that the U.S. fell short of its 2005 goal to reduce cases of

foodborne listeriosis by 50 percent. In light of this recent announcement it seems clear that there are still contributions and improvements that can be made to control *L. monocytogenes* in foods. As mentioned, this may include exploring newer technologies, food preservation methods, and novel naturally-produced compounds as consumer opinion and needs evolve.

DISSERTATION ORGANIZATION

This dissertation is a compilation of work that includes a literature review (chapter 2) pertaining to the research described here as four journal articles (chapters 3 – 6), followed by general conclusions (chapter 7). It is the author's intent to submit chapters 3 and 6 to Applied and Environmental Microbiology, and chapters 4 and 5 to the Journal of Food Protection. The paper entitled, "Antimicrobial efficacy of methanobactin against *Listeria monocytogenes* Scott A in laboratory medium" was presented at the International Association for Food Protection 2005 Annual Meeting in Baltimore, MD (August 14-17, 2005), and the Iowa State University Institute for Food Safety and Security's 1st Annual Symposium in Ames, IA (April 19, 2006). The paper entitled, "Bactericidal activity of methanobactin in combination with various surfactants against *Listeria monocytogenes* Scott A" was also presented at the latter event, and has been accepted for presentation at the International Association for Food Protection 2006 Annual Meeting in Calgary, Alberta, Canada (August 13-16, 2006). References can be found at the end of each chapter with the exception of this one and chapter 7, and follow the format of the aforementioned journals.

CHAPTER 2. LITERATURE REVIEW

LISTERIA MONOCYTOGENES

History

The first listeria-like microorganism found in human tissue was reported by Hayem in 1881 (Gray, 1957). A similar bacterium was observed in Germany by Henle in 1893 and in Sweden by Hülphers in 1911. It wasn't until 1926 that Murray and coworkers first described the bacterium, *Bacterium monocytogenes*, as the cause of monocytosis in infected laboratory rabbits and guinea pigs (Murray *et al.*, 1926). The following year, the same bacterium was isolated by Pirie from tissues of gerbils in South Africa and he called it *Listerella hepatolytica* in honor of Lord Lister (Pirie, 1927). When it became clear that it was the same microorganism, Murray and Pirie agreed to call it *Listerella monocytogenes* (Seeliger and Jones, 1986), but it was given its present name by Pirie in 1940 (Pirie, 1940). *Listeria monocytogenes* was first isolated from sheep by Gill in 1929 and then from humans by Nyfeldt (Gray and Killinger, 1966). *L. monocytogenes* is now recognized as one of the most problematic and dangerous human foodborne pathogens. Countless studies have been conducted to understand the nature of this microorganism and ways by which it can be controlled in the food processing environment.

Identification

L. monocytogenes is a small (0.5 × 1.0-2.0 μm), regular, gram-positive, non-sporeforming, rod-shaped bacterium with rounded ends that can be found as single cells as well as in short chains. It can also appear spherical, and may be confused with streptococci.

L. monocytogenes, a microaerophilic, facultative anaerobe, tests positive for catalase, negative for oxidase, and also produces a β-hemolysin which results in zones of clearing on blood agar (Rocourt, 1999). The CAMP (Christie-Atkins-Munch-Peterson) test is considered as a definitive test for the presence of *L. monocytogenes*. A positive CAMP test in the presence of *Staphylococcus aureus* or *Rhodococcus equi* is a positive presumptive test for a *L. monocytogenes* isolate. When *L. monocytogenes* is grown in the presence of these two organisms, hemolysin is produced synergistically. This is possibly due to the action of a phosphatidylinositol-specific or phosphatidylcholine-specific phospholipase C from *L. monocytogenes*, and a sphinogmyelinase from *S. aureus* (McKellar, 1993).

L. monocytogenes also hydrolyzes sodium hippurate and esculin, and tests negative for H_2S. When grown on nutrient agar with Henry illumination, the bacterial colonies appear mostly smooth, 0.2-0.8 mm in diameter, and bluish-gray with a blue-green hue after 24-48 hours at 35-37°C (Lachica, 1990). A commonly used selective media used for its isolation is modified oxford (MOX) agar, where selectivity and differentiation is based on the presence of lithium chloride (LiCl), two antibiotics, and esculin hydrolysis. These are all tests that can help identify a *Listeria* spp. isolate. The organism is highly motile where peritrichous flagella allow it to exhibit tumbling motility in a narrow growth range. When the organism is grown between 20 and 25°C, flagellin is produced and assembled on the outside of the cell, but production is drastically reduced at 37°C (Peel *et al.*, 1988). *L. monocytogenes* is a mesophile that has psychrotrophic properties displaying growth between -0.4 and 50°C (Walker and Stringer, 1987; Junttila *et al.*, 1988)

Growth Requirements and Characteristics

L. monocytogenes is not a fastidious microorganism and the nutritional requirements are typical of many other gram-positive bacteria. It can grow well in common media such as brain heart infusion, trypticase soy, and tryptose broths, where reduced oxygen and 5-10% carbon dioxide have been found to enhance growth. Growth factors include four B-vitamins: biotin, riboflavin, thiamine, and thioctic acid, and six amino acids: arginine, cysteine, glutamine, isoleucine, leucine, and valine (Premarante *et al.*, 1991; Glaser *et al.*, 2001; Welshimer, 1963). While the biosynthetic pathways for the four vitamins are missing or incomplete, the pathways for the listed amino acids have been identified. Thus, the requirement for amino acids may be due to repression of some amino acid biosynthetic pathways in laboratory growth conditions (Glaser *et al.*, 2001).

Primary sources of nitrogen and carbon for *L. monocytogenes* are glutamine and glucose, and it lacks a complete tricarboxylic acid (TCA) cycle due to the absence of α-ketoglutarate dehydrogenase, succinyl CoA synthetase, and succinic dehydrogenase (Trivett and Meyer, 1971; Kim *et al.*, 2006). Examination of a schematic of the TCA cycle in this organism (Kim *et al.*, 2006) shows that it is split into two independent half-cycles, with a reductive portion and an oxidative portion. The right side links central pathways of carbon catabolism and nitrogen assimilation and the left functions counterclockwise and is used for respiration coupled to fumarate reduction. *L. monocytogenes* has several fermentation pathways and can produce ATP through a complete respiratory chain (Patchett *et al.*, 1991; Glaser *et al.*, 2001). It is able to metabolize glucose to L(+)-lactic acid by the Embden-Meyerhof pathway, and studies on carbohydrate fermentations revealed that under

anaerobic conditions only hexoses and pentoses support growth, while lactose and maltose could also be used when grown in aerobic conditions (Pine *et al.*, 1989; Daneshvar, 1989; Romick *et al.*, 1996).

As mentioned earlier, *L. monocytogenes* has a wide temperature range (-0.4-50°C) for growth while its optimum is between 30 and 37°C. It can survive freezing, and adapt to low temperatures by adjusting membrane fluidity and/or by the accumulation of compatible solutes such as glycine, betaine, proline, and carnitine (Annous *et al.*, 1997; Beumer *et al.*, 1994; Ko *et al.*, 1994). However its versatility in terms of temperature is not the only attribute that gives this microorganism its "hardy" reputation.

L. monocytogenes is notably resistant to osmotic stress, and can survive at water activity below 0.93, second only to *S. aureus* with respect to pathogenic microorganisms in foods, though the growth range depends on interactions with acidity and temperature (Farber *et al.*, 1992). It can grow in broth containing 10% (w/v) NaCl, and can survive in even higher salt concentrations (McClure *et al.*, 1989; Sorrells and Enigl, 1990). Like that of cold shock stress adaptation, a major and well-characterized aspect of the salt tolerance of *L. monocytogenes* is the intracellular accumulation of glycine betaine (Sleator *et al.*, 1999). In addition, this pathogen can grow in the presence of 10% (w/v) bile, 0.025% (w/v) thallous acetate, 1.5% (w/v) lithium chloride and 0.04% (w/v) potassium tellurite. Unlike most other gram-positive bacteria, *L. monocytogenes* is able to grow on MacConkey agar (Jay, 2000).

The influence of acidity on the growth and survival of *L. monocytogenes* is dependent on pH, acid type, temperature, water activity, salt concentration and nutrient

availability. Optimum pH for growth is 6-8, and the organism displays moderate tolerance to low pH, ranging from 4.1-9.6 (Jay, 2000; Conner *et al.*, 1986).

L. monocytogenes is able to utilize numerous exogenous siderophores for iron acquisition despite its inability to produce them (Coulanges *et al.*, 1998). In fact esculetin, a hydrolysis breakdown product of esculin, has been shown to function as a siderophores for the organism (Coulanges *et al.*, 1996). *L. monocytogenes* utilizes a citrate inducible iron uptake system (Adams *et al.*, 1990), surface-bound reductase (Deneer *et al.*, 1995), and the first reported putative extracellular iron reductase (Barchini and Cowart, 1996).

Distribution and Transmission

L. monocytogenes is ubiquitous in nature and widely found in plants, soil, dust, and surface water samples (McCarthy, 1990; Weis and Seeliger, 1975). It does not multiply in soil but can be transmitted to the soil from human and animal feces, sewage sludge, silage and decaying vegetation. Welshimer (1960) demonstrated that survival of *L. monocytogenes* in soil is dependent upon soil type and moisture content. Lakes, rivers, and inshore marine waters can also be contaminated via sewage, which can then infect animals directly (Dijkstra, 1982). The gastrointestinal tract of healthy animal species can harbor *L. monocytogenes* and since decaying vegetation is a natural environment for this microorganism, it is not surprising that the majority of grazing animals such as sheep, goats and cattle carry *L. monocytogenes.* The presence of the pathogen in feces of birds, pigs, rodents and other domestic animals is well documented (Gray and Killinger, 1966). These animals can carry the bacteria for several months without symptoms of listeriosis and can

contaminate foods of plant and animal origin such as fruits, vegetables, meats and dairy products (Gray and Killinger, 1966). The incidence of *L. monocytogenes* shedding in animals has been shown to be affected by diet (Low *et al.*, 1995) and stress level (Fenlon, 1999). Like many other mammals, humans can harbor *L. monocytogenes* in the intestinal tract without any apparent systems, where it is estimated that around 5 to 10% of the human population are carriers (Salyers and Whitt, 1994).

It is well established that any fresh food product of animal or plant origin could potentially harbor varying numbers of *L. monocytogenes*, due to its widespread distribution in animals and the environment. There are, however, certain foods that have been associated with *L. monocytogenes* contamination such raw milk, soft cheeses, fresh and frozen meat, poultry, seafood products, and fruits and vegetables. Its prevalence in milk and dairy products has received much attention because of early outbreaks (Jay, 2000; Farber and Peterkin, 1991), but attention has now shifted to post-process contamination of ready-to-eat (RTE) meat products (Tompkin, 2002; Glass and Doyle, 1989; Samelis and Metaxopoulos, 1999; Beumer *et al.*, 1996; Fenlon *et al.*, 1996). Because *L. monocytogenes* is an intracellular pathogen it can be found in interior muscle cores of animals as well. For example, Johnson *et al.* (1988) demonstrated that the pathogen can be present in muscle tissues of Holstein cows at levels of approximately 140-280 CFU/g when animals were inoculated intravenously two days before slaughtering. Thus, in addition to previously mentioned potential post-processing contamination, inadequately processed meats from infected animals could be a threat to the consumer, and subsequent consumption can result in human listeriosis.

Listeriosis in Humans

As previously mentioned, *L. monocytogenes* is an intracellular pathogen. It can enter the blood stream through the intestinal wall, thereby allowing the infection to spread anywhere in the body. Overall, the symptoms of the disease are variable depending on the susceptibility of the host, where typically in the average healthy adult, *L. monocytogenes* infections are usually asymptomatic or at most, produce mild influenza-like symptoms (Salyers and Whitt, 1994). In addition, the symptoms (fever, headache, fatigue, vomiting, and less commonly nausea and diarrhea) of infection typically occur within 7-60 days after consuming contaminated food (Wallace *et al.*, 2000). These are some reasons why it has been difficult to establish an infective dose; however, it is suggested that <100 cells can cause disease in humans depending on the individual's immune system. The organism causes serious infections in children or adults with underlying conditions that compromise their immune responses (i.e. AIDS, cancer, diabetes, old age, and alcoholism). In such persons, listeriosis can cause central nervous system infections (i.e. encephalitis and meningitis) and fatal bacteremia. It seems that pregnancy increases a woman's susceptibility to infection. *L. monocytogenes* is one of the few bacteria that can cross the placenta, which normally acts as a very effective filtration barrier that prevents bloodborne pathogens from gaining access to the fetus. This organism can potentially infect the fetus resulting in miscarriage, stillbirth, preterm labor, or an infant born alive with a systemic listeriotic infection. About 25% of infected babies die from this disease (Salyers and Whitt, 1994; Silver, 1998).

There are two types of neonatal listeriosis: early-onset disease and late-onset disease. Early-onset usually causes premature birth of infected infants showing signs of sepsis and sometimes granulomatosis infantiseptica. When a full-term infant shows symptoms several weeks after birth, the disease is called late-onset listeriosis (Visintine, 1977). Late-onset disease includes meningitis about 93% of the time, but has a lower mortality rate than early-onset disease (McLauchlin, 1990).

L. monocytogenes has several virulence factors and PrfA is the regulatory activator protein responsible for the coordination the virulence factors (Sheehan *et al.*, 1994). The most significant virulence factor found in *L. monocytogenes* is listeriolysin O (LLO), due to overwhelming evidence that this molecule is responsible for the β-hemolysis of erythrocytes and the destruction of phagocytic cells that engulf them. LLO has been shown to be highly homologous to streptolysin O (SLO) and pneumolysin (PLO). LLO has a molecular weight of 60,000 D and consists of 504 amino acids (Geoffroy *et al.*, 1987; Mengaud *et al.*, 1988). It is produced mainly during the exponential phase, with maximum levels after 8-10 hours of growth (Geoffroy *et al.*, 1989). The gene that controls its production is the chromosomal *hly* gene. Purified LLO is activated by SH-compounds such as cysteine and inhibited by low quantities of cholesterol. LLO is active at a pH of 5.5 but not at pH 7.0, suggesting the possibility of its activity in macrophage phagosomes which can have a pH of 4.6 (Karp, 1996). The LD_{50} of LLO for mice is about 0.8 μg, and it induces an inflammatory response when injected intradermally (Geoffroy, 1987). It is suggested that LLO and the other poreforming toxins (i.e. SLO and PLO) evolved from a single progenitor gene (Salyers and Whitt, 1994).

When *L. monocytogenes* is contracted orally, it first colonizes the intestinal tract, however the mechanisms are poorly understood. From the intestines, the organism invades tissues, including the placenta in pregnant women, and then enters the blood stream, from which it can invade other susceptible tissues. *L. monocytogenes* is an intracellular pathogen, therefore it has the capability to enter target cells and replicate intracellularly (Salyers and Whitt, 1994). It is thought that internalin (Inl), encoded by the *inlA* gene, plays a role in adherence of the organism to the outside of an undifferentiated target cell (either intestinal crypt cells or M cells) and then stimulates phagocytosis into the cell. This protein is probably not essential for entry of the organism into phagocytes because these cells normally perform phagocytosis. Once inside the cell and surrounded by the vesicle membrane, *L. monocytogenes* uses LLO to create pores in the membrane, thereby escaping into the host cell's cytoplasm, where it can multiply rapidly (once every 50 minutes). Phosphatidylinositol-specific phopholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC) are two other hemolysins that enable *L. monocytogenes* to escape one host cell and move into another neighboring host cell. These hemolysins are produced by the genes *plcA* and *plcB*, respectively. These are different from LLO by not forming pores, but instead by hydrolyzing membrane phospholipids like PI and PC (Salyers and Whitt, 1994).

L. monocytogenes can then invade neighboring cells by the polymerization of actin at one end of the bacterium, by means of the ActA protein, encoded by the *actA* gene. This propels *L. monocytogenes* forward while the tail that is left behind, is depolymerized by the

host cell in a tug-of-war fashion (Salyers and Whitt, 1994). This is the process by which *L. monocytogenes* can systematically infect humans, thereby resulting in listeriosis.

Major Outbreaks

The first confirmed outbreak of listeriosis in North America was documented in 1981 when 41 people became ill and 11 died after consuming contaminated coleslaw in Nova Scotia, Canada. The coleslaw had been prepared from cabbage that had been fertilized with raw sheep manure (Schlech *et al.*, 1983). Another large outbreak that occurred in Boston, Massachusetts in 1983, was linked to pasteurized milk which resulted in 49 cases and 11 deaths. The milk came from a farm where cows were known to be infected with listeriosis, however no defects in the pasteurization process were found. It was concluded that post-processing contamination might have taken a place (Fleming *et al.*, 1983). The largest outbreak of listeriosis in North America occurred in California in 1985 and apparently came from a Mexican-style soft cheese made from a mixture of inadequately pasteurized milk and raw milk. The outbreak resulted in 142 cases with 37% mortality rate of nonpregnant adults and 32% mortality rate of perinatals (Linnan *et al.*, 1988).

L. monocytogenes is currently considered a post-processing contaminant of RTE meats because of its persistence in the processing plant environment and more recent outbreaks associated with RTE meats. In late 1998, a multi-state outbreak of listeriosis was attributed to the consumption of contaminated frankfurters produced by Bil Mar Foods, owned by Sara Lee. This outbreak caused 21 deaths and sickened one hundred people

(CDC, 1999). In December 2000, another major multi-state outbreak of listeriosis occurred, this time involving deli meats produced by Cargill Inc. Twenty-nine cases were reported, including 4 deaths, and 3 miscarriages (CDC, 2000). The most recent multi-state listeriosis outbreak occurring in the United States was associated with Pilgrim's Pride pre-sliced turkey deli meat and occurred in northeastern United States. There were 46 confirmed cases, 7 deaths, and 3 miscarriages or stillbirths. A poultry processing plant had two isolates from floor drains that were indistinguishable from the outbreak strains, based on pulsed-field gel electrophoresis (PFGE) profiles. Approximately 27.4 million pounds of fresh and frozen RTE turkey and chicken products were voluntarily recalled due to this last outbreak.

RTE Meat Products and *L. monocytogenes*

L. monocytogenes outbreaks have been associated with the consumption of raw or pasteurized milk, dairy products (especially soft Mexican cheeses), fresh meat and poultry, vegetables, paté, wieners, smoked mussels, goat, sausages, turkey franks, coleslaw, shrimp, and RTE meats (Jay, 2000). It is conceivable that *L. monocytogenes* could potentially be found on raw food products that enter a processing plant. Therefore, food processors are faced with a challenging task to control foodborne contamination by this organism because of its ubiquitous nature.

As mentioned, RTE meat products have been implicated in major listeriosis outbreaks. Due to the nature of these meat products, they are at a particular high risk for contamination by *L. monocytogenes*. For example, normal frankfurter processing

conditions have been shown to be sufficient to eliminate the pathogen from the finished product (Zaika *et al.*, 1990), if well controlled (Kaczmarek *et al.*, 2005). However, this does not guarantee consumer safety because frankfurters may be consumed without further cooking or reheating. This risk of contamination of frankfurters with *L. monocytogenes* is attributed to post-processing contamination which occurs during peeling of the casings and before packaging of the product (Wenger *et al.*, 1990; Palumbo and Williams, 1994). *L. monocytogenes* is a particular problem in these cured RTE meat products because, in general, it can grow to high numbers despite refrigerated storage, the presence of sodium chloride and nitrite salts, and the absence of atmospheric oxygen when vacuum-packaged (Lou and Yousef, 1999). Also, the pH of these meat products (~5.8-6.2) is not sufficiently low enough to suppress growth despite the presence of these other aforementioned hurdles, nor has the natural spoilage microflora shown to play a major role in retarding the growth of *L. monocytogenes* (Radin *et al.*, 2006).

Several surveys, which confirm the physiological evidence just mentioned, have been conducted to determine the incidence of the pathogen on RTE meats. In the United States, Wang and Murina (1994) examined 20 brands of retail wieners and found that for 19 of the brands, the overall incidence of *L. monocytogenes* was 7.5%, where products of 20th brand was 71% positive. The liquid exudates harbored most of the pathogen indicating that contamination occurred after cooking, because these products are removed from their packages after cooking and repackaged for sale; this allows post-processing contamination from food handlers, equipment, or air. In Alberta, Canada, Tiwari and Alrenrath (1990) reported a 17% incidence of *L. monocytogenes* in 38 samples of retail wieners and 67

samples of sliced deli meat. Several surveys conducted in Europe revealed the presence of *Listeria* spp. (including *L. monocytogenes*) in a significant portion of RTE meats in retail. In Denmark, out of 304 samples of four types of packaged meat products, the percentage positives for *L. monocytogenes* shortly after packaging and at the sell-by date ranged from 6-23% and 10-21%, respectively (Qvist and Liberski, 1991). These authors also studied the incidence of the pathogen in 15 vacuum-packaged product types, where overall there was a 13.5% incidence (Qvist and Liberski, 1992). A nine-year survey from a coordinated food sampling program in the United Kingdom, established in 1995, revealed that 1.7% of sliced RTE meats were contaminated with *Listeria* spp. which was the highest contamination rate among other RTE products (Meldrum *et al.*, 2005). Numerous surveys in other countries have also indicated similar contamination rates (Farber and Peterkin, 1999).

The United States has the most rigid policy mandating a zero-tolerance for *L. monocytogenes* in RTE meats, meaning that any RTE meat containing this organism will be considered adulterated and, thus, subject to recall and/or seizure. In 2003, following the major outbreaks of listeriosis involving the consumption of RTE meat products, the United States Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) established an interim final rule (FSIS, 2003), mandating food processors to choose from three alternatives to control *L. monocytogenes* in RTE meat and poultry products. Alternative 3 relies only on sanitation and has the most risks associated with it compared to the other alternatives. It is also the most demanding in terms of demonstrating compliance with regulatory authorities. This has encouraged food processors to implement Alternatives 1 and 2, where the former states that the establishment is required to apply a postlethality

treatment (may be an antimicrobial agent) to reduce or eliminate the pathogen and an antimicrobial agent or process to limit or suppress growth of the pathogen. The latter is less stringent requiring the establishment to employ either a postlethality treatment or a growth inhibitor.

Despite the very strict regulations food processors must comply, the consuming public places even higher demands on food companies. Favor for more minimally-processed, more natural, nutritious, convenient, and yet safe food products is forcing processors to meet such demands. This has prompted research efforts to uncover and develop multi-hurdle approaches involving the use of naturally-occurring or naturally-produced antimicrobials to control the growth of foodborne pathogenic microorganisms. Investigations on these types of compounds, in relation to *L. monocytogenes* and mainly meat products are discussed in the following section.

USE OF NATURALLY-PRODUCED AND NOVEL ANTIMICROBIAL TREATMENTS TO CONTROL *L. MONOCYTOGENES*

Plant- and Animal-Derived Compounds

The most widely used antimicrobials that are found in plants are sorbic acid, benzoic acid, and their salts. Sorbic acid was first isolated from the oil of unripened rowanberries (Sofos and Busta, 1993) and is mainly used as a fungistat, but can inhibit germination and/or outgrowth of *Clostridium botulinum* (Sofos *et al.*, 1979). When used alone, effectiveness is somewhat limited against *L. monocytogenes* (Dorsa *et al.*, 1993; Lungu and Johnson, 2005). There are extensive reviews (Sofos and Busta, 1981) on the use of this antimicrobial; therefore, a comprehensive review on the antimicrobial effect of this

antimicrobial is not presented here. Benzoic acid is one of the oldest preservatives used, thus there is also a large body of literature on this antimicrobial as well. Benzoic acid occurs naturally in many plants, and cranberries are the most well recognized source of this organic acid (Chipley, 1993). While it performs much better at low pH, concentrations of 1000-3000 ppm are bacteriostatic against *L. monocytogenes*, but modestly bactericidal (El-Shenawy and Marth, 1988; Yousef *et al.*, 1989). Activity is much improved when combined with other organic acids (El-Shenawy and Marth, 1989). Benzoic acid and other organic acids that can be found in plants are not necessarily restricted to plant sources, thus it should be kept in mind that there is cross-over among producer-groups (animal or microbial); nevertheless, these are naturally occurring substances. For example, benzoic acid can be a by-product of microbial degradation of hippuric acid or phenylalanine in fermented dairy products (Stijve and Hischenhuber, 1984). Caprylic, citric, fumaric, malic, succinic, and tartaric acids all have moderate activity, as well. Effectiveness against organisms is based on the proportion of undissociated to that of dissociated acid and lipophilicity of the substances (Doores, 1993). Soybean extracts metabolized by two molds generated phenols and acids that were found to be active against *L. monocytogenes* in fish and meat systems (McCue *et al.*, 2005); anacardic acid from the cashew also displayed potent activity against gram-positive pathogenic bacteria (Himejima and Kubo, 1991). Additionally, smoke wood extracts have also been shown to be highly active against *Listeria* spp. (Suñen, 1998).

A wide variety of plant spices, extracts, and essential oils have also been investigated for activity against *L. monocytogenes*. Aureli *et al.* (1992) tested 32 plant

essential oils against the pathogen and found that cinnamon, clove, origanum, pimento, and thyme were effective. Pimento oil displayed the most rapid activity while thyme reduced populations by 2 logs on pork meat after the first week of storage. On cooked beef, pimento extract was shown to inhibit the growth of *L. monocytogenes* for 14 days, as well (Hao *et al.*, 1998). Fyfe *et al.* (1997) found significant growth reduction (4-8 logs) in broth by using a combination of oils including anise, fennel, and basil. Also, garlic, clove, red hot chili (Leuschner and Ielsch, 2003), and mustard flour (Rhee *et al.*, 2003) have been shown to have moderate activity against the pathogen. On chicken frankfurters, clove oil (1 and 2%) inhibited *L. monocytogenes* growth however the study lasted only 2 weeks. On meat and cheese, it was shown to have moderate activity as well (Vrina Menon and Garg, 2001). On beef, four garlic compounds were tested against *L. monocytogenes* with greatest activity being shown to occur with the two sulfide compounds (Yin and Cheng, 2003). Cranberry/oregano surface treatments on fish and beef systems displayed low initial reductions and moderate inhibition during an 8-day study. Eugenol (an oregano essential oil) and cinnamaldehyde of cinnamon were bactericidal against *L. monocytogenes* at low concentrations in broth media (Gill and Holley, 2004). On meat and cheese, cinnamon displayed reductions of *L. monocytogenes* by 1-2 logs with limited inhibitory activity (Vrinda Menon *et al.*, 2002). In laboratory media oregano, mint, dictamus, and sage inhibited the growth of *L. monocytogenes* (Flair-Flow Europe, 1999), while on beef oregano essential oil reduced populations of *L. monocytogenes* by 2-3 logs (Tsigarida *et al.*, 2000). Carvacrol and eugenol (Hao *et al.*, 1998) are effective growth inhibitors of *L. monocytogenes* but encapsulation of them in surfactant micelles improves activity

(Gaysinsky *et al.*, 2005a; Gaysinsky *et al.*, 2005b). Incorporation of pure essential oils anise, basil, coriander, and oregano into chitosan films for meat packaging effectively reduced *L. monocytogenes* populations. In this study, oregano at 1 or 2% performed the best reducing populations by 3.6-4.0 logs (Zivanovic *et al.*, 2005). Also, vanillin has been shown to be active against *Listeria* spp. (Fitzgerald *et al.*, 2004), while hop extracts were highly inhibitory in broth, coleslaw, milk, and cheese (Larson *et al.*, 1996). It is apparent that many studies have investigated the use of spices and essential oils to control *L. monocytogenes* and other pathogens (Hirasa and Takemasa, 1998), but the application for many of the aforementioned compounds may be limited to amounts that will not result in overpowering sensory profiles. As a result of this, these antimicrobials may be present in concentrations too low for effectiveness, thus requiring the use of additional preventive measures.

Plants and animals also produce antimicrobial peptides as defense mechanisms, referred to as defensins. Thionin (abundant in leaves and seeds of plants), lipid transfer protein, snakin, and potato defensin were evaluated for activity against *L. monocytogenes* by López-Solanilla *et al.* (2003), in addition to some animal-derived peptides. Thionin and snakin were the most inhibitory, displaying MIC values of 2 and 10 μg/ml, respectively. Fabatins have been classified as novel plant peptides from the broad bean *Vicia faba* and noted for their activity of against bacteria; however *L. monocytogenes* was not included in the study (Zhang and Lewis, 1997). Maize kernels have been shown to possess novel antimicrobial peptides as well (Duvick *et al.*, 1992). This demonstrates that there are likely many uncharacterized peptides yet to be discovered and tested for antimicrobial efficacy.

Protamine is a highly basic, polycationic peptide found in fish spermatozoan nuclei where its role is to compact genomic DNA. Studies indicate it is highly inhibitory to bacteria, including *L. monocytogenes* (Johansen *et al.*, 1996; Johansen *et al.*, 1997). Determined by broth dilution, protamine and magainin (from amphibian skin) MICs were both 10 μg/ml (López-Solanilla *et al.*, 2003), however using agar dilution, Hansen and Gill (2000) showed that protamine could not inhibit *L. monocytogenes* until the media was at pH 6.0 or greater. The lowest MIC (50 μg/ml) occurred at pH 8.0. In fish and curry sauce, protamine at 5000 ppm inhibited development of *L. monocytogenes*, however no activity was found in meat or poultry products. They concluded that protamine is not ideally suited for high-protein foods because its highly positive charge can interact with negative groups in proteins (Uyttendaele and Debevere, 1993). Insect defensins and cecropins have also displayed activity against bacteria (Kagan *et al.*, 1990; Cociancich *et al.*, 1993; Christensen *et al.*, 1988).

Chitosan is a polysaccharide found in crustaceans that has been frequently studied for its use in packaging films. For example, *L. monocytogenes* could be inhibited on fresh chicken breast (Cooksey, 2005) and reduced (2 logs) on processed meats (Zivanovi *et al.*, 2005) by these means. When used in dips for skinless sausage yeast and molds were inhibited, however antimicrobial activity could be improved against bacteria, including *L. monocytogenes* when the compound was enzymatically and chemically degraded to smaller oligomers (Flair-Flow Europe, 1999).

Lysozyme is a lytic enzyme found in many natural systems including tears, plant tissues, milk, and eggs (Conner, 1993) that breaks down peptidoglycan in the bacterial cell

wall, resulting in cell lysis. It is active against *L. monocytogenes* (Hughey and Johnson, 1987), with bactericidal activity at 9.27 μg/ml in broth media (Proctor and Cunningham, 1993). A study done by Hughey *et al.* (1989) demonstrated a marked increase in lysozyme activity against *L. monocytogenes* when used in conjunction with EDTA. Additionally, results showed that 4-log reductions could be achieved on vegetables, with lesser activity on meat products. Another enzyme that has been studied fairly extensively is lactoperoxidase. It is part of the lactoperoxidase system (includes an oxidizable substrate and H_2O_2) that generates hypothiocyanous acid and hypothiocyanate as the major active antimicrobials (Conner, 1993). Activity against *L. monocytogenes* has been shown (Kamau *et al.*, 1990a) and it can sensitize cells to heat by as much as 10-fold (Kamau *et al.*, 1990b). Although it has been traditionally used in milk, applications of the lactoperoxidase system in red meat (Kennedy *et al.*, 2000) and a beef cube system (Elliot *et al.*, 2004) prevented the growth of *L. monocytogenes*. Interestingly, both studies found that activity was highest at temperatures at which the pathogen can grow, but not optimally.

Lactoferrin is an iron-binding glycoprotein present in cow's milk that has antimicrobial activity. It is reported to be effective against *L. monocytogenes* (Nagasawa *et al.*, 1972) where activity is attributed to binding of iron thereby reducing accessibility of this micronutrient to organisms. A patent was issued to Naidu in 1999 to control the growth of *L. monocytogenes* and other pathogens on RTE meats by using a surface treatment that includes the use of immobilized lactoferrin (IM-LF). Lactoferricin is an antimicrobial peptide produced from lactoferrin by gastric pepsin cleavage. This breakdown product was found to be even more active against *L. monocytogenes* displaying

at least 4-log reductions in 30 minutes (31 μg/ml) and MIC values ranging from 0.3-9 μg/ml depending on strain and media type (Wakabayashi *et al.*, 1992). Additionally, chloride salts were found to be antagonistic, thus increased MIC values. Conalbumin is another iron-binding protein found in egg whites that restricts the growth of bacteria by means similar to that of lactoferrin. It does not seem to be as effective, however, and it typically only extends the lag phase of the target organism (Conner, 1993). It has been reported that no single component of raw egg albumen can account for its antibacterial activity toward *L. monocytogenes* (Sionkowski and Shelef, 1990), and that along with high pH, lysozyme, conalbumin, and other compounds must work in concert for effectiveness (Wang and Shelef, 1991).

Microbial-Derived Compounds

Discussion on various types of antimicrobials that are produced by bacteria will be focused on those pertaining to potential food use. Although there are numerous types of antibiotics produced across the microbial world that surely are active against *L. monocytogenes*, they will not be discussed in this review because they lack relevance for use in foods. Sources of bacterial-derived compounds to control pathogenic microorganisms, including *L. monocytogenes*, have primarily come from lactic acid bacteria (LAB). Organic acids, and their salts, are among the most well studied compounds used to control the presence of this pathogen in foods. It is well established that the undissociated form of organic acids, such as lactic, acetic, and citric are 10-600 times more effective than the dissociated forms (Helander *et al.*, 1997). Amezquita and Brashears

(2002) attributed the inhibition of *L. monocytogenes* on five different RTE meat products, in part, to acid production by protective LAB cultures when they were co-inoculated with the pathogen. This *in situ* production of organic acids by fermentation of LAB has also been shown to enhance the efficacy of bacteriocins as well (Winkowski and Montville, 1992).

Direct addition of these organic acids has been extensively studied for use as or in carcass washes (Tamblyn and Conner, 1997; Yang *et al.*, 1998; Dorsa, 1997). There are also a vast number of studies that have been published that report the efficacy of post-processing organic acid surface treatments against *L. monocytogenes* on RTE meat products. In line with the discussion on current problems associated with controlling the pathogen on RTE meats, the objective of these studies are in attempt to control the pathogen in the event of post-processing contamination. One of the earliest studies on the matter was done by Palumbo and Williams (1994). They reported that dipping frankfurters with 5% lactic or acetic acids gave ~1-log reductions and growth inhibition of *L. monocytogenes* (2-minute dips followed inoculation). When used alone, citric acid was not found to work as well when used alone. Similarly, *L. monocytogenes* on frankfurters that were dipped for 30 seconds with 3.4% (of an 88% solution) lactic acid displayed an extended lag phase (~6 weeks), while an aqueous solution of potassium lactate (KL) was less effective (Nuñez de Gonzalez *et al.*, 2004). Similar results were found by Samelis *et al.* (2001) on pork bologna. In another study, dipping frankfurters formulated with antimicrobials has been shown to generate minor reductions during storage (Barmpalia *et al.*, 2004). Other studies demonstrate high initial reductions like the previously mentioned,

but growth inhibition effectiveness was less dramatic. For example, dipping frankfurters and bratwurst in 6% lactate and 3% diacetate solutions did not have any effect on delaying the growth of *L. monocytogenes* during storage (Glass *et al.*, 2002). A study conducted by Lu *et al.* (2005) demonstrated that when frankfurters were dipped for 2 minutes in 2.5% solutions of lactic or acetic acid, initial populations of *L. monocytogenes* were reduced by 0.7-2.1 log CFU/cm^2, but also had little inhibitory effect during storage. Geornaras *et al.* (2006) demonstrated 1.0-1.8 log CFU/cm^2 initial reductions of *L. monocytogenes* on frankfurters when dipped for 2 minutes in solutions of acetic acid (2.5%), lactic acid (2.5%), or potassium benzoate (5%), but inhibitory action during storage was limited. A study published a year earlier by these researchers reported similar findings for bologna and ham (Geornaras *et al.*, 2005). Islam *et al.* (2002a) found that spraying chicken luncheon meat with very high concentrations of sodium benzoate and propionate had little effect on initial reductions and inhibition during storage of *L. monocytogenes*. When they used these solutions on turkey frankfurters, growth of the pathogen was delayed, but the storage study only lasted for 14 days (Islam *et al.*, 2002b), which may indicate cured RTE meat products may be a more appropriate food for these applications. Perhaps the best demonstration of organic acid use on frankfurters was done by Murphy *et al.* (2006), where a combination of acetic, lactic, propionic, and benzoic acids along with steam pasteurization inhibited *L. monocytogenes* growth for 19 weeks. This indicates the importance of hurdle technology and potential synergistic behavior of organic acids. While the effectiveness of these compounds for use as surface treatments seems variable, collectively, there is a good indication that initial reductions of *L. monocytogenes* seems fair, however overall

bacteriostatic activity is limited. Sources of variation may be a result of differences in product formulation, product type, cure status, acid type and concentration, among others. In addition, organic acid salts have been extensively studied as antimicrobials in RTE meat formulations to prevent the growth of *L. monocytogenes*. Literature on this subject is discussed in the following section.

Metabolic activities of LAB and others produce fermentation by-products and novel compounds in addition to organic acids. The fermentation by-product, diacetyl has broad antimicrobial activity at concentrations of 300-1000 ppm, but because it produces a marked butter flavor at 2-4 ppm, and *in situ* production by LAB is slightly lower yet, its contribution to preservation is limited (Helander *et al.*, 1997). Reuterin (2-hydroxypropionalaldehyde) is a non-proteinaceous metabolite of glycerol produced by *Lactobacillus reuteri* having a broad antimicrobial activity spectrum (Nout and Rombouts, 1992). El-Ziney *et al.* (1999) investigated the efficacy of reuterin solutions on pork products, inoculated with *L. monocytogenes* and *Escherichia coli* O157:H7. Reductions after 1 (500 Arbitrary Units/ml on cooked pork surfaces) and 7 (250 Arbitrary Units/ml in raw ground pork) days of storage at 7°C were 0.63 and 3 log CFU/cm^2, respectively. In addition, *E. coli* O157:H7 was far more sensitive to reuterin activity, and combination with 5% lactic acid improved activity against both pathogens. On sausage surfaces, reuterin was reported to inhibit the growth of *L. monocytogenes* during storage at 5°C, however storage periods for both of these studies only lasted for 7 days (Kuleasan and Cakmakci, 2002). Production of H_2O_2 is a typical by-product of LAB because they lack peroxidase. Most work has investigated its use in raw milk and for sanitizing packaging materials (Lou and

Yousef, 1999). Interestingly, *in situ* production of this has been shown to activate the endogenous lactoperoxidase system in meats (Oyarzabal, 1998). Three novel antimicrobials were isolated from the culture filtrate of the LAB, *Lactobacillus plantarum*, including benzoic acid. Methylhydantoin, mevalonolactone, and cyclo(glycyl-L-leucyl) inhibited the growth of other LAB by 10-15% when used alone, but when all three were combined with 1% lactic acid, complete inhibition was achieved (Niku-Paavola *et al.*, 1999). Cyclic lipopeptides are compounds produced by many different groups of bacteria (Katz and Demain, 1977; Moffitt and Neilan, 2000); they are thought to have biosurfactant (Nielsen *et al.*, 2002) and antimicrobial properties (Gerard *et al.*, 1997), as well as promote swarming.

Siderophores and siderophore-peptides are compounds produced by a variety of bacteria, where the former is well characterized. Studies determining potential use in foods are limited but will be discussed later in the chapter.

The final major group of antimicrobials that warrants discussion regarding its use against *L. monocytogenes* in RTE meat products is bacteriocins. Other than organic acids, this class of compounds, defined as antimicrobial peptides produced by bacteria that are active against reasonably closely related strains, has been widely studied. Detailed discussion of their uses in RTE meats to destroy and inhibit the growth of *L. monocytogenes*, are discussed in a later section.

Lactates in Cured RTE Meat Formulations

The incorporation of lactic acid salts (i.e. K^+ and Na^+) in RTE meat formulations are known to increase processing yields by increasing water holding capacity, but also increase flavor, shelf life and product safety. Formulation of RTE meats with lactates, in particular cured RTE meats, and its combination with sodium diacetate to produce synergistic activity are well established (Mbandi and Shelef, 2001). In-depth discussion of this latter compound is out of the scope of this review. In addition, there may be a trend in processors removing diacetates from formulations due to its strong vinegar-like odor and taste. RTE meat products, in particular frankfurters and sliced deli meats, were involved in the most recent listeriosis outbreaks. In this regard, most work has focused on the formulation of lactates in cured RTE meats versus non-cured meats, thus discussion is focused on the former. The USDA-FSIS presently permits the use of lactates at concentrations up to 4.8% by weight of total formulation in RTE meats (CFR, 2006). The mechanism of bacterial inhibition of lactates is not well understood, although there is some evidence that it can reduce water activity enough to sufficiently inhibit bacteria (Debevere, 1989). However, Weaver and Shelef (1993) found that 4% sodium lactate (NaL) in liver sausage did not lower the a_w sufficiently enough to inhibit the growth of *L. monocytogenes*. Possible mode of action may arise from lowering the cytoplasmic pH by undissociated lactic acid and possibly by metal chelation (Shelef, 1994). Feedback inhibition and interference with proton transfer across the cell membrane also seem likely (Sofos, 1995).

Most, but not all studies indicate that when used alone, >2% NaL is needed to reliably inhibit the growth of *L. monocytogenes* on RTE meats for the length of the

recommended commercial shelf life (~90 days in the case of frankfurters). Lactate formulation concentrations reported here are adjusted after taking into account that most of these studies use 60% solutions of lactate. A study conducted by Geornaras *et al.* (2006) showed that formulating frankfurters with 1.5% NaL did not inhibit the growth of *L. monocytogenes* on frankfurters for more than eight days. In addition, stress-hardened biofilm cultures were used as one of the three test inocula and 0.05% sodium diacetate was also included in the formulation. Stekelenburg (2003) demonstrated that unless diacetate was included, 1.8% KL inhibited *L. monocytogenes* for only 2 weeks on frankfurters when stored at 4°C, which agrees with studies by Glass *et al.* (2002). In this study 2.1% lactate did inhibit growth for at least 60 days. However, Nuñez de Gonzalez *et al.* (2004) reported inhibition for 6 weeks using 2% NaL but higher concentrations were not evaluated. Formulating frankfurters with 1.8% NaL inhibited the growth of *L. monocytogenes* for almost 40 days at 4°C (Samelis *et al.*, 2002). In the same study, combination with antimicrobial dips did not improve formulation activity; however addition sodium diacetate or glucon-δ-lactone to the formulation resulted in inhibition for 120 days. In another study, at 10°C, this NaL concentration inhibited *L. monocytogenes* for approximately only 1 week in frankfurters, unless antimicrobial dips or other formulation ingredients were also employed (Barmpalia *et al.*, 2004). An earlier study by this laboratory (Bedie *et al.*, 2001) showed inhibition for approximately 50 days by 1.8% NaL at the same storage temperature, while 3.6% NaL inhibited growth for 120 days. In contrast to the aforementioned studies, Porto *et al.* (2002) demonstrated that when frankfurters were formulated to have a final concentration of 2% KL, growth of *L. monocytogenes* could be inhibited for 90 days at both

4 and 10°C. It should be noted that inoculum concentrations used in this study were also very low. Similar findings have been shown by previous work in our laboratory as well (unpublished data). Choi and Chin (2003) also found similar results with 2% NaL, however the storage study only lasted for 8 weeks.

When 2% NaL was added to RTE ham, growth of *L. monocytogenes* was inhibited <7 days during storage at 4°C, while the addition of other formulation ingredients did not have a marked effect (Zhu *et al.*, 2005). Similar findings were reported on incorporation of 1.8% NaL into pork bologna formulations (Barmpalia *et al.*, 2005). Incorporation of 2.5% NaL into beef bologna formulations inhibited the pathogen for the length of the storage study (45 days at 5°C), however populations started to proliferate at this point (Mbandi and Shelef, 2002). Models developed by Seman *et al.* (2002) demonstrated that formulating bologna, wieners, ham, or cotto salami with 1.5% KL with 0.15% sodium diacetate resulted in little to no growth of *L. monocytogenes* for both predicted and actual values. Other work done by this group also demonstrated that cure status has a dramatic effect on the efficacy of these antimicrobial formulations in RTE meat products, where the presence of curing agents increases activity (Legan *et al.*, 2004). It is worth noting that many of these studies mentioned, show inhibition using 2% lactate for a major portion of the 3-month storage periods. This may indicate that this concentration borders on the threshold of the desired inhibitory action, because others have shown this formulation concentration to be sufficient. It would seem that to ensure the safety of RTE meat products, other interventions should be included, such as post-process surface treatments or other antimicrobial ingredients. Collectively these studies indicate that effectiveness of lactates in meat formulations can

depend on inoculum concentration, strain, inoculum source, product type (Shelef and Yang, 1991), product formulation (Hu and Shelef, 1996), cure status, storage temperature, and the employment of other interventions.

Use of Bacteriocins to Control *L. monocytogenes* in RTE Meat Products

Bacteriocins are antimicrobial compounds that have a peptide or protein component essential for activity. Although most bacteriocins have a narrow spectrum of inhibition and only inhibit closely related species, some bacteriocins, such as nisin and pediocin, have a relatively broad spectrum and can inhibit some less closely related organisms (Lou and Yousef, 1999). Although their modes of action vary, bacteriocins usually destabilize the cytoplasmic membrane of sensitive cells, increase membrane permeability, and dissipate the proton motive force by forming water-filled transmembrane pores or channels (Jack *et al.*, 1995). Bacteriocins of LAB are divided into 4 distinct classes: (I) Lantibiotics, lanthionine-containing peptides, such as nisin, (II) small (<10 kD), non-lanthionine-containing, relatively heat-stable bacteriocins, such as pediocin PA-1, PO2, or AcH, (III) large (>30 kD) heat-labile molecules, and (IV) bacteriocins with nonpeptide moieties (Klaenhammer, 1993).

Drawbacks of bacteriocin use in biopreservation includes limited large-scale application in the food industry. Additionally, when bacteriocins are added to foods, they usually show only a modest antimicrobial effect, a common observation among other antimicrobials. Also, LAB bacteriocins are typically limited to activity against only gram-positive bacteria, due to their exclusion by the outer membrane (OM) of gram-negatives

(Stevens *et al.*, 1992). Therefore, a bacteriocin is usually applied to food systems in conjunction with other compounds from a hurdle approach. In the United States, nisin is the only bacteriocin currently permitted in foods in its purified form; allowed up to 250 ppm in various types of pasteurized cheese spreads (CFR, 2005a). However, there are many published studies investigating the efficacy of bacteriocins in/on other foods. Because a large number of these compounds are active against *L. monocytogenes*, and the association of this pathogen with RTE meat products is well-recognized, discussion will be confined to investigations of bacteriocins to control *L. monocytogenes* on RTE meat products. Typical applications (as bacteriocin-containing fermentates, crude extracts, or purified forms) for RTE meat products and other foods include: (1) Direct addition of bacteriocin-producing bacteria, (2) food surface treatment, (3) formulation as antimicrobial ingredients, or (4) incorporation into primary food packaging materials (active packaging).

Several criteria for selection of suitable biocontrol microorganisms for use in meat or meat products were proposed by McMullen and Stiles (1996). Biopreservation microorganisms should be psychrotrophic, produce bacteriocins early in the growth cycle, and exhibit little negative effect on product quality. In addition, bacteriocins produced by these bacteria should be bactericidal and stable in the food environment. The authors concluded that nisin-producing lactococci are poor biocontrol organisms, since they do not grow well at chill temperatures or in meat products. They also noted that pediocin-producing pediococci are also poor meat biopreservatives, because antilisterial activity occurs only at abuse temperatures (Degnan *et al.*, 1992). In this study pediocin production was not observed on wieners stored at refrigeration temperature, but at 25°C, production of

pediocin by the producer organism inhibited the pathogen for 8 days. Yousef *et al.* (1991) found similar results; however, *L. monocytogenes* counts decreased by 5.84 log CFU/ml after 3 days when wiener exudates were coinoculated with *Pediococcus acidilactici* H. In contrast Berry *et al.* (1991) found that *P. acidilactici* JD1-23 controlled the growth of *L. monocytogenes* for 15 days at 15°C and 60 days at 4°C on vacuum-packaged frankfurters. Also, a combination of 3 LAB, including *P. acidilactici*, inhibited the growth of *L. monocytogenes* for 28 days on frankfurters and ham stored at refrigeration temperature. Bactericidal activity was noted on frankfurters as well (Amezquita and Brashears, 2002). Other LAB have also been studied for their potential use as bioprotectives on refrigerated meats. Bredholt *et al.*, (1999) isolated 5 uncharacterized, indigenous LAB from meat products and applied them in challenge studies ($\geq 10^4$ CFU/g) against *L. monocytogenes* on cooked, sliced, vacuum- and gas-packaged ham. Results showed that growth of the pathogen could be inhibited for 30 days at 8°C. Very similar results were shown by Mataragas *et al.* (2003) on sliced cooked cured pork shoulder (4°C), using *Leuconostoc mesenteroides* L124 and *Lactobacillus curvatus* L442 isolated from dry fermented sausages. When *Enterococcus faecium* A-48-32 (10^7 CFU/g) was coinoculated with *L. monocytogenes* (10^3/g) in cured sausages, pathogen counts were undetectable after 9 days of storage. Also *Lactobacillus sakei* CTC494 (Hugas *et al.*, 1998) and *Lb. sake* 706 (Schillinger *et al.*, 1991) were found to have moderate inhibition of listerial growth on cooked pork and in sausages, respectively.

Probably the most promising application for use of bacteriocins in RTE meat products is their inclusion into surface treatment solutions. For example, Nisaplin (0.5%),

the commercial form of nisin equivalent to 5000 IU/ml of nisin, was used as a surface treatment on vacuum-packaged bologna and ham stored at 10°C in combination with organic acids, to control the presence of *L. monocytogenes* (Geornaras *et al.*, 2005). Initial reductions were 2.4 to 2.9 log CFU/cm^2 when Nisaplin was used alone or followed organic acid treatments. Nisaplin alone had little to no effect on growth inhibition, however when combined with lactic acid (2.5%), pathogen counts were reduced to undetectable levels at the end of the storage (48 days). It should be noted that inoculation occurred before treatment in this study. On sausage, activity of 100-200 ppm nisin against *L. monocytogenes* increased when combined with the chelator tert-butylhydroquinone (TBHQ) or high-pressure processing (Chung *et al.*, 2005). Results to these studies demonstrate the importance of combining bacteriocins with other hurdles for effective use. More studies involving bacteriocins in multi-hurdle approaches will also be discussed, with exception of surfactants, as they will be discussed separately in the next section. On beef frankfurters, Uhart *et al.* (2004) studied the effects of a 6000 AU-pediocin dip (5 min) on *L. monocytogenes* before inoculation during 3 weeks of storage at 4°C. Results indicated that pediocin alone displayed almost no initial lethality, but lowered counts by ~0.5 log CFU/g after 3 weeks of storage. Combining pediocin with NaL and sodium diacetate improved reductions at 3 weeks by ~2 log CFU/g. Similar studies were conducted by Chen *et al.* (2004a; 2004b; 2004c) using ALTA 2341, a food-grade fermentation product containing significant pediocin activity. In these studies, pediocin alone (3000 or 6000 AU/link) had little effect at 10 or 25°C, but after 7 weeks of storage at 4°C pediocin reduced *L. monocytogenes* counts by approximately 0.5 log CFU/g, which is in agreement with the

aforementioned study. However, the pathogen grew to high numbers, but below that of the control, by the end of storage (12 weeks), which indicates the importance of conducting storage studies for appropriate lengths of time. Combining pediocin with postpackaging thermal pasteurization (PPTP; Chen *et al.*, 2004a) or postpackaging irradiation (Chen *et al.*, 2004b) drastically improved the control of *L. monocytogenes*, where some treatments lowered counts to undetectable numbers by the end of storage, depending on storage temperature, packaging type, and severity of heat or irradiation treatments. Pediocin AcH on sliced cooked sausages reduced populations of *L. monocytogenes* by >1 log CFU/g during 21 days of storage, however, the control group did not increase in numbers on the product (Mattila *et al.*, 2003). Also, studies on a bacteriocin produced by *Carnobacterium piscicola* revealed potential use on vacuum-packaged beef muscle meat, where growth of *L. monocytogenes* was inhibited for 14 days at refrigeration temperature (Schöbitz *et al.*, 1999).

Investigations of using bacteriocins as RTE meat formulation ingredients are limited, which may imply a lack of appropriateness for this as an efficient antimicrobial application. However, significant use was shown by Aymerich *et al.* (2000) who challenged a close relative of *L. monocytogenes*, *L. innocua,* with enterocins A and B by formulating it into ham, pork mince, and liver pate. Results showed that 4800 AU/g reduced populations by 7.98 log cycles in cooked ham and 9 log cycles in pate during refrigerated storage for 37 days. In pork mince, 1600 AU/g inhibited growth of the organism. These researchers also found that nisin had excellent inhibitory against *L. monocytogenes* on sliced cooked ham for 84 days, when combined with lactates and high

hydrostatic pressure (Aymerich *et al.*, 2005). In contrast to ham, formulating enterocin CCM 4231 (12,800 AU/g) into dry salami had very little effect on *L. monocytogenes*, where after initial reductions of 1.67 log cycles, the pathogen grew rapidly to 10^7 CFU/g after 1 week. The authors suggested there was limited bacteriocin diffusion into the meat (Lauková *et al.*, 1999). Overall effectiveness of bacteriocins as part of meat formulations seems limited, possibly due to the higher pH of meat (Rayman *et al.*, 1983; Abee *et al.*, 1995), inability to uniformly distribute the bacteriocin (Cleveland *et al.*, 2001), and interference by meat components such as phospholipids, fat content (de Vuyst and Vandamme, 1994; Davies *et al.*, 1999) or other components (Chung *et al.*, 1989). These antagonistic intrinsic food factors are not limited to situations in which bacteriocins are applied as formulation ingredients, but also to bioprotective use and post-processing surface treatments.

Incorporation of bacteriocins into packaging or edible films (active packaging) has also been an active area of research, with promising applications for nisin in particular. Antimicrobial films prevent microbial growth on food surface by direct contact of the package with the surface of foods, such as meats. For this reason, and for it to work, the antimicrobial packaging or edible film must contact the surface of the food so that bacteriocins can diffuse to the surface. Gradual release of bacteriocins from a film to the food may have an advantage over dipping or spraying foods with bacteriocins. In the latter processes, antimicrobial activity may be lost or reduced due to inactivation of the bacteriocins by food components or dilution below active concentration due to migration in the foods (Appendini and Hotchkiss, 2002; Chen and Hoover, 2003). For example, Fang *et*

al. (1996) demonstrated that immobilized nisin inhibited the growth of *L. monocytogenes* longer than free nisin on cooked pork. Grower *et al.* (2004) found that film coatings formulated with nisin, along with various acids did not affect water vapor transmission rates of low density polyethylene (LDPE) film, while tensile strength increased. Activation of polyethylene films by soaking, spraying, and coating with antilisterial bacteriocin 32Y, for use against *L. monocytogenes* on meat products differed in terms of activity, where coating was the best (Mauriello *et al.*, 2004). Lungu and Johnson (2005a) determined the effects using nisin in combination with NaL and sodium diacetate in zein coatings for model turkey pieces. Zein coatings are edible films made of corn protein. It was reported that nisin alone reduced populations by 6.6 log CFU/g after 28 days of refrigerated storage, and diacetate alone was similar. Combination of the two actually produced slightly lower reductions, and although the authors concluded that no synergies were found among the treatments, reduction of *L. monocytogenes* to undetectable numbers could be achieved depending on the coating material. In another study, these researchers investigated the use of incorporating nisin with and without potassium sorbate in zein coatings for use on full fat turkey frankfurters. Results showed that nisin alone significantly reduced *L. monocytogenes* counts after 28 days of storage at 4°C. Using a larger pathogen inoculum, counts were 6.1 logs lower than the controls, but undetectable when lower inoculum concentrations were used. Inclusion of sorbate did not improve nisin effectiveness (Lungu and Johnson, 2005b). Similarly, Dawson *et al.* (2002) evaluated the use of 2.5% nisin and 8% lauric acid-impregnated soy-based films to control *L. monocytogenes* growth on turkey bologna at 4°C. After 21 days of storage nisin, lauric acid, or a combination of the two

reduced populations by 1 log after 21 days of storage, indicating there was no benefit using this hurdle approach, however synergistic behavior was noted in a broth system. Using nisin-coated cellulose-based films, Franklin *et al.* (2004) investigated effectiveness in controlling listerial growth on frankfurters during refrigerated storage for 60 days. At the end of storage nisin at 10,000, 7500, 2500, and 156.3 International Units/ml (250-3.9 μg/ml) reduced pathogen counts by >2, >2, 1, and 0 log-cycles respectively. In contrast, Luchansky and Call (2004) incorporated nisin (50,000 IU/in^2) into cellulose casings for frankfurter processing. After 90 days of refrigerated storage, *L. monocytogenes* counts were reduced by 1.15 log CFU/package using nisin-coated casings on frankfurters formulated with lactate and diacetate. However lactate and diacetate without nisin reduced populations by 0.95 log CFU/package at 90 days, and nisin alone reduced populations by only 0.88 log CFU/package, but increased to high numbers by 60 days. This indicated that when incorporating nisin into frankfurter casings, antimicrobial formulations are needed. Excellent pediocin activity has also been shown by incorporating the bacteriocin into food packaging films for use against *L. monocytogenes* on meat and poultry. Using a 7.75 μg/cm^2 concentration, growth of the pathogen could be inhibited for 12 weeks at refrigerated storage (Ming *et al.*, 1997).

In summary, results to these studies demonstrate very promising use of bacteriocins as a means of controlling *L. monocytogenes* on RTE meat products. In addition, the use of incorporating other hurdles in combination with bacteriocins seems necessary for reliable activity against the pathogen. While it is well established that the use of divalent metal cation chelators (i.e. EDTA) improve the activity of bacteriocins against gram-negative

(Stevens *et al.*, 1991; Stevens *et al.*, 1992) and gram-positive bacteria (Parente *et al.*, 1998; Mok *et al.*, 1999), less is known regarding the potential role of surfactants in potentiating the efficacy of antimicrobial compounds.

Use of Surfactants as Antimicrobial Adjuvants to Control *L. monocytogenes*

Surfactants (surface-active agents) function by decreasing the surface tension between polar and nonpolar groups thereby allowing them to come in closer approximation to one another. It is thought that surfactants may heighten bacterial sensitivity to certain antimicrobials by destabilizing the cell membrane, however, the mechanism is not fully understood (Li *et al.*, 2002). In addition, if surfactants are added to antimicrobial dip or spray formulations for foods, there may also be improved "wettability" of the food surface allowing more uniform distribution of the antimicrobial substance (Shefet *et al.*, 1995). Although there are a vast number of surfactants that fall within different classes (i.e. anionic and nonionic), there are a small number that have been intensely studied for the control of foodborne pathogens in various foods (Salager, 2002). Discussion on surfactants will be confined to nonionic surfactants Tween 20 and 80 (polyoxyethylene sorbitan monoesters), and the acid-anionic surfactant sodium lauryl sulfate (SLS), because of their approval for food use, and selection for our studies. Tween 20 and 80 are used in a variety of foods ranging from frozen desserts to vitamins (CFR, 2005b; CFR, 2005c), while SLS is used in products such as edible fats and oils, egg whites, and fumarate-acidified beverages (CFR, 2005d).

It is well established that Tween 20 and 80 have negligible effects on the survival of *L. monocytogenes* and other organisms (Dockstader and Groomes, 1970). Despite the absence of inhibitory action of Tween 20 and 80 against *L. monocytogenes*, researchers have reported they do have an effect on bacterial membrane lipid composition. Li *et al.* (2002) demonstrated a Tween 20-induced increase in the C_{15}/C_{17} and anteiso/iso ratios of the membrane fatty acids of *L. monocytogenes*, indicating a lowering of membrane phase transition temperature. Lactococci grown in the presence of Tween 80 were also shown to have decrease in C_{19} fatty acids accompanied by an increase of fatty with shorter chain lengths, indicating increased membrane fluidity (Kimoto *et al.*, 2002). SLS has antimicrobial activity against *L. monocytogenes* (unpublished data) and other bacteria (Raiden *et al.*, 2003), with heightened effectiveness at acidic pH (Dychdala, 1983). The mechanism of action is not well understood; however, there is evidence for: (i) general denaturation of proteins, (ii) inactivation of essential enzymes, and (iii) disruption of cell membranes, resulting in alterations in permeability (Cords and Dychdala, 1993). The bacterial OM is known to be necessary for SLS resistance, but not entirely impervious to it (Rajagopal *et al.*, 2003), thus gram-negative bacteria are much more resistant to the direct effects of SLS, compared to gram-positive bacteria.

There are several reports in the literature evaluating the use of surfactants in combination with other antimicrobials; however, there is a scarcity of published information involving the use of surfactants against *L. monocytogenes* in RTE meat products. In regard to the use of Tween 20 and 80, variable results are in the scientific literature reporting their use in combination with antimicrobials.

Studies conducted in food systems, that demonstrate improved effectiveness of bacteriocin/Tween treatments, have attributed it to improved food surface wettability, removal of bacterial cells, and a reduction of non-specific binding that would otherwise result in less available bacteriocin amounts (Shefet *et al.*, 1995; Bhatti *et al.*, 2004). For example, *in vitro* studies have indicated that Tween 20 (Mazzotta *et al.*, 1996) and Tween 80 (Sip and Grajek, 2001) enhance nisin and non-lantibiotic divercin activity, respectively. In broth media, Li *et al.* (2002) attributed an increase in nisin sensitivity of *L. monocytogenes* cells to Tween 20-induced improved nisin-membrane binding efficiency where no changes in membrane fluidity were observed. Nisin activity (10 or 50 IU/ml) against *L. monocytogenes* was improved by the use of Tween 80 in milk (Jung *et al.*, 1991) but was antagonized in tomato juice against *Bacillus coagulans* (Henning *et al.*, 1986). Because fat reduces nisin activity, as previously mentioned, the authors of the former study suggested that the surfactant displaced nisin from fat globules thereby making it available for activity. This was supported by previous studies demonstrating the ability of Tween 80 to displace proteins from the milk fat globule (Mulder and Walstra, 1974). These two studies indicate that depending on food type, surfactants can either improve or reduce activity of bacteriocins. In regards to pediocin, Degnan *et al.* (1993) found that liposome encapsulation of it in Tween 80 increased activity against *L. monocytogenes* in dairy products and on beef. Differences in the type of antimicrobial used can also be affected by use of Tween 80 and other surfactants. For example, surfactants form micelles in which solute molecules may be solubilized within, thereby preventing the agent from interacting with bacterial cells. This was suggested when Tween 20 and 80 were shown *in vitro* to

compromise the antimicrobial activity of bile, essential oils, phenols, surfactants, organo-tin compounds, and anilides (Kimoto *et al.*, 2002; Hammer *et al.*, 1999; Inouye *et al.*, 2001; Juven *et al.*, 1994; Ishizeki *et al.*, 1973) against organisms other than *L. monocytogenes*. In contrast, encapsulation of carvacrol and eugenol in other nonionic surfactants has been shown to improve activity against *L. monocytogenes* (Gaysinsky, 2005a; Gaysinsky, 2005b).

Utilization of SLS has mainly been applied to poultry carcass washes, however results in our laboratory have shown synergistic activity with lactic acid on frankfurters (unpublished data). Tamblyn and Conner (1997) used an aqueous solution of 125 ppm SLS and 0.5% lactic acid against *Salmonella* Typhimurium on broiler chicken skin surfaces, demonstrating reductions between 0.52 and 1.54 log CFU/skin depending on temperature and bacterial attachment. Hill and Ivey (1988) patented a method for controlling *Salmonella* spp. on meat carcasses during processing that involves immersing the carcass in an aqueous solution of surfactants including SLS at pH below 4.0 at 45-60°C. They also suggested that this treatment can be used at lower temperatures for an appropriate amount of time. A similar acid-synergist approach was tested against various bacterial strains in a study of Restaino *et al.* (1994) where the antimicrobial properties of a buffered organic acid anionic surfactant (BOAAS), containing SLS, citric acid, and EDTA, was compared with six other conventional sanitizers. This antimicrobial treatment displayed superior activity compared to other sanitizers against foodborne pathogens, including *L. monocytogenes*. The initial numbers of the pathogens were all reduced by >5 log-cycles on the surface of artificially contaminated Formica countertop after 30-sec exposure to a 0.6% solution of

BOAAS. Results to the aforementioned investigations indicate that depending on the components used, surfactants can heighten the efficacy of antimicrobial compounds.

METHANOBACTIN

Methanotrophic Bacteria

Methanotrophic bacteria, or methanotrophs, are a subset of a physiological distinct group of bacteria known as methylotrophs. Methanotrophs are gram-negative bacteria that are unique in their ability to utilize methane as a sole carbon and energy source. Methylotrophic bacteria are those aerobic bacteria that utilize one-carbon compounds more reduced than formic acid as sources of carbon and energy and assimilate formaldehyde as a major source of cellular carbon (Hanson and Hanson, 1996; Hou, 1984). Classification of methanotrophs into five genera was proposed by Wittenbury *et al.* (1981; 1984; 1970), with a sixth genus added more recently (Bowman *et al.*, 1993; Bowman *et al.*, 1995). Separations are based on differences in morphology, resting stage type, fine structures of intracytoplasmic membranes, and physiological characteristics, such as formaldehyde assimilation pathway and complete TCA cycle status (Hanson and Hanson, 1996). Methanotrophs are further divided into groups I, II, and X, where type X assimilates formaldehyde primarily via the ribulose monophosphate (RuMP) pathway like type I, but is distinguishable by having low levels of enzymes of the serine pathway. Type X can also grow at higher temperatures (i.e. 45°C) than the other types (Wittenbury *et al.*, 1981; Wittenbury *et al.*, 1984; Hanson and Hanson, 1996). Cell morphology of methanotrophs ranges from cocci and rods to ellipsoid and pear-shaped. Intracytoplasmic membrane

arrangements can be bundles of vesicular disks or paired membranes aligned to the periphery of cells. In addition, many form resting stages of which there are three types: exospheres, and two types of cyst (lipid and azotobacter-type), which are desiccation- and/or heat-resistant (Hou, 1984).

Methanotrophic bacteria can be found in mud, swamps, water, and vegetation (Hanson and Hanson, 1996) and are responsible for the oxidation of biologically-generated methane (Soehngen, 1906); therefore they are of great environmental importance to reduce the amount of this greenhouse gas released to the Earth's atmosphere. In addition to these ubiquitous organisms playing an integral part of global carbon cycling, their potential use in single-cell protein (SCP) production has been evaluated (Tusé, 1989). SCP can be produced directly from the methane or methanol fraction of natural gas by cultivating methanotrophic bacteria. Methanol seems to be a preferable substrate due to safety considerations, mass transfer problems, and lesser yield associated with growth on methane (Powell and Rodgers, 1984). Some advantages of using methanotrophic bacteria versus methanol-utilizing yeasts in SCP production include: (1) The yield of yeast can be as much as 25% lower than the yield of bacteria per gram of methanol, (2) the yield of yeast is lower than yield of bacteria per mole of oxygen, (3) yeast methanol dehydrogenase (DH) has a lower affinity for oxygen and methanol, thus it can become limiting, and (4) lower overall efficiency of the yeast reaction leads to a requirement for extra cooling in the fermentor (Powell and Rodgers, 1984).

The enzymes used in the first step of methane metabolism are known as methane monooxygenases (MMOs). These enzymes catalyze the oxidation of methane to methanol.

It is well established that there are two types of MMOs: membrane-associated or particulate MMO (pMMO) and the cytosolic or soluble MMO (sMMO; Lontoh and Semrau, 1998; Lieberman and Rosenzweig, 2005a; Ward *et al.*, 2004; Lieberman and Rosenzweig, 2005b; Murrell *et al.*, 2000; Hanson and Hanson, 1996). While virtually all methanotrophs employ pMMO, not all methanotrophs possess the genes for sMMO. This competitive advantage is known to be carried in a number of type II and X methanotrophs (Hanson and Hanson, 1996), as well as a couple of type I methanotrophs (Fuse *et al.*, 1998; Shigematsu *et al.*, 1999). Particulate MMO is found in the extensive intracytoplasmic membranes and has a more narrow substrate specificity than sMMO. The latter can oxidize alkanes, alkenes, and aromatics of up to eight carbons, while the former cannot oxidize aromatics (Murrell *et al.*, 2000). Expression of the two enzymes is highly dependent on copper availability. Under conditions in which the copper-to-biomass ratio is low (under "low-copper" growth conditions), sMMO is expressed. In addition, sMMO activity is more pronounced in high cell density fermentations and may be repressed by the presence of copper (Murrell *et al.*, 2000; Jahng and Wood, 1996). This enzyme is well characterized and its active site has been elucidated, in contrast to pMMO. The more ubiquitous enzyme, pMMO, is expressed under "high-copper" growth conditions. While the crystal structure of *Methylococcus capsulatus* (Bath) pMMO was recently determined to a resolution of 2.8 Å, its active site is still under considerable debate despite 20 years of research (Lieberman and Rosenzweig, 2005a). In non-methanotrophic organisms, copper metabolism is geared towards accumulation, exclusion, extrusion, and/or detoxification. These mechanisms are well established for *E. coli*, *Pseudomonas syringae*, and *Enterococcus hirae*. Extensive reviews

can be found on this subject (Cooksey, 1993; Rensing and Grass, 2003; Lu *et al.*, 2003; Harrison *et al.*, 2000; Nies, 1999; Vulpe and Packman, 1995; Puig *et al.*, 2002); therefore, no further details will be discussed in this literature review. In contrast to most other microorganisms, copper plays a very important role in methanotroph physiology; however, it is believed they are not typically starved for copper in the environment (Berson and Lidstrom, 1996; Fitch *et al.*, 1993). Despite this, it has been suggested by numerous researchers that these organisms may possess a specific copper-trafficking system due to their high copper dependencies. In addition, difficulty in determining the pMMO active site has likely stemmed in part from the presence of the recently characterized and currently recognized compound, methanobactin.

Chronological Characterization of Methanobactin

To better understand the physiological role and ecological significance of copper regulation in methanotrophic *M. trichosporium* OB3b, Fitch *et al.* (1993) used five previously isolated mutants (Phelps, *et al.*, 1992) that exhibited constitutive sMMO activity in the presence of copper; an activity that would normally be repressed in wild-types. It was suggested that the lack of effects on growth rate and sMMO expression due to copper in these mutants was not a result of increased stability of sMMO to copper deactivation. They proposed that the mutant phenotype arose from defects in copper uptake and metabolism rather than from changes in sMMO expression or enzyme stability. Furthermore, the researchers noted that extracellular copper solubilization occurred in the media of mutant cultures and suggested that the organism was excreting a Cu(II)-

complexing agent(s), perhaps analogous to siderophores in Fe(III) metabolism (Bagg and Neilands, 1987). They further suggested that the increase in soluble copper in the mutant cultures may be due to either the overproduction of such agents or to a defect that prevents the cells from internalizing the complexes with copper. This is the first report demonstrating phenomenon indicative of methanobactin existence.

Zahn and DiSpirito (1996) investigated procedures to solubilize and isolate pMMO from another methanotroph, *M. capsulatus* (Bath), and subsequently analyzed the active preparations. Their findings included the identification of three pMMO polypeptides that were expressed during the switchover from low- to high-copper media. The study provided evidence that both iron and copper are part of the active site in pMMO and most of the copper was found to be loosely bound to pMMO by association with the first described, copper-binding cofactor (CBC). Those researchers first identified the presence of an extracellular copper-complexing agent that co-purified with pMMO and, at that time, was thought to be a dimer composed of two 618-Da peptides (~1200 M_r) that bound 2-3 copper atoms. In copper-free medium, the CBC was predominately observed in the extracellular fraction, and when concentrations of CBC decreased in the spent media, this difference was attributed to association with membrane fractions. It was concluded that CBC may provide a secondary function, such as stabilize pMMO, maintain a particular redox state, or sequester copper. These hypotheses fueled the need for future research.

Further work (DiSpirito *et al.*, 1998) on these so-called copper-binding compounds/cofactors (CBCs) from *M. trichosporium* OB3b provided preliminary structural evidence and spectral properties. Two were identified (CBC-L_1 and CBC-L_2) and were

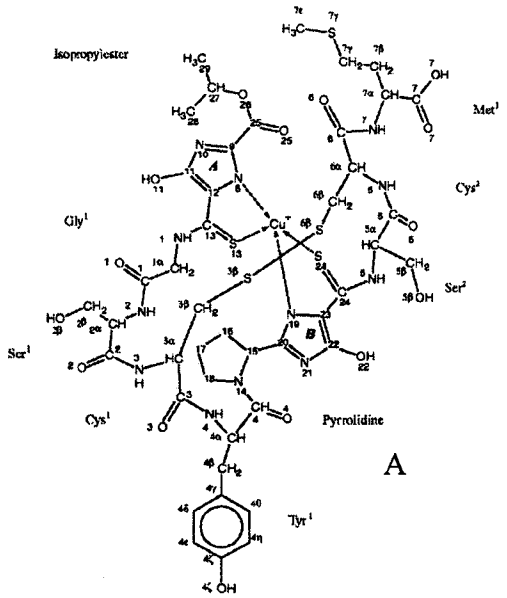
found to be identical to the CBC from *M. capsulatus* (Bath) previously isolated by this group. Results showed that the concentration of CBC in the spent media was highest in cells expressing the pMMO and stressed for copper (5 μM $CuSO_4$), which is at the switchover point between the expression and subsequent activity of the two MMOs. These growth conditions led to a light yellow colored-media due to high concentrations of CBC, where it was even more pronounced in mutant cultures. In addition, yellow halos could be observed around $sMMO^c$ mutant colonies as a result of extracellular copper-bound CBC. Complete amino acid sequencing could not be achieved due to the presence of non-peptide components, and fractions were generated as breakdown components of CBC-L_1. In addition, copper titration experiments revealed that the larger CBC bound one copper ion and that their previous studies overestimated the binding stoichiometry. In agreement with the aforementioned studies, $sMMO^c$ mutants were deficient in copper uptake. While nonprecipitable copper increased in the growth media, little to no expression of the three pMMO polypeptides was observed, and when cultured in high-copper media, mutant CBC was found to be copper-bound. It was concluded that the mutants may be deficient in copper uptake by the inability to bind copper-containing CBCs from the extracellular media, possibly through pMMO. In addition, because pMMO activity was lost when CBCs were removed from the pMMO polypeptides, it was suggested that CBCs may function as a cofactor for its activity or stability, scavenge oxygen radicals, as well as fulfilling possible secondary roles previously mentioned. Earlier that same year, Téllez *et al.* (1998) isolated what they referred to as a copper-binding ligand (CBL) from *M. trichosporium* OB3b, thought to be a same compound described by DiSpirito *et al.* (1998). This study described

a ~500-MW compound that had a ligand preferring copper over nickel despite 20-fold concentrations of the latter. Wild-type cultures produced it maximally when growth conditions were copper-limited while it was found in high concentrations in the spent of mutants regardless of copper concentration. In addition, when copper was added to the growth media of wild-types, the CBL was found to be reinternalized or utilized by the organism suggesting it being part of a copper-acquisition system, whereas the mutants appeared incapable of this activity.

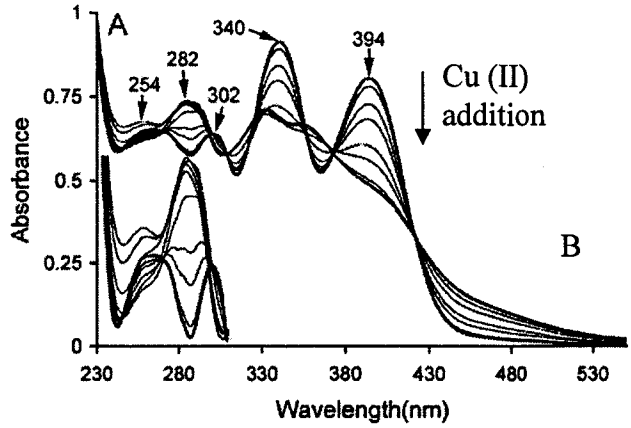
The development of improved purification procedures with higher activity for pMMO of *M. capsulatus* (Bath) by Choi *et al.* (2003) led to further description of the copper role in morphological changes, copper acquisition, fatty acid concentration, and pMMO and sMMO expression in methanotrophs. Continued study on CBC revealed that the compound displayed strong superoxide dismutase-like activity similar to or greater than that of the actual enzyme or copper complexes used for this activity. It was found that copper-free CBC did not scavenge $O_2^{\cdot-}$ while copper-bound CBC did.

Ph. D. research work on *M. trichosporium* OB3b by Kim (2003) led to the isolation, structural elucidation, and characterization of this copper-complexing agent (Kim *et al.*, 2004; Kim *et al.*, 2005). Studies revealed that previously identified CBCs and CBL were actually breakdown products of the primary molecule, with the exception of CBC-L_1—now called methanobactin for its similarities with pyoverdin class siderophores (pseudobactin and azotobactin). Previous attempts to isolate the compound used low-pH conditions leading to breakdown, which has been reported to also occur during purification of other compounds as well (Demange *et al.*, 1988). Shown below (Figure 1) is the schematic

drawing of copper-bound methanobactin (Mb-Cu) and UV-Vis absorption spectra of Mb-Cu with Cu:Mb molar ratios ranging from 0.01 to 1.0 Cu(II) atom per Mb.



Adapted from Kim *et al.* (2004)



Adapted from Choi *et al.* (2006)

Figure 1. Schematic drawing of methanobactin (A) and UV-Vis absorption spectra of methanobactin as isolated and upon successive copper (as CuCl or $CuSO_4$) additions from 0.01 to 1.0 molar equivalents (B).

These two studies (Kim *et al.,* 2004; Kim *et al.*, 2005) revealed that Mb-Cu is a small (1217 Da), fluorescent chromopeptide with a coordination environment containing one Cu^+ ion in a dual nitrogen- and sulfur-donating reducing ligand system derived from the thionyl imidazolate moieties. The primary sequence of Mb-Cu is *N*-2-isopropylester-(4-thionyl-5-hydroxy-imidazole)-Gly^1-Ser^2-Cys^3-Tyr^4-pyrrolidine-(4-hydroxy-5-thionyl-imidazole)-Ser^5-Cys^6-Met^7, with an empirical formula of $C_{45}N_{12}O_{14}H_{62}Cu$. The authors described the overall structure as a compact pyramid-like shape with the metal complexation site located at the base of the pyramid and not buried. The isopropylester

group folds underneath creating a tail-like projection and cleft, and obscures the metal site somewhat. The pyrrolidine allows a bend in the overall chain similar to that of proline. It was also noted that the thioamide bonds associated with the imidazole groups resemble those found in the antibiotics promoinducin and thiostreptone, which are inhibitors of protein synthesis (Chiu *et al.*, 1999). Specifically, EDTA-dialyzed methanobactin revealed UV-Vis spectrum characteristics such as: absorption maximum at 340 nm, a shoulder at 394 nm, featureless absorption in the 240-280 nm range, and no features beyond 400 nm. Exposure to UV light for extended periods of time resulted in complete loss of 394 nm absorbance, suggesting methanobactin is photosensitive in the apo-form (i.e. not bound to copper). Successive additions of $CuCl_2$ resulted in the loss of three characteristic peaks at 394, 340, and 281 nm and the appearance of new features at 335, 290, and 255 nm, where loss was most evident at 388 nm. The 255-nm peak indicates the presence of di-sulfide bond between the two cysteine residues upon copper addition. Saturation with copper also revealed peaks at 333 and 356 nm, where at pH $\leq$5.0 the latter peak was lost relative to former indicating reformation of apo-methanobactin. Fluorescence emission spectra revealed that λ_{ex}= 282 and 342 nm corresponded to tyrosine and chromophore component, respectively. In regards to its effect on growth parameters of *M. trichosporium* OB3b, downshifting copper availability during culturing shortened lags and increased growth rates as the Cu:Mb supply ratio approached 1:1, further supporting that this is the optimum binding stoichiometry. These authors proposed that because methanobactin fulfills the analogous role of iron-binding siderophores, it should be the first to be placed into a new class of compounds called chalkophores (copper in Greek).

Up until this point, studies on methanobactin suggest that it fulfills roles in copper-trafficking: (1) Accumulates to high amounts in the growth media, but is rapidly internalized when copper is provided, (2) stimulates growth in copper-grown cultures with an optimal 1:1 copper:Mb binding stoichiometry, (3) copper uptake-deficient mutants accumulate it in growth media in the presence of copper, and (4) removal of it from pMMO results in irreversible loss of pMMO activity in cell-free systems. Furthermore, the compound, when bound to copper, may be internalized to the cell, possibly in association with pMMO (6-8 Mb-Cu per pMMO), and also may fulfill a secondary roles as a oxygen radical scavenger, pMMO stabilizer, and/or maintain the appropriate redox state of the enzyme. In 2004, the genome sequence of *M. capsulatus* (Bath) was solved (Ward *et al.*, 2004). This project revealed the presence of a non-ribosomal peptide synthetase (NRPS), suggested to be involved in the production of methanobactin. The starting module contained an adenylation domain, a thiolation domain, and unusual acetyltransferase domain. They suggested that the starting module may interact with a second module that contains a condensation domain and terminal thioesterase needed for peptide release, which would lead to the release of methanobactin.

More recent studies (Hakemian *et al.*, 2005; Choi *et al.*, 2005; Choi *et al.*, 2006; Choi *et al.*, submitted for publication) have helped further characterize the physiological role(s) of methanobactin. For example, studies have indicated that apo-methanobactin from *M. trichosporium* OB3b can load Cu(I) or Cu(II), where only Cu(I) is found in Mb-Cu. It was suggested that Cu^{+2} reduction to Cu^{+1} may be coupled to disulfide bond formation in methanobactin (Hakemian *et al.*, 2005). Choi *et al.* (2005) established improvements in the

purification of methanobactin from either *M. trichosporium* OB3b or *M. capsulatus* (Bath). Studies revealed that Mb is a redox-active compound that could stimulate methane-oxidation activity of both whole-cell and cell-free fractions from the latter organism expressing pMMO, where apo-methanobactin and copper-to-Mb ratios <0.5 Cu(II) per Mb did not. Electron paramagnetic resonance (EPR) spectra differed depending on copper-to-Mb ratios, where <0.4, Cu(II) addition to Mb showed an initial coordination by both sulfur and nitrogen. This was followed by reduction to Cu(I) in <2 minutes, but at ratios between 0.4 and 0.9 (Cu(II):Mb) there was more nitrogen coordination. Preliminary experiments revealed that Mb may bind copper as a homodimer (i.e. Mb-Cu-Mb, followed by the binding of a second Cu(II), resulting in a final molar ratio of 1:1). When Mb-Cu was a homodimer, (i.e. copper:Mb ratios <0.6), pMMO activity was inhibited, but at 0.6-0.8 molar ratios, Mb-Cu stimulated pMMO. In addition, this study was the first to report solid evidence of Mb-Cu-pMMO interaction (possibly between Mb-Cu and the type II Cu(II) centre of pMMO) based on increased electron flow to pMMO, increased free radical formation following O_2 addition, and decreased free radical formation when O_2 and CH_4 were added; all due to Mb-Cu activity.

Further studies by this group (Choi *et al.*, 2006; Choi *et al.*, submitted for publication) have revealed that unlike siderophores, methanobactin can bind a variety of transition metals but to a lesser extent than Cu(I) or Cu(II), including: Au(III), Co(II), Cd(II), Fe(III), Hg(II), Mn(II), Ni(II), U(VI), and Zn(II), but not Ca(II), Cr(VI) and Mg(II). Most metals were found to bind methanobactin differently than that of copper, where the proposed copper-methanobactin binding model is as follows: methanobactin binds Cu(II)

initially as a dimer via the 4-thiocarbonyl-5-hydroxy imidazolate (THI) moieties, Cu(II) is reduced to Cu(I), change in coordination to involve the two 4-hydroxy-5-thiocarbonyl imidazolate (HTI) groups, addition of a second Cu(II) to the dimer formation changing coordination to a dual N and S system with subsequent release of two Mb-Cu monomers. These authors also suggested that methanobactin may be a so-called "moonlighting protein" due to the lack of success in assigning a singular role for this molecule. As mentioned, Mb has been shown to act as an oxygen radical scavenger and increase the rate of electron flow to pMMO, participate in a specialized copper-trafficking system by sequestering copper for subsequent uptake, and possibly serve as a copper chaperone or regulatory protein for pMMO. Moonlighting proteins are defined as proteins that can carry out more than one unrelated function (Jeffery, 1999). Roles can differ depending on cell location, oligomeric state, ligand or substrate concentration, change in physical environment, and/or complex formation with other proteins. With the unique structure and the possibility of multiple physiological roles of this compound, it is tempting to speculate that it may also possess some antimicrobial properties as well. Siderophores are known to possess antimicrobial properties, where they typically involve reducing iron availability to target organisms. Some of the literature pertaining to this is discussed in the following section.

Although methanobactin was first isolated ten years ago, its characterization has come about within the last few years. Work will continue to further characterize its physiological roles and potential applications. In addition, methanobactin is found in *M. trichosporium* OB3b, *M. capsulatus* (Bath), and more recently, *Methylomicrobium album* BG8 (Choi *et al.*, 2005). This suggests that methanobactin may be a commonality among

methanotrophic bacteria, and the possibility exists that other organisms might produce similar compounds.

Antimicrobial Activity of Siderophores

There are a limited number of studies specifically investigating the antimicrobial properties of the iron-chelating agents, siderophores, in purified and known quantities. Much of the related research deals with the use of natural and synthesized siderophore-antibiotic conjugates for improved delivery in medical applications (Miller *et al.*, 1991; Braun, 1999; Braun and Braun, 2002), rather than use for controlling microorganisms in foods. Furthermore, in these applications, little antimicrobial activity is associated with the siderophore component when used as a carrier. Similarly, Thomas *et al.*, (2004) discovered the first of a new class of bacterial-derived antimicrobials, called siderophore-peptides. It was found that producer strains (*K. pneumoniae* or recombinant *E. coli*) possessed the machinery to post-translationally modify the bacteriocin, Microcin E492, into a catechol-type siderophore-mimicking compound. Activity against sensitive bacteria was attributed to uptake through any three catechol-type siderophore receptors located on the OM.

In vitro investigations of the antimicrobial activity of siderophores have led to varying results. Hartzen *et al.* (1989) found that the siderophore deferoxamine, alone at 200 µg/ml, had no effect on *S. aureus*, unless it was used in combination with ascorbic acid. They also noted that inhibitory concentrations of these combinations only had an effect for 6 h, where bacterial growth could overcome the compounds at 24 h, unless additional test compounds were added during the assay. The use of the pyoverdin-producing (a

siderophore) bacterium, *Pseudomonas fluorescens*, has been shown to be suppressive towards the disease-causing wheat fungus, *Gaeumannomyces graminis* var. *tritici* (Hamdan *et al.*, 1991), however, the importance of siderophore production was found to be negligible for the inhibition of the pathogen. In contrast, Cheng *et al.* (1995) isolated 3 strains of this siderophores-producing organism from chicken and ground pork. They found that the siderophores inhibited the growth of *L. monocytogenes* and several gram-negative foodborne pathogenic bacteria by way of restricting iron availability. In regards to *L. monocytogenes*, Coulanges *et al.* (1998) demonstrated that the iron-chelating agents, tropolone and 8-hydroxyquinoline, could also inhibit the growth of *L. monocytogenes* but could be antagonized if other siderophores were included. Buyer and Leong (1986) demonstrated antagonism between various *Pseudomonas* spp. on solid growth media, due to the production of siderophores, however, the actual amounts involved were not mentioned. Similar findings were reported by Tapia-Hernández *et al.* (1990) where siderophores produced by *Azospirillum brasilense* were attributed to possess some antibacterial activity against strains of *E. coli* and *Pseudomonas* spp. Manwar *et al.* (2004) used paper disks soaked in cell-free filtrate, containing 0.24 mg/ml siderophore, produced by *Pseudomonas aeruginosa*. They found satisfactory zones of inhibition on plates containing various plant-pathogenic fungi. Studies such as these have found a minimal antimicrobial effect for siderophores alone, and observed activity attributed to the siderophores binding iron, rendering it unavailable for susceptible organisms, thereby causing iron starvation.

Peptide antibiotics have been suggested to normally act as carriers of ions through membranes in the producer organisms, which agrees with the strong metal binding properties of peptide antibiotics, and their ability to modify membrane permeability (Haavik and Froyshov, 1975; Weinberg, 1957; Bodanszky and Periman, 1969; Katz and Demain, 1977). Ornibactin, yersiniabactin, mycobactin, and pyochelin are both siderophores and virulence factors for the producer-organisms *Burkholderia cepacia*, *Yersinia pestis*, *Mycobacterium tuberculosis*, and *Pseudomonas* spp., respectively (Moffitt and Neilan, 2000). Another compound found in pseudomonads is pyocyanin of *P. aeruginosa*. Interestingly, this compound functions as an iron-chelator, electron transfer agent, and virulence factor in this organism. It was suggested that the redox activity associated with it may be responsible for overlapping activities (Hernandez and Newman, 2001). Although methanobactin is unique and the first in its class, similarities of methanobactin with the aforementioned compounds may reveal that antimicrobial activity may be another role of such a dynamic compound.

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CHAPTER 3. ANTIMICROBIAL EFFICACY OF METHANOBACTIN AGAINST *LISTERIA MONOCYTOGENES* SCOTT A IN LABORATORY MEDIUM

A paper to be submitted to Applied and Environmental Microbiology

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ABSTRACT

Methanobactin is a novel extracellular fluorescent chromopeptide produced by *Methylosinus trichosporium* OB3b, a methane-oxidizing bacterium. This easily culturable organism is important in global carbon cycling and for single-cell protein production. This study investigated the antimicrobial efficacy of copper-bound methanobactin (Mb-Cu) against *Listeria monocytogenes* Scott A in brain heart infusion (BHI) broth and the effect of pH (5.5 to 7.3) on its activity. Minimum inhibitory concentrations (MICs) were determined at 24 h for stationary-phase *L. monocytogenes* cultures in BHI broth (32°C) via use of a broth microdilution method. Growth was monitored spectrophotometrically (595 nm) in 6-h increments and viability was determined after 24-h exposure via surface-plating samples

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onto BHI agar and enumerating colonies after 72 h at 32°C. There was a non-linear relationship between the MIC of Mb-Cu and pH, with *L. monocytogenes* being most sensitive to Mb-Cu at pH 6.0. The MICs of Mb-Cu were 6.58, 7.40, 4.11, and 7.40 mM at pH values 7.3, 6.5, 6.0, and 5.5, respectively. MICs of Mb-Cu exhibited a bactericidal action and resulted in 3.34-, 3.96-, 4.87-, and 4.87-log reductions in populations of *L. monocytogenes* at pH 7.3, 6.5, 6.0, and 5.5, respectively. Overall, reductions in numbers of the pathogen increased with increasing concentration of the peptide. At pH 6.0, Mb-Cu concentrations greater than the MIC, consistently resulted in undetectable levels (>5.00-log reductions) of *L. monocytogenes*. Based on the results of this study, methanobactin, a natural antimicrobial peptide, is bactericidal to *L. monocytogenes* and has good potential for use in food applications to destroy this pathogen.

INTRODUCTION

Listeria monocytogenes is a deadly foodborne pathogen (20-30% mortality rate) that is capable of causing listeriosis in immuno-compromised persons (31). This organism is ubiquitous in nature and can contaminate foods from various environmental sources during food processing, distribution, and storage. The ubiquity of *L. monocytogenes* in the environment and its ability to grow at refrigeration temperatures have made this pathogen a major food safety concern to food processors and public health regulatory agencies (13). *L. monocytogenes* can persist in various niches in meat processing facilities, even after vigorous cleaning and sanitation (30). Consequently, cooked meat products manufactured in well-managed processing plants may be contaminated with *L. monocytogenes* before

packaging (35). Current challenges faced by food processors to control *L. monocytogenes* increase the need for new intervention strategies to destroy this pathogen on ready-to-eat (RTE) meat products.

A wide variety of chemical food additives are used to help prevent the outgrowth of pathogens and spoilage microorganisms in foods. There is, however, an increasing consumer demand for foods that are natural, minimally-processed, and convenient. This challenge is being met by an increase in food preservation research involving the use of naturally-produced antimicrobials, as opposed to conventional chemically-synthesized compounds. Some of the prospective biopreservative candidates for food use include: organic acids (32), plant-derived compounds, such as, peptides (38), spices (24), essential oils (16), and other extracts (11), and compounds derived from bacteria (29). A variety of bacterial metabolites have been evaluated for their antimicrobial properties. Non-proteinaceous compounds include: organic acids, diacetyl, hydrogen peroxide, alcohols, carbon dioxide, and reuterin. Proteinaceous compounds include: various bacteriocins and siderophores (17). Siderophores are iron-chelating agents produced by a wide variety of bacteria, and several research efforts have focused on their potential medical applications for iron deprivation and as antibiotic carriers. Bacteriocins, however, have received a great deal of attention for their capacity to destroy target microorganisms (1, 10). The food industry has paid the most attention to bacteriocins such as nisin, pediocin, lactocin, enterocin, leucocin, and colicin; however, nisin is currently the only bacteriocin that is allowed in foods, in its purified form. In light of the ongoing research involving bacterial-derived proteinaceous compounds for the suppression and destruction of spoilage and

pathogenic foodborne microorganisms, it is apparent that other novel compounds similar to that of bacteriocins and siderophores may also possess antibacterial properties, as well. One of these potential compounds is methanobactin.

Methanobactin is a novel extracellular fluorescent chromopeptide (1154 Da), produced by the methane-oxidizing bacterium, *Methylosinus trichosporium* OB3b. Under copper-limiting growth conditions, this compound accumulates to high amounts in the growth media, however, when copper is provided, copper-bound methanobactin (Mb-Cu) is rapidly internalized (36, 7). Methanotrophic bacteria have high copper requirements and Mb-Cu is thought to bind copper with 1:1 methanobactin:copper binding stoichiometry. It is thought to help mediate copper transport via a copper trafficking system, thus it has been termed a "chalkophore" for fulfilling the analogous role of iron-binding siderophores (23). Methane-oxidizing bacteria have been examined for their use in single-cell protein (SCP) production due to their ease of cultivation. Thus it seems feasible to explore the potential of this compound from an organism such as this, to be used as a biopreservative in foods. Methanobactin has a unique structure, and the characterization of this novel compound has only been recently elucidated (7, 9, 21, 22, 23). To our knowledge there is no published research on antibacterial characteristics of methanobactin. Accordingly, the first objective of this research was to determine the minimum inhibitory concentration (MIC) of copper-bound methanobactin (1217 Da) against *L. monocytogenes* and the effect of pH on its antimicrobial activity. In addition, growth rates were monitored to verify the MIC results and determine if there was any effect on growth. The second objective was to determine if

the effect, if any, was bactericidal or bacteriostatic. Copper-bound methanobactin is referred to as methanobactin throughout this paper unless indicated otherwise.

MATERIALS AND METHODS

Microorganism and inoculum preparation. *L. monocytogenes* Scott A NADC 2045 serotype 4b (human isolate from a 1983 milk outbreak), obtained from the National Animal Disease Center (NADC), Agricultural Research Service (United States Department of Agriculture, Ames, IA), was used throughout the experiment. The culture was maintained as frozen (-20°C) stock in tryptic soy broth (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.6% (wt/vol) yeast extract (Difco; TSBYE) and 10% (vol/vol) glycerol. Prior to each experiment, the stock culture was transferred twice in 10 ml of brain heart infusion (BHI; Difco) broth and incubated at 32°C for 18 h. The inoculum was then diluted 100-fold to achieve a population of ~6.50 log CFU/ml (assay concentration) in BHI (pH 5.5, 6.0, 6.5, or 7.3) that had been previously adjusted with 5 N HCl.

Methanobactin preparation. Lyophilized copper-bound methanobactin samples (referred to as methanobactin throughout this paper) were prepared using a modified protocol previously described by Choi *et al.* (8). Methanobactin samples were transferred to sterile 125-mm screw-capped test tubes, placed in Seward stomacher bags (Seward Ltd., London, England), and held on ice for one hour prior to irradiation. Samples were sterilized via electron-beam irradiation at the Iowa State University Linear Accelerator

Facility, which has a MeV CIRCE III Linear Electron Accelerator (MeV Industrie S. A., Jouy-en-Josas, France). Samples were irradiated at ≥30 kGy in the electron beam mode at an energy level of 10 MeV and an average dose rate of 58 kGy/min. Absorbed radiation doses were determined by the use of 5 (diameter) by 5 mm (length) dosimeter alanine pellets (Bruker Analytische Messtechnik, Rheinstetten, Germany) placed on the top and bottom surfaces of one stomacher bag containing Mb-Cu samples. Immediately after irradiation, the pellets were placed in a Bruker EMS 104 EPR Analyzer to measure absorbed doses by electron paramagnetic resonance. The average absorbed dose was obtained from the arithmetic average of the top and bottom surface readings. Sterile samples were stored at ≤-20°C and held for no longer than 2 months.

Susceptibility assay. The bioassays were performed using a broth microdilution method with some modification (27). Stock solutions of Mb-Cu (1.2%, wt/vol; 9.86 mM) were aseptically prepared in BHI (pH 5.5, 6.0, 6.5, or 7.3) and diluted (2 ml) to final assay concentrations of 8.22, 7.40, 6.58, 5.76, 4.93, and 4.11 mM Mb-Cu followed by two-fold dilutions down to 0.06 mM Mb-Cu. All pH adjustments were performed using 5N HCl. Mb-Cu dilutions were transferred (100-µl aliquots) to 96-well, round-bottom, polystyrene microtiter plates (Beckton Dickinson, Franklin Lakes, NJ). Twenty microliters of freshly cultured *L. monocytogenes* Scott A culture was added to the microtiter wells to give a total well volume of 120 µl. Negative controls used for sterility tests were non-inoculated BHI with or without Mb-Cu, while positive controls were inoculated BHI without Mb-Cu. Copper controls were also used by adding copper sulfate to BHI, in equimolar amounts to

that of Mb-Cu. Plates were covered and incubated statically at 32°C for 24 h. Absorbance was monitored spectrophotometrically (595 nm), after agitation, in 6-h increments using a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). The MIC values for Mb-Cu were determined at four pH values (5.5, 6.0, 6.5, and 7.3) where they were designated as the lowest Mb-Cu concentration at which no turbidity was observed in the wells.

Microbiological analysis. After incubation for 24 h, the contents of the microtiter plate wells were serially diluted in 0.1% peptone water and aliquots of appropriate dilutions were surface-plated, in duplicate, onto plates of BHI agar. All inoculated plates were incubated aerobically at 32°C and bacterial colonies were counted at 72 h.

Data analysis. All experiments were repeated at least twice and the absorbance readings and microbiological counts ($\log_{10}$ CFU/ml) are reported as means. Statistical Analysis System software program (SAS Institute Inc., Cary, NC) was used to identify the presence of significant differences, where data was compared using the mixed procedure. All pairwise differences were adjusted using the Bonferroni method.

RESULTS

Effect of pH on MIC. The broth microdilution assay demonstrated that Mb-Cu inhibits the growth of *L. monocytogenes* Scott A in BHI broth. In addition, antimicrobial effectiveness clearly depended upon the pH of the growth medium. A non-linear

relationship was observed between the pH of the growth medium and MIC of Mb-Cu. The most conducive pH for antimicrobial efficacy was pH 6.0, where the MIC was 4.11 mM Mb-Cu. The normal BHI broth (pH 7.3) proved to be the second-most effective of the pH values tested, displaying an MIC of 6.58 mM Mb-Cu. The highest MIC of Mb-Cu (7.40 mM Mb-Cu) was observed at pH 6.5 and 5.5. These results demonstrate that there was an optimum pH in the middle of the range tested, for improving the performance of Mb-Cu to inhibit *L. monocytogenes* in growth media. Copper sulfate controls displayed no inhibition of the pathogen at 8.22 mM, or below, at any pH, thus indicating that the inhibition of copper-bound methanobactin could not be attributed to the effect of copper ions in solution (data not shown).

Effect of methanobactin on growth of *L. monocytogenes*. Figure 1 displays selected growth rates of *L. monocytogenes* as affected by sub-lethal Mb-Cu concentrations and media pH. As the pH of the media decreased, control growth curves became less pronounced, indicating that the population concentrations were not as high and that the growth rates were also decreased. Overall, this observation held true when culture conditions included Mb-Cu. At pH 7.3, the two highest Mb-Cu concentrations, 5.76 and 4.93 mM, displayed the slowest growth rates; however final absorbance (595 nm) measurements at 24 hours were higher than the control and other treatments. Methanobactin at 4.11 mM displayed an absorbance "bump", higher than other samples, at 18 hours. The lowest 3 Mb-Cu concentrations (0.51, 1.03, and 2.06 mM) had similar profiles, below that of the control. pH 6.5 growth curves show the Mb-Cu treatments,

where the control, as expected, displayed the highest profile, while the 3 lowest Mb-Cu concentrations were similar. The highest concentration (6.58 mM) produced the slowest growth rate of the pathogen; however, based on the absorbance at 24 hours, the final culture population was similar to the control. Methanobactin samples of 5.76 and 4.93 mM also produced slower growth rates (pH 6.5); however, 24-h absorbance readings were greater than the control. The most effective pH tested was 6.0. The pH 6.0 growth curves display substantially lower Mb-Cu concentrations than at other pH values because the MIC was much lower. At this pH, the control group displayed better growth than the treatment group, where growth rates decreased with increasing Mb-Cu concentration. However, *L. monocytogenes* grew nearly as well in the presence of the two highest Mb-Cu concentrations (2.06 and 1.03 mM); similar to the growth trend displayed by the higher concentrations at pH 7.3 and 6.5. *L. monocytogenes* grew the slowest at pH 5.5 where the control in this group grew the fastest. As Mb-Cu concentration increased, the growth rates decreased. The two highest concentrations (6.58 and 5.76 mM) displayed the slowest growth rates, where 6.58 mM displayed the lowest overall absorbance values for all curves. Overall, based on absorbance, sub-lethal amounts of Mb-Cu slowed growth of *L. monocytogenes* in some cases; however, the organism was still able to grow to high populations, similar to that of the control.

Two other observations were made in regards to the absorbance data. The first was that lines displaying higher Mb-Cu concentrations tended to become negative in slope before growth occurred. The other was when final 24-h absorbance readings were plotted against Mb-Cu concentration (Figures 2 to 5), a peculiar trend became apparent. Final

absorbance readings of *L. monocytogenes* exposed to Mb-Cu concentrations in the middle of the range tested, displayed a consistent "dip" but as the concentrations increased to near that of the MIC, an increase was observed. At pH 6.0 and 5.5 this increase was near to or lower than that of the control but at pH 7.3 and 6.5, this increase resulted in a higher absorbance than the control. This does not fully agree with the viability data displayed on the same plots (Figures 2 to 5).

Effect of methanobactin on viability of *L. monocytogenes*. Figures 2 to 5 show log numbers of *L. monocytogenes* survivors after 24-h incubation in BHI (pH 7.3, 6.5, 6.0, and 5.5) containing varying amounts of Mb-Cu. In general, as the pH of the media decreased, *L. monocytogenes* was unable to grow to as high a population, but this difference was slight. At sub-inhibitory methanobactin concentrations, there seemed to be no decrease in viability, after 24 h. In fact, *L. monocytogenes* was able to grow to as a high population as the control groups (~10^9 CFU/ml). The mean initial population was 6.44 log CFU/ml; therefore these group populations increased by ~2.50 log CFU/ml.

Methanobactin appeared to be highly bactericidal towards *L. monocytogenes* but possessed little to no bacteriostatic activity. There was no decrease in viability until the concentration reached the MIC, the lowest concentration at which there was no observed growth. At pH 7.3, Mb-Cu concentrations of 6.58, 7.40, and 8.22 mM reduced *L. monocytogenes* populations by 3.28-, 3.72-, and 4.28-log cycles, respectively. Growth media at pH 6.5 containing 7.40 and 8.22 mM Mb-Cu reduced populations by 3.90- and 3.82-log cycles, respectively. The most promising pH value tested, 6.0, resulted in drastic

reductions in *L. monocytogenes* populations. At 4.11 mM (the MIC) methanobactin, a 4.81-log reduction was observed. At the higher concentrations tested at pH 6.0, all viable counts fell below the detection limit, indicating a >5.00 log reduction. BHI at pH 5.5, containing 7.40 mM Mb-Cu, reduced *L. monocytogenes* counts by 4.87 logs, and 8.22 mM lowered viability below detection (<1.00 log CFU/ml).

DISCUSSION

Mb-Cu is hypothesized to be an agent for copper sequestration and uptake in *Methylosinus trichosporium* OB3b, an analogous function of the iron-binding siderophores, produced by a vast number of microorganisms (21, 22, 23). Studies involving the use of siderophore-antibiotic conjugates have proven quite effective for drug delivery into pathogenic microorganisms (26); however, these applications are not acceptable for use in foods as a means of controlling foodborne microorganisms, nor is the antimicrobial activity associated with the siderophore when used as a carrier. *In vitro* investigations of the antimicrobial activity of siderophores have led to varying results. Hartzen *et al.* (15) found that the siderophore deferoxamine, alone at 200 μg/ml, had no effect on *Staphylococcus aureus*, unless it was used in combination with ascorbic acid (pH 7.3-7.6). They also noted that inhibitory concentrations of these combinations only had an effect for 6 h, where bacterial growth could overcome the compounds at 24 h, unless additional siderophore was added during the assay. Cheng *et al.* (6) isolated 3 strains of this siderophores-producing organisms from chicken and ground pork. They found that the siderophores inhibited the growth of *L. monocytogenes* and several gram-negative foodborne pathogenic bacteria by

way of restricting iron availability. Several studies have demonstrated a minimal antimicrobial effect for siderophores alone, and observed activity has been attributed only to the siderophores binding iron, rendering it unavailable for susceptible organisms, thereby causing iron starvation (5, 14, 25, 33). However, very few studies have used purified siderophores in known amounts to study their antimicrobial effectiveness. This proves difficult when attempting to compare purified amounts of copper-bound methanobactin to non-purified iron-binding siderophores needed to effectively inhibit target microorganisms. Bacteriocins are another class of compounds worth comparing to Mb-Cu due to the fact that they are also proteinaceous compounds produced by many bacteria, and many of which have similar molecular weights.

There have been many bacteriocins evaluated for the use in the inhibition and/or destruction of *L. monocytogenes* but we will focus our comparisons to the most rigorously studied bacteriocins for use in foods. Nisin is well known to possess antilisterial activity and its effect is strain dependent. MICs for nisin on tryptic soy agar (TSA) have ranged from 18.5 to 3000 μg/ml (740 to 120,000 IU/ml), where the value for *L. monocytogenes* Scott A was 300 μg/ml (3). These values are quite lower than the MICs of methanobactin, which were 4.11 to 7.40 mM (5000-9000 μg/ml) depending on the pH of the media; however, a difference in media and methodology may play a role. Pediocin PA-1 inhibited the growth of *L. monocytogenes* at 54.7 AU/ml; however initial inoculum concentrations were only ~10^3 cells/ml (28). Hoover *et al.* (18) demonstrated that 8 different *L. monocytogenes* strains were susceptible to pediocin-producing *Pediococcus* spp. The

authors also noted that one *L. monocyotogenes* strain generated a resistant sub-population after continued exposure to the growth extracts.

In the present study, Mb-Cu has been clearly shown to inhibit *L. monocytogenes* Scott A; however, effectiveness may also differ based on pathogen strain. There are limited similarities of Mb-Cu to other naturally-produced biopreservatives, such as siderophores and bacteriocins. Taking this into account, Mb-Cu is one of the first reported bacterial-derived compounds that has a concrete metabolic function (to provide methanotrophic bacteria with copper under limited conditions), yet also possess such good antilisterial activity. This is what sets this novel natural biopreservative apart from the many others that have been evaluated for controlling foodborne pathogens. The effective concentrations needed to inhibit *L. monocytogenes* may be economically feasible and practical, depending on the pH of the system.

The pH values used in this study were selected because of their relevance in many food systems, especially RTE meats where pH values range from 4.7 to 6.5 (19). We demonstrated that the pH of the suspending medium (adjusted with HCl) influenced the MIC of Mb-Cu against *L. monocytogenes*, where it was effective from pH 5.5 to 7.3, with an optimum at pH 6.0, and slightly increased effectiveness at pH 7.3. This peculiar trend is difficult to explain given the limited knowledge of Mb-Cu's effect on bacterial physiology. According to Kim *et al.* (22), lowering the pH to 5.0 altered the UV-Vis spectrum of Mb-Cu in the 356 nm region to that of metal-free, or apo-methanobactin (Mb), suggesting a loss of copper. Copper coordination is likely prerequisite for stability and antibacterial activity. It is possible that some Mb-Cu activity is lost as the pH approaches 5.5, which could

increase the MIC. Acid sensitivity has also been demonstrated for the iron-binding ligand of the siderophore azotobactin D, produced by *Azotobacteri vinelandii* (12). It is well known that most bacteriocins have improved antimicrobial effectiveness at acid pH, however, this has not always been shown to be true. Interestingly, pH 6.0 has also been shown to be optimal for bacteriocin activity, such as: bavaricin MN-influenced carboxyfluorescein efflux in *L. monocytogenes* lipid vesicles (20), nisin-Z induced K^+ efflux in *L. monocytogenes* (2), and nisin-induced cell death of *Staphylococcus aureus* (34). These researchers did not offer any possible structure-function explanation for this phenomenon. Although it may influence the way in which Mb-Cu interacts with the cell envelope, nothing is known regarding the oxidation state of Mb-Cu and its relationship to pH. Solubility of Mb-Cu in BHI, adjusted to various pH values, did not seem to differ based on visual inspection. Other factors that the pH of the suspending medium may influence are: overall net charge of media proteinaceous constituents, Mb-Cu net charge, media-methanobactin interaction, cell membrane-methanobactin interaction, antimicrobial mode of action chemistry of Mb-Cu, and pH-influenced physiological changes of *L. monocytogenes* cells.

Periodic absorbance (595 nm) measurement of the bacterial suspensions in the microtiter wells during the 24-h incubation period were not only useful for the verification of the MIC values attained, but also provided some information on how Mb-Cu affects the growth rate of *L. monocytogenes*. Spectophotometric monitoring of *L. monocytogenes* growth during exposure to Mb-Cu at various pH values demonstrated that overall there was little significant effect on the growth profiles compared to that of the controls. The growth

curves in Figure 1 show that high sub-lethal concentrations of Mb-Cu seem to increase the lag phase during growth except a pH 6.0. This exception may be attributed to the very low concentrations displayed compared to those at other pH values. Overall, outgrowth of cultures (~10^9 CFU/ml) occurred at all sub-lethal concentrations of Mb-Cu, regardless of pH; this is further supported by the viability data (Figures 2-5). Negative absorbance readings were frequently observed for cultures exposed to sub-lethal Mb-Cu concentrations, and for most absorbance measurements at lethal concentrations (data not shown). Although this may indicate that *L. monocytogenes* was undergoing cell lysis, further studies in buffer have not been able demonstrate this, indicating this could be an artifact of the analysis or possibly due to some cell clumping (data not shown). As previously mentioned, when final absorbance readings were plotted against Mb-Cu concentration (Figures 2-5), a "dip" was observed followed by an increase just before inhibitory concentrations were used. This was consistently observed with limited variation throughout the experiment, as shown by the standard deviations found on these plots. Changes in overall cell shape, size, and aggregation were not seen when examined under a bright-field light microscope (data not shown). This trend might reflect the behavior of *L. monocytogenes* cultures when exposed to Mb-Cu and may help elucidate the mechanism of action of Mb-Cu on *L. monocytogenes* cells.

In general, sub-inhibitory methanobactin concentrations did not affect the viability of *L. monocytogenes*, after 24 h, where results showed growth similar to that of the controls (~10^9 CFU/ml). However, pH of the growth media has a profound effect on the efficacy of this compound for use in destroying *L. monocytogenes* and depending on the pH, Mb-Cu

inhibitory concentrations and higher, resulted in population reductions ranging from 3.28 to >5.00 log-cycles of the pathogen. This indicates that Mb-Cu is bactericidal towards *L.* monocytogenes and displays little to no bacteriostatic activity. These results also demonstrate that in order for methanobactin to have an effect on *L. monocytogenes*, a threshold methanobactin concentration must be exceeded, where *L. monocytogenes* cannot tolerate the presence of the compound at concentrations higher than this value, and is consequently destroyed. In short, the most effective treatment was 4.93 mM Mb-Cu at pH 6.0, resulting in a >5.00-log reduction, however, 4.11 mM Mb-Cu at this pH decreased counts by 4.81 log-cycles. Such drastic cell death may point to methanobactin-cell membrane interaction, however follow-up studies have not shown significant lysis, but only slight leakage of UV-absorbing material (data not shown). Destabilization of the bacterial cell membrane is a pH-dependent mode of action for many antimicrobial peptides, including nisin (1, 2), pediocin (4) and other bacteriocins (20, 37). This notion is further supported by preliminary studies demonstrating that the respiration of *L. monocytogenes* cultures is affected by methanobactin, and at 8.22 mM concentrations, rapid cell death occurs within 1 h (data not shown).

The natural compound methanobactin, produced by *Methylosinus trichosporium* OB3b, has proven to be a very effective biopreservative for the destruction of *Listeria monocytogenes*. The fact that this compound fulfills an essential metabolic role for this ecologically-friendly, producer-organism, sets it apart from many other naturally-produced biopreservatives used to control foodborne pathogens in foods. To our knowledge, this is the first published report of Mb-Cu's antimicrobial activity, leaving many questions to be

answered. Further optimization of the test conditions may allow lower levels of Mb-Cu needed to achieve a desired antimicrobial effect. This may include Mb-Cu in combination with surfactants, chelators, or other food preservation methods (i.e. thermal, irradiation). Studies need to determine the antimicrobial efficacy of Mb-Cu against other foodborne pathogens, especially gram-negative organisms. Safety studies need to be conducted to determine if this compound may have allergenic properties or other toxicological effects. Finally, these results need to be corroborated in real food systems because the required levels to inhibit microbial growth will likely be considerably higher for foods than for culture media. However, Mb-Cu shows promise as a biopreservative to control *L. monocytogenes* given that initial inoculum levels were unrealistically high in this study. There is a good possibility of Mb-Cu isolation on an industrial scale, which will reduce the overall costs of its manufacture. Mb-Cu may be potentially used as a spray or dip for various RTE meat products based on its activity at moderate acidic pH values, or possibly for incorporation into food packaging (active packaging).

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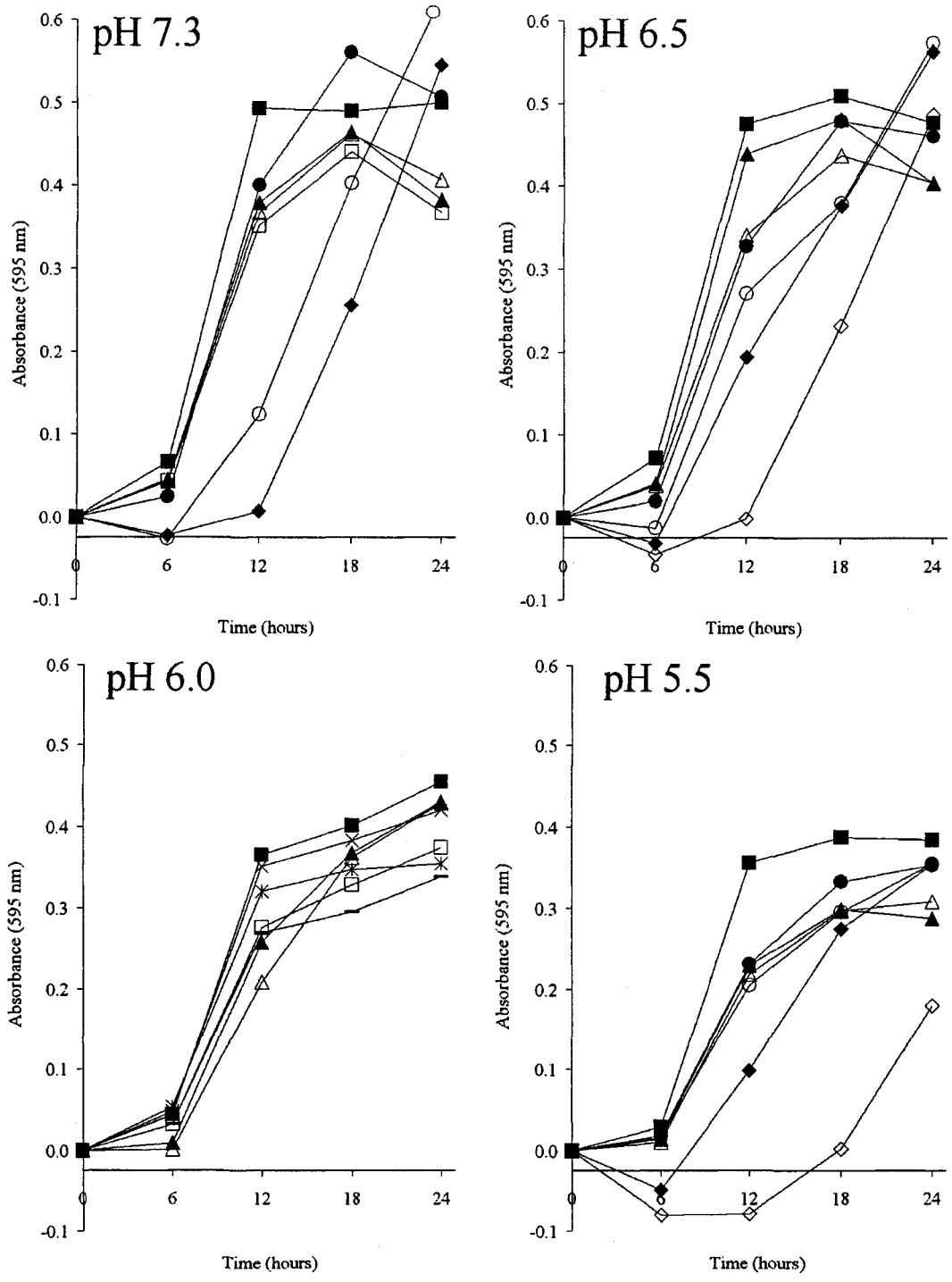

Figure 1. Selected growth curves determined by absorbance (595 nm) of *L. monocytogenes* Scott A bacterial suspensions in BHI at different pH at sub-lethal concentrations (mM) of methanobactin: Control (■), 0.06 (✕), 0.13 (✱), 0.26 (—), 0.51 (□), 1.03 (▲), 2.06 (△), 4.11 (●), 4.93 (○), 5.76 (◆), and 6.58 (◇).

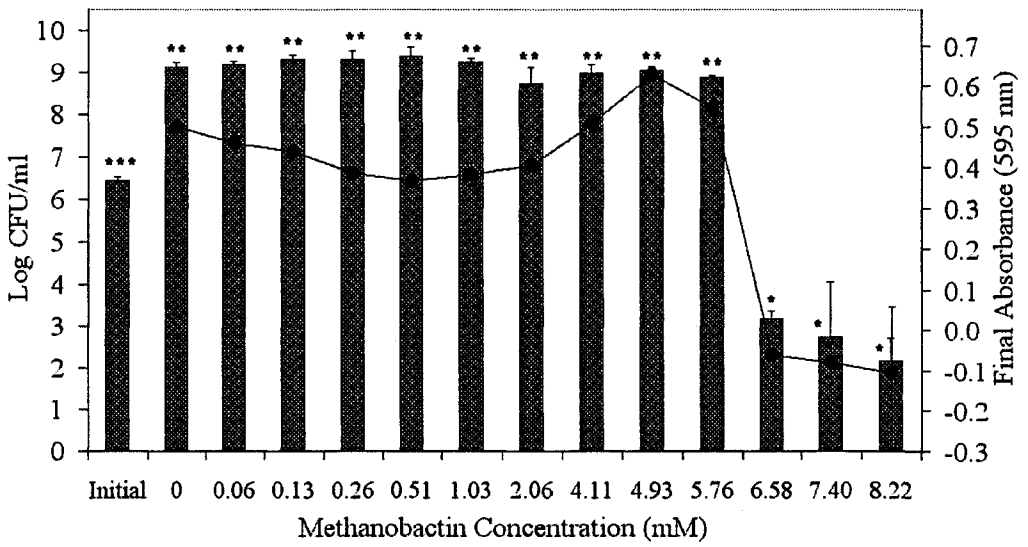

Figure 2. *Listeria monocytogenes* Scott A survivors (bars) following 24-h incubation (32°C) in BHI (pH 7.3) with varying amounts of methanobactin and the final, 24-h absorbance (595 nm) readings (—●—) of the bacterial suspensions in the microtiter wells. Values represent the means of at least three replicates ± SD (error bars) and asterisks indicate significant differences ($P<0.05$) between treatment means.

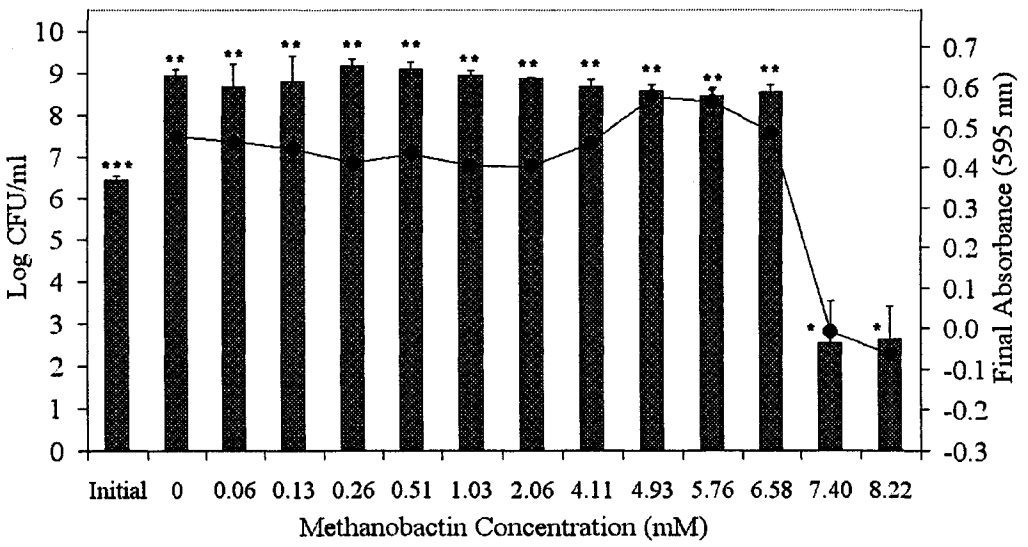

Figure 3. *Listeria monocytogenes* Scott A survivors (bars) following 24-h incubation (32°C) in BHI (pH 6.5) with varying amounts of methanobactin and the final, 24-h absorbance (595 nm) readings (—●—) of the bacterial suspensions in the microtiter wells. Values represent the means of at least three replicates ± SD (error bars) and asterisks indicate significant differences ($P<0.05$) between treatment means.

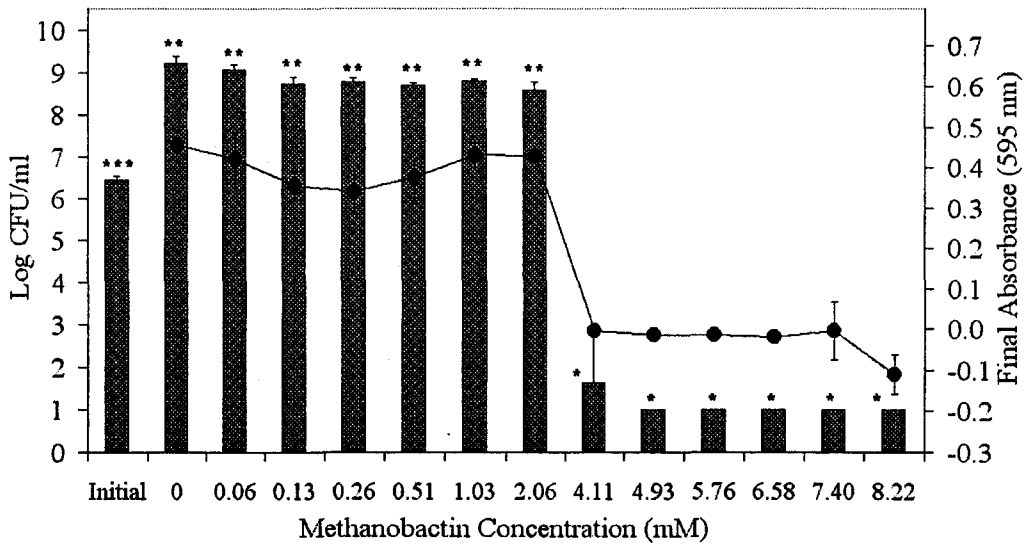

Figure 4. *Listeria monocytogenes* Scott A survivors (bars) following 24-h incubation (32°C) in BHI (pH 6.0) with varying amounts of methanobactin and the final, 24-h absorbance (595 nm) readings (—●—) of the bacterial suspensions in the microtiter wells. Values represent the means of at least three replicates ± SD (error bars) and asterisks indicate significant differences ($P<0.05$) between treatment means.

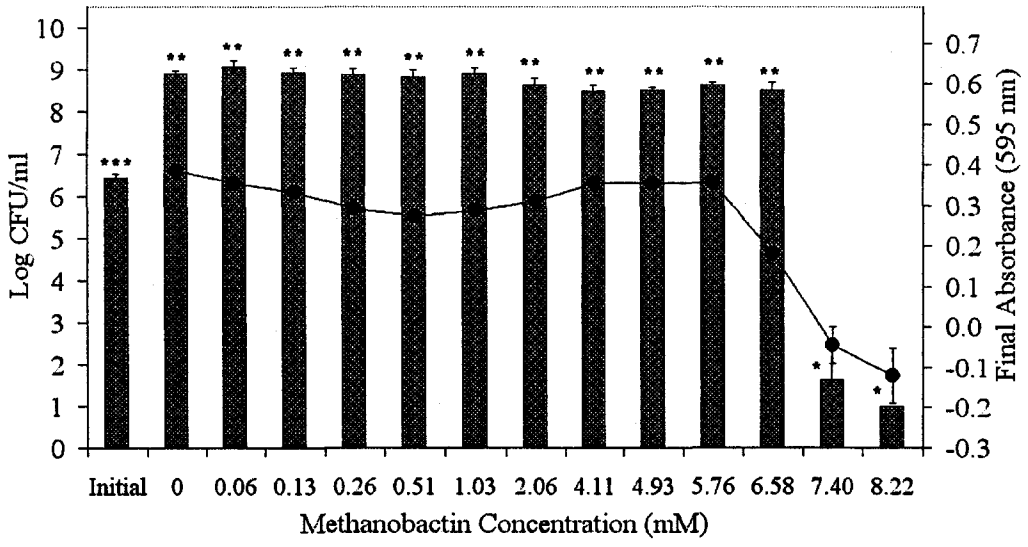

Figure 5. *Listeria monocytogenes* Scott A survivors (bars) following 24-h incubation (32°C) in BHI (pH 5.5) with varying amounts of methanobactin and the final, 24-h absorbance (595 nm) readings (—●—) of the bacterial suspensions in the microtiter wells. Values represent the means of at least three replicates ± SD (error bars) and asterisks indicate significant differences ($P<0.05$) between treatment means.

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CHAPTER 4. BACTERICIDAL ACTIVITY OF METHANOBACTIN IN COMBINATION WITH VARIOUS SURFACTANTS AGAINST *LISTERIA MONOCYTOGENES* SCOTT A

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ABSTRACT

Methanobactin is a novel extracellular chromopeptide produced by the methanotroph, *Methylosinus trichosporium* OB3b, a nonfastidious bacterium important in global carbon cycling and single-cell protein production. This study investigated the effect of pH (5.75 to 6.25) and surfactants (Tween 20, Tween 80, and sodium lauryl sulfate) at 0.25 and 0.50% on the antimicrobial efficacy of copper-bound methanobactin against *Listeria monocytogenes* Scott A in brain heart infusion (BHI) broth. Minimum inhibitory concentrations (MICs) were determined at 24 h for stationary-phase *L. monocytogenes* cultures in BHI (32°C) via use of a broth micro-dilution method. Growth was monitored spectrophotometrically (595 nm) and viability was determined after 24-h exposure via

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surface-plating samples onto BHI agar and enumerating colonies after incubation (72 h, 32°C). The MIC of methanobactin alone was 2.06 mM regardless of pH. Tween surfactants, especially Tween 80, were antagonistic to methanobactin activity. This antagonistic effect was more pronounced with increasing pH and surfactant concentration. In contrast, growth did not occur in samples containing sodium lauryl sulfate (SLS) with or without methanobactin. Synergistic combinations of methanobactin (1.03 mM) and SLS (0.25%), at pH 5.75, reduced *L. monocytogenes* populations by 5.33-log cycles, whereas 2-fold higher concentrations of methanobactin (2.06 mM) without SLS were required to achieve this same reduction. Increasing the pH only slightly reduced the effectiveness of the methanobactin/SLS treatments. These results clearly demonstrate that surfactants, depending on type, can have an antagonistic or synergistic effect on methanobactin activity. A combination of SLS with methanobactin showed improved listericidal activity, demonstrating that this system has good potential for use in food applications.

INTRODUCTION

Listeria monocytogenes is a psychrotrophic foodborne pathogen and the serious illness associated with it has a 20-30% mortality rate (*25*). In addition, food processors find this "hardy" contaminant particularly difficult to eliminate from the processing environment due to its ubiquity in nature. In 2004, the United States Department of Agriculture – Food Safety and Inspection Service (USDA-FSIS) reported that the *L. monocytogenes* Interim Final rule had improved the safety of ready-to-eat (RTE) meats and poultry, where recalls dropped one-third from 2002 to 2004 (*8*). However, earlier this year, the United States

Center for Disease Control and Prevention (CDC) reported that the U. S. fell short of its 2005 goal to reduce cases of foodborne listeriosis by 50% (*9*). In light of this recent announcement it seems clear that there are still improvements to be made in controlling *L. monocytogenes* in foods. Control measures may include exploring newer technologies and food preservation methods as consumer tastes, preferences, and lifestyles evolve. There is an increasing consumer demand for foods that are natural, minimally-processed, and convenient. This trend has sparked research interest involving the use of naturally-produced antimicrobials as a replacement for chemically-synthesized compounds for the preservation of foods.

Some of these biopreservative candidates for food use include: organic acids (*26*), plant-derived compounds, such as, peptides (*29*), spices (*18*), essential oils (*13*), and other extracts (*5*). A variety of bacterial metabolites have been evaluated for their antimicrobial properties where proteinaceous compounds include various bacteriocins and siderophores (*14*). Siderophores are iron-chelating agents and most of the research on these compounds have focused on potential medical applications. Bacteriocins, however, have received a great deal of attention for their capacity to destroy target microorganisms in foods (*4*). Both of these classes of compounds are found widespread in nature, however, nisin is the only bacteriocin currently allowed in foods, in its purified form.

Recent work in our laboratory indicated that methanobactin, a compound produced by the methane-oxidizing bacterium, *Methylosinus trichosporium* OB3b (*15*), possesses antilisterial properties in broth laboratory media. Methanobactin is a novel extracellular fluorescent chromopeptide (1154 Da), that accumulates to high amounts in growth media

under copper-limiting conditions; however, when copper is provided, methanobactin is rapidly internalized (*3, 28*). This compound, now termed a "chalkophore" for its analogous role to iron-binding siderophores (*16*), shows similarity to bacteriocins due to its antimicrobial activity and possession of unique constituents; a characteristic of some bacteriocins. Methane-oxidizing bacteria have been examined for their use in single-cell protein (SCP) production due to their ease of cultivation, thus it seems practical to pursue the potential use of this biopreservative for the destruction of *L. monocytogenes* in foods. We previously found that pH 6.0 permitted the use of copper-bound methanobactin in the lowest amount (4.11 mM), to inhibit the growth of *L. monocytogenes* Scott A. Although this amount is practical and economically reasonable, researchers have used a variety of surfactants and other compounds to help lower antimicrobial concentrations needed to display the same effect (*7, 12, 27*).

Surfactants (surface-active agents) are known to decrease the surface tension between polar and nonpolar groups thereby allowing them to come in closer proximity to one another. It is thought that surfactants may heighten bacterial sensitivity to certain antimicrobials by destabilizing the cell membrane, however, the mechanism is not fully understood (*19*). In addition, if surfactants are added to antimicrobial dip or spray formulations for foods, there may also be improved "wettability" of the food surface allowing more uniform distribution of the antimicrobial substance (*24*). Although there are a vast number of surfactants that fall within different classes (i.e. anionic and nonionic), there are a small number that have been intensely studied for the control of foodborne pathogens in various foods. The surfactants Tween 20 and 80 (polyoxyethylene sorbitan

monoesters), and sodium lauryl sulfate were selected for this study because of their generally-recognized-as-safe (GRAS) status and approval for food use. The objectives of this study were: (1) Determine the effect of different surfactants (at 0.25 and 0.50%), and pH (5.75-6.25) on the antimicrobial efficacy of copper-bound methanobactin (1217 Da) against *L. monocytogenes* Scott A, as measured by minimum inhibitory concentrations (MICs); (2) Monitor growth curves to verify the MIC results and determine if there was any effect on growth via turbidometric measurement; and (3) Evaluate viability of *L. monocytogenes* after exposure to methanobactin treatments by spread plating onto microbiological media.

MATERIALS AND METHODS

Microorganism and inoculum preparation. *L. monocytogenes* Scott A NADC 2045 serotype 4b (human isolate from a 1983 milk outbreak), obtained from the National Animal Disease Center (NADC), Agricultural Research Service (United States Department of Agriculture, Ames, IA), was used throughout the experiment. The culture was maintained as frozen (-20°C) stock in tryptic soy broth (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.6% (wt/vol) yeast extract (Difco; TSBYE) and 10% (vol/vol) glycerol. Prior to each experiment, the stock culture was transferred twice in 10 ml of brain heart infusion (BHI; Difco) broth and incubated at 32°C for 18 h. The inoculum was then harvested by centrifugation (10,000 × *g*, 10 min) in a refrigerated (4°C) centrifuge (Sorvall Super T21; DuPont Instruments, Wilmington, DE). The cells were washed once in 0.02M 2-morpholinoethanesulfonic acid (MES) buffer (pH 5.75, 6.00, or

6.25; Sigma, St. Louis, MO) and diluted 100-fold by resuspension in 0.1M MES buffer (pH 5.75, 6.00, or 6.25) to achieve a population of ~6.50 log CFU/ml (assay concentration) when transferred in 20-µl aliquots.

Surfactant preparation. Stock solutions (1.2%) of Tween 20 (vol/vol; Fisher Scientific, Pittsburgh, PA), Tween 80 (vol/vol; Fisher), and sodium lauryl sulfate (wt/vol; Sigma) were prepared in 0.1 M MES buffer (pH 5.75, 6.00, or 6.25), diluted 2-fold to make 0.6% solutions, and sterilized by autoclaving (121°C, 15 psi, 15 min). From these solutions, 50-µl aliquots were used to achieve assay concentrations of 0.25 and 0.50% surfactant. This was performed for all surfactants at all pH values.

Methanobactin preparation. Lyophilized copper-bound methanobactin samples (referred to as methanobactin throughout this paper) were prepared using a modified protocol previously described by Choi *et al.* (*3*). Methanobactin samples (12 mg) were transferred to 1.5-ml polystyrene microcentrifuge tubes (Eppendorf, Westbury, NY), placed in Seward stomacher bags (Seward Ltd., London, England), and held on ice for one hour prior to irradiation. Samples were sterilized via electron-beam irradiation at the Iowa State University Linear Accelerator Facility, which has a MeV CIRCE III Linear Electron Accelerator (MeV Industrie S. A., Jouy-en-Josas, France). Samples were irradiated at ≥30 kGy in the electron beam mode at an energy level of 10 MeV and an average dose rate of 58 kGy/min. Absorbed radiation doses were determined by the use of 5 (diameter) by 5 mm (length) dosimeter alanine pellets (Bruker Analytische Messtechnik, Rheinstetten,

Germany) placed on the top and bottom surfaces of one stomacher bag containing methanobactin samples. Immediately after irradiation, the pellets were placed in a Bruker EMS 104 EPR Analyzer to measure absorbed doses by electron paramagnetic resonance. The average absorbed dose was obtained from the arithmetic average of the top and bottom surface readings. Sterile samples were stored ≤-20°C and held for no longer than 2 months.

Susceptibility assay. The bioassays were performed according to a broth microdilution method with some modification (*21*). Irradiated methanobactin samples were used to aseptically prepare 1.2% (wt/vol) stock methanobactin solutions (9.86 mM) in 2.4×BHI (pH 5.75, 6.00, or 6.25) that had been previously adjusted with 5 N HCl. Two-fold dilutions of these stock solutions (1 ml) were performed in 2.4×BHI (pH 5.75, 6.00, or 6.25) to give a series of test concentrations ranging from 4.11 to 0.06 mM. Methanobactin dilutions were transferred (50-µl aliquots) to 96-well, round-bottom, polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ). Addition of 50 µl of the surfactant solutions and 20 µl of freshly prepared *L. monocytogenes* Scott A culture were then added to the wells to give a total well volume of 120 µl. An appropriate aliquot of MES buffer (0.1M, pH 5.75, 6.00, or 6.25) was used when treatments required 0% surfactant. Negative controls used for sterility tests were uninoculated BHI with or without methanobactin and surfactant, while positive controls were inoculated BHI without methanobactin. Plates were covered and incubated statically at 32°C for 24 h. Absorbance was monitored spectrophotometrically (595 nm), after agitation, in 6-h increments using a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA). The MIC values for

methanobactin were determined at three pH values (5.75, 6.00, and 6.25) and three different surfactants (each at 0.25 and 0.50%) where they were designated as the lowest methanobactin concentration at which no turbidity was observed in the wells. In all, there were 168 unique treatment combinations tested.

Microbiological analysis. After incubation for 24 h, certain microtiter plate wells were selected for viability determination by aseptically removing the contents and serially diluting them in 0.1% peptone water. Aliquots of appropriate dilutions were surface-plated, in duplicate, onto plates of BHI agar. All inoculated plates were incubated aerobically at 32°C and bacterial colonies were counted at 72 h.

Data analysis. All experiments were repeated at least twice and the absorbance readings and microbiological counts ($\log_{10}$ CFU/ml) are reported as means. Statistical Analysis System software program (SAS Institute Inc., Cary, NC) was used to identify the presence of significant differences, where data was compared using the mixed procedure. All pairwise differences were adjusted using the Bonferroni method.

RESULTS

Treatment effects on the inhibition of *L. monocytogenes*. All MIC results are summarized in Table 1. Regardless of pH, the MIC of methanobactin alone was 2.06 mM, where this was confirmed by no change in absorbance after 24 h. The combination of Tween 20 (T20) and Mb-Cu displayed lesser inhibition of *L. monocytogenes* cultures. As

media pH increased, the MIC of Mb-Cu increased, indicating a higher concentration needed to inhibit the pathogen. This trend also seemed to be dependent on surfactant concentration, as well. At pH 5.75, the MICs of Mb-Cu were 2.06 mM for both concentrations of T20. However, the MIC increased to 4.11 mM with the addition of 0.50 and 0.25% T20, at pH 6.00 and 6.25, respectively. The addition of Tween 80 (T80) had an even more antagonistic effect on the MIC of Mb-Cu than T20 with the MIC seemingly dependent on both pH and surfactant concentration, similar to that of T20. At pH 5.75, the MIC of Mb-Cu with 0.25% T80 was 2.06 mM; however, this increased to 4.11 mM at pH 6.00 and 6.25. The MIC of Mb-Cu increased to 4.11 mM, when combined with 0.50% T80, at all pH values. The use of sodium lauryl sulfate (SLS), at either concentration, did not allow the growth of *L. monocytogenes* cultures, therefore final absorbance and MIC results were unfortunately not obtainable.

Final 24-h absorbance readings (Abs_{final}-Abs_{intial}) of the bacterial suspensions, in the presence of methanobactin with or without T20 or T80, in different pH conditions are shown in Figure 1. A peculiar trend in final absorbance as influenced by Mb-Cu concentration was also observed (Figure 1). As the curves approached the MIC values, there seemed to be a "dip" followed by an upward trend in the final absorbance of the cultures. This was even more pronounced as pH increased and with samples containing T20 and T80. Plotting 6-, 12-, and 18-h absorbance values in this way also displayed the same profile (data not shown). This consistent trend did not correspond with viability data (not shown), where control populations were nearly the same as those exposed to these sub-inhibitory Mb-Cu concentrations. Changes in overall cell size, shape, and aggregation were

not apparent during examination of the cells under a bright-field light microscope. In addition to determining the MIC of Mb-Cu as influenced by pH, surfactant type, and surfactant concentration, growth of cultures exposed to sub-inhibitory concentrations of Mb-Cu with or without surfactants, were also monitored.

Sub-inhibitory treatment effects on the growth of *L. monocytogenes*. Figure 2 displays an example of the growth profiles of *L. monocytogenes* in the presence of sub-inhibitory concentrations of methanobactin without surfactants, in different pH conditions. Overall, control (0 mM) absorbance values decreased with decreasing pH indicating slower growth in more acidic BHI. Populations exposed to the experimental treatments followed the same pattern. The profiles exhibited a reasonable trend, displaying overall reduced growth with increasing Mb-Cu concentration. Culture growth was slowest at 1.03 mM Mb-Cu, but increased with increasing pH, indicating that pH 5.75 was the most effective. The addition of T20 to the growth medium, without Mb-Cu, resulted in only slight decreases in absorbance compared to the control groups, whereas T80 had no effect (data not shown). Growth profiles of *L. monocytogenes* exposed to treatments containing both Mb-Cu and T20 appeared similar to Figure 1; however, the lines displaying different Mb-Cu concentrations were in closer proximity to one another. This was similar for samples containing T80, but to a lesser extent. The concentration of these surfactants did not seem to have an impact on the growth curves, as well (data not shown). As mentioned, growth of *L. monocytogenes* in the presence of SLS could not be examined due to a lack of increase in

absorbancy of the samples. It is worth noting that many of the inhibitory treatments, including SLS, resulted in very noticeable negative absorbance readings.

Treatment effects on the survival of *L. monocytogenes*. Figure 3 displays the survivors for the T20/Mb-Cu treatments in different pH conditions. Control samples grew from the 6.59 log CFU/ml-initial inoculum concentration to 8.97, 9.07, and 9.18 log CFU/ml ($P<0.05$) at pH 5.75, 6.00, and 6.25, respectively. Survivors were not detectable (<1.00 log CFU/ml) at the Mb-Cu MIC (2.06 mM) and above, when used alone at any pH, indicating a >5.59-log reduction for these samples ($P<0.05$). Methanobactin alone, at 1.03 mM, resulted in growth to 8.03, 8.75, and 9.10 log CFU/ml at pH 5.75, 6.00, and 6.25, respectively. Growth at and below 0.51 mM Mb-Cu and below, were similar those of the control groups. T20 alone was similar to the control groups in that it did not affect the growth of *L. monocytogenes*, at any pH. Combining T20 with Mb-Cu increased the tolerance of *L. monocytogenes* to Mb-Cu. In agreement with the inhibition results, T20 antagonism was higher with both increasing T20 concentration and pH. For example, at pH 6.00, addition of 0.50% T20 to 2.06 mM Mb-Cu did not reduce *L. monocytogenes* populations, where 2.06 mM alone resulted in undetectable numbers. At pH 6.25, outgrowth occurred in samples containing 0.25 and 0.50% T20 with 2.06 mM Mb-Cu.

L. monocytogenes survivors following exposure (24 h) to Mb-Cu, T80, and combination in different pH conditions are shown in Figure 4. Viability results demonstrated that T80 was very antagonistic to Mb-Cu activity, and that T80, alone, did not prevent growth in any pH condition. No cells were recovered from the 2.06 mM Mb-Cu

alone. The combination of 0.25% T80 with 2.06 mM Mb-Cu, at pH 5.75, resulted in a 4.07-log reduction of *L. monocytogenes*. However, culture outgrowth occurred when 0.50% T80 was used. When 0.25% T80 was used at pH 6.00, the population increased to 6.92 log CFU/ml, whereas the higher concentration of T20 was required to have this effect at pH 6.00. Complete outgrowth was observed at pH 6.25 when T80 was used in combination with 2.06 mM Mb-Cu.

Contrary to T20 and T80, SLS was effective at enhancing the bactericidal effect of methanobactin against *L. monocytogenes* (Figure 5). SLS (0.25%) reduced initial populations by 1.59-, 2.24-, 2.09-log cycles at pH 5.75, 6.00, and 6.25, respectively ($P<0.05$). The higher concentration (0.50%) of SLS was slightly more effective where it reduced populations by 1.74-, 2.38-, and 2.45-log cycles, with increasing pH; however, these results were not significantly different from those obtained from using 0.25% SLS. The combination of SLS at either concentration, with ≥2.06 mM Mb-Cu, resulted in undetectable numbers at all pH values ($P<0.05$), showing no difference from results produced by using ≥2.06 mM Mb-Cu alone. Outgrowth occurred in samples containing 1.03 mM Mb-Cu alone, however, when combined with 0.25% SLS, viability was reduced by 5.33-, >4.59-, and 4.33-log cycles at pH 5.75, 6.00, and 6.25, respectively. Combination of 1.03 mM Mb-Cu with 0.50% SLS resulted in 5.06-, >4.59-, and >4.59-log reductions, with increasing pH. Overall differences in results were not significant between SLS concentrations. More importantly, synergy occurred within these treatments because outgrowth occurred in the presence of 1.03 mM Mb-Cu alone, but combinations of 1.03 mM Mb-Cu with 0.25% SLS resulted in counts that were 3.74-, 2.35-, and 2.24-log cycles

lower than samples containing only 0.25% SLS, at pH 5.75, 6.00, and 6.25, respectively ($P<0.05$). Once again, increasing the pH lowered the effectiveness of these treatments, and overall differences were not significant between SLS concentration ($P>0.05$). Outgrowth was observed in samples containing 0.51 mM Mb-Cu alone, however, reductions occurred when combined with SLS. The higher SLS concentration (0.50%) was not significantly different than 0.25%, when combined with 0.51 mM Mb-Cu. Populations were 0.58-, 1.85-, and 1.50-log cycles lower than 0.50% SLS alone, at pH 5.75, 6.00, and 6.25, where the mean at pH 5.75 was not significantly lower. Interestingly, the optimum pH was 6.00 for these treatments. Regardless of pH, treatments containing 0.26 mM Mb-Cu and SLS were similar to samples containing only SLS. Overall, the destruction of *L. monocytogenes* was greatest when using 2.06 mM Mb-Cu with or without SLS. However, 1.03 mM Mb-Cu could be used when in combination with 0.25% SLS (pH 5.75), and still achieve 5.33-log reductions. Another practical treatment was 0.51 mM Mb-Cu/0.50% SLS (pH 6.00), where it reduced populations by 4.23-log cycles.

DISCUSSION

We demonstrated that methanobactin effectively inhibited *L. monocytogenes* Scott A at a concentration of 2.06 mM, regardless of the pH conditions tested. The selected pH range was based on results of our previous findings, where at pH 6.00 in BHI, the MIC of Mb-Cu was 4.11 mM (data not shown). The results to this study demonstrated a 2-fold lower MIC value, at this pH, compared to our original study. This difference may be attributed to a difference in experimental conditions. By adding two different test

compounds (Mb-Cu and surfactant), buffers were needed as the diluent for stock solutions and for replacing a compound when particular treatments did not require either Mb-Cu or a surfactant. The use of a buffered system may be responsible for this discrepancy because this was the only difference in experimental conditions. Methanobactin shares limited similarities with other biopreservatives that have been studied for use in controlling foodborne microorganisms. As previously mentioned, methanobactin is hypothesized to be an agent for copper sequestration and uptake in *M. trichosporium* OB3b, an analogous function of the iron-binding siderophores, produced by a vast number of microorganisms (*16*). Very few studies have used purified siderophores in known amounts to study their antimicrobial effectiveness. Those that have used cultures or undefined culture filtrates containing siderophores, have shown little effectiveness (*10, 12*). However, Manwar *et al.* (*20*) found satisfactory zones of inhibition on plates containing various plant-pathogenic fungi, when using paper disks soaked in cell-free filtrate, containing 0.24 mg/ml siderophore, produced by *Pseudomonas aeruginosa*. Bacteriocins are another class of compounds worth comparing to methanobactin due to the fact that they are also proteinaceous compounds produced by many bacteria; many of which have similar molecular weights. Benkerroum and Sandine (*1*) demonstrated that the MICs of nisin against *L. monocytogenes* strains ranged from 18.5 to 3000 μg/ml (740 to 120,000 IU/ml) on tryptic soy agar (TSA). This is fairly comparable, to some to degree, to the MIC of methanobactin, which was 2.06 mM (2500 μg/ml), however the methodologies differed. Pediocin PA-1 inhibited the growth of *L. monocytogenes* at 54.7 AU/ml (*22*), however,

initial inoculum concentrations were only ~10^3 cells/ml, which were over 1000-fold lower than the inoculum concentration used in this study.

Researchers have used a variety of surfactants and other compounds to help lower antimicrobial concentrations needed to inhibit and/or destroy microorganisms. In the present study, we determined the impact of combining T20, T80, or SLS with methanobactin, on the fate of *L. monocytogenes* Scott A. Our data agrees with the well established notion that T20 and T80 have negligible effects on the survival of *L. monocytogenes*. Despite the absence of inhibitory action of T20 and T80 against *L. monocytogenes* reported in the present study, researchers have reported that these surfactants do have an effect on bacterial membrane lipid composition. Li *et al.* (*19*) demonstrated a T20-induced increase in the C_{15}/C_{17} and anteiso/iso ratios of the membrane fatty acids of *L. monocytogenes*, indicating a lowering of membrane phase transition temperature. Lactococci grown in the presence of T80 were also shown to have decrease in C_{19} fatty acids accompanied by an increase of fatty with shorter chain lengths, indicating increased membrane fluidity (*17*). The present study clearly demonstrates that surfactants may have an antagonistic or synergistic effect on the activity of Mb-Cu, dependent upon surfactant type (i.e., nonionic versus acid anionic). Variable results have ensued from literature reporting the use of Tweens, in combination with antimicrobials.

We report that T20 and T80 lower the antimicrobial activity of copper-bound methanobactin against *L. monocytogenes* Scott A. Studies conducted in food systems, demonstrating improved effectiveness of bacteriocin/Tween treatments, have attributed it to improved food surface wettability, removal of bacterial cells, and a reduction of non-

specific binding that would otherwise result in less available bacteriocin amounts (*2, 7, 24*). In broth media, Li *et al.* (*19*) attributed the increase in nisin sensitivity of *L. monocytogenes* cells to T20-induced improved nisin-membrane binding efficiency where no changes in membrane fluidity were observed. Given what is known regarding membrane-induced changes attributed to T20 and T80, it is possible that nonionic surfactants may bind methanobactin rendering it unavailable for activity. For example, surfactants may form micelles in which solute molecules (i.e., Mb-Cu) may be solubilized within, thereby preventing the agent from interacting with bacterial cells. This was suggested when T20 and T80 were shown to compromise the antimicrobial activity of essential oils, *in vitro* (*11*). It is also possible that nonionic surfactants may compete with Mb-Cu for the same site on the bacterial cell. T80 was more antagonistic to Mb-Cu activity in relation to T20. The only structural difference between these two compounds is the type of fatty acid ester they contain. T20 contains laurate (C12:0) while T80 contains oleate (C18:1). This structural difference may be responsible for differences in micellar formation and size, and resultant solubilization of Mb-Cu.

SLS was shown to synergistically enhance the antimicrobial efficacy of Mb-Cu against *L. monocytogenes*. This surfactant is known to be more effective at lower pH, which our data agrees with. The mechanism of action is not well understood; however, the three most commonly cited hypotheses are: (i) general denaturation of proteins, (ii) inactivation of essential enzymes, and (iii) disruption of cell membranes, resulting in alterations in permeability (*6*). The bacterial outer membrane is known to be necessary for SLS resistance, but not entirely impervious to it (*23*), thus gram-negative bacteria are much

more resistant to the direct effects of SLS, compared to gram-positive bacteria. This was shown to be the case for *L. monocytogenes*, where 0.25 and 0.50% SLS reduced populations by 1.59-2.24 and 1.74-2.45 log-cycles, respectively, depending on pH. SLS has also been shown to increase the activity of organic acids against *Salmonella* Typhimurium on chicken broiler skins (*27*) and to enhance the antimicrobial activity of essential oils against *S.* Typhimurium and *Escherichia coli* O157:H7 (*13*).

Compared with neutral surfactants, like T20 and T80, SLS ionizes in solution to where the sulfate groups carry a negative charge. In addition, SLS are linear (straight alkane) in nature compared with the polyoxyethylene sorbitan monoesters. As a result of this, Mb-Cu/SLS interactions exhibit greater antimicrobial activity when compared with Tween surfactants. In fact, the combination of SLS and Mb-Cu may result in mixed micelle formation, in which micellar shells would be composed of both the solute (Mb-Cu) and the surfactant monomers (SLS). This would bring Mb-Cu closer to target sites on bacterial cells because the solute would be at the surface of the micelles. This may also increase the rate of Mb-Cu uptake by *L. monocytogenes* cells, followed by accelerated destabilization and loss of biological activity of the bacterial cytoplasmic membrane.

Methanobactin, a natural bacterial-produced biopreservative, was shown to effectively destroy *L. monocytogenes*. The addition of SLS to the treatments permitted lowering Mb-Cu concentrations 2-fold or more, and still maintain high antilisterial activity. This is the first published work demonstrating increased antimicrobial effectiveness of methanobactin when used in combination with a surfactant. Methanobactin fulfills important physiological roles in the producer organism; setting this compound apart from

most other biopreservatives that are used to control foodborne pathogens in foods. More research is needed to determine the effect of Mb-Cu on other foodborne pathogens (including gram-negatives), and any potential allergenic issues. Information regarding the effects of temperature, cations, and food constituents would prove to be useful in determining potential food applications for methanobactin/SLS treatments. Given the fact that inoculum levels used in this study were unrealistically high for foods, the application of Mb-Cu/SLS in the form of a spray or dip, for various RTE meat products, may be a promising antimicrobial intervention for control of *L. monocytogenes*.

ACKNOWLEDGEMENTS

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Table 1. [a]Minimum inhibitory concentrations (MICs) of methanobactin against *L. monocytogenes* Scott A as influenced by pH and surfactant.

pH	No Surfactant	Tween 20		Tween 80		Sodium Lauryl Sulfate	
		0.25%	0.50%	0.25%	0.50%	0.25%	0.50%
6.25	2.06	4.11	4.11	4.11	4.11	ND[b]	ND
6.00	2.06	2.06	4.11	4.11	4.11	ND	ND
5.75	2.06	2.06	2.06	2.06	4.11	ND	ND

[a]MIC expressed in millimoles (mM)
[b]Not Determined

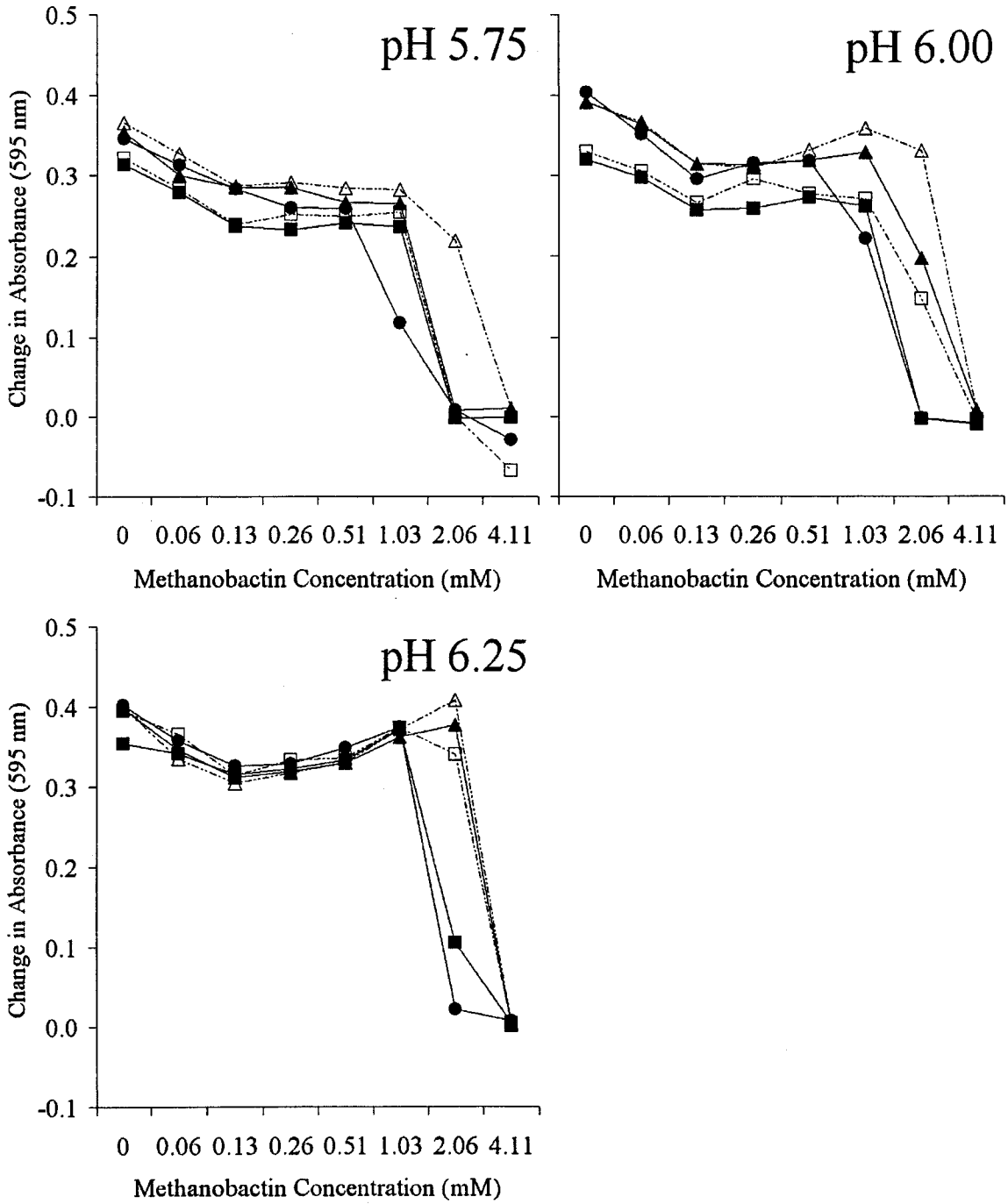

Figure 1. Final 24-h absorbance readings (595 nm) of *L. monocytogenes* Scott A cultures after exposure to Mb-Cu without surfactant (●), and in combination with 0.25% T20 (■), 0.50% T20 (□), 0.25% T80 (▲), or 0.50% T80 (△), in different pH conditions.

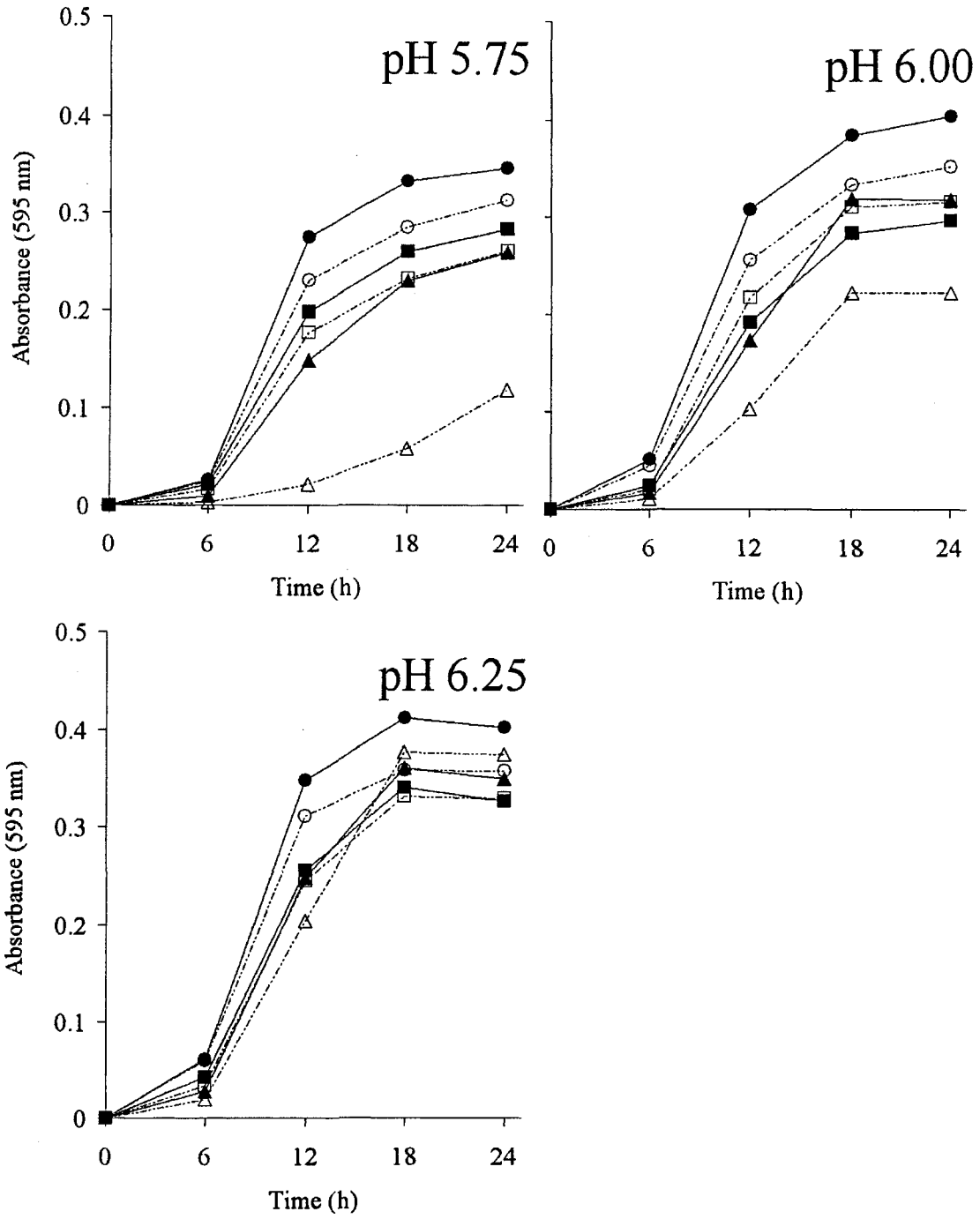

Figure 2. Growth of *L. monocytogenes* Scott A, at 32°C and in different pH conditions, as measured by absorbance (595 nm) in the presence of sub-inhibitory concentrations of Mb-Cu: 0 (●), 0.06 (○), 0.13 (■), 0.26 (□), 0.51 (▲), and 1.03 (△) mM.

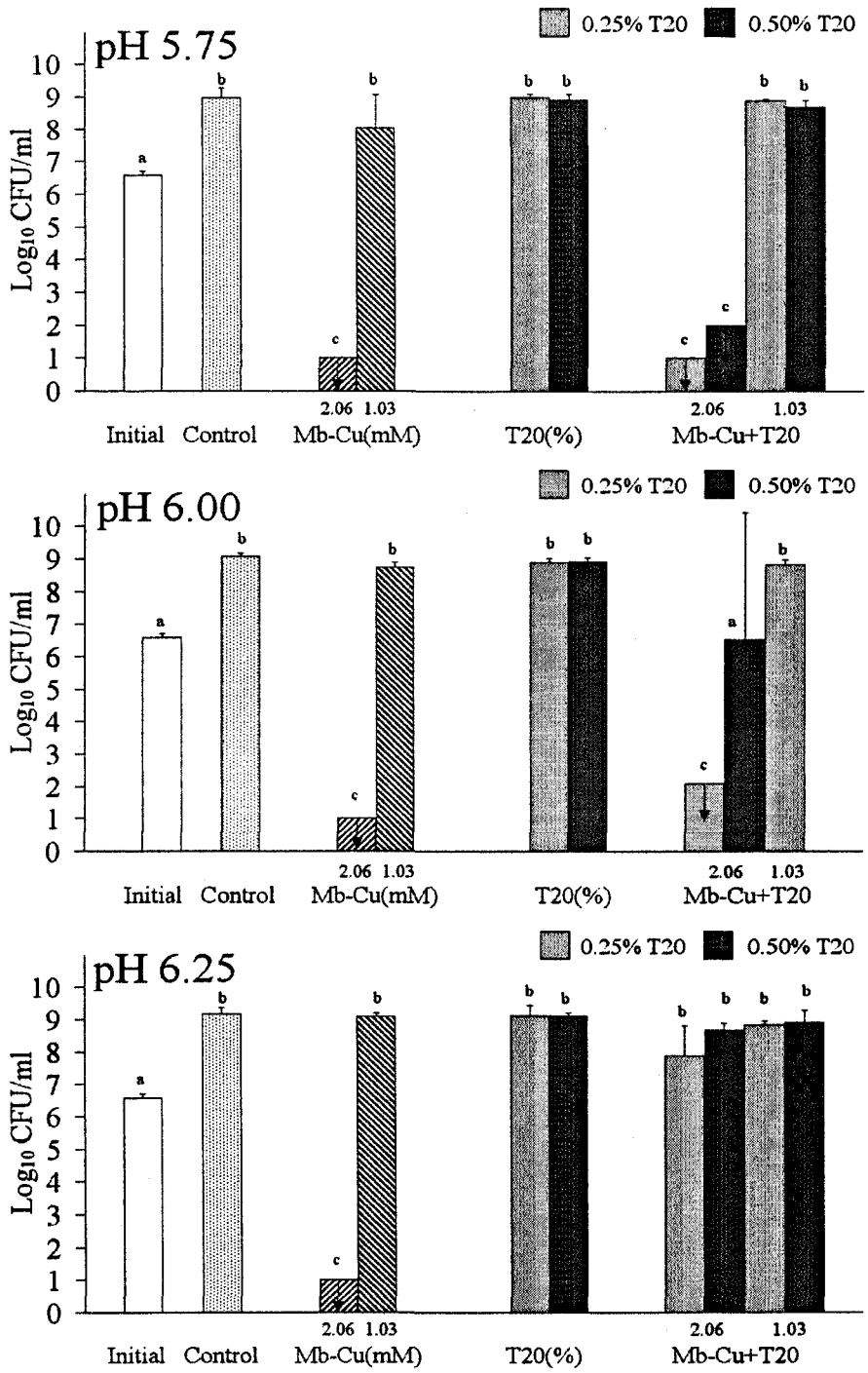

Figure 3. Viability of *L. monocytogenes* Scott A following exposure (24 h) to methanobactin, Tween 20, and their combination in different pH conditions, when enumerated on BHI agar. Values represent the means of at least three replicates ± SD (error bars). Letters indicate significant differences ($P<0.05$) between treatment means within a given pH value.

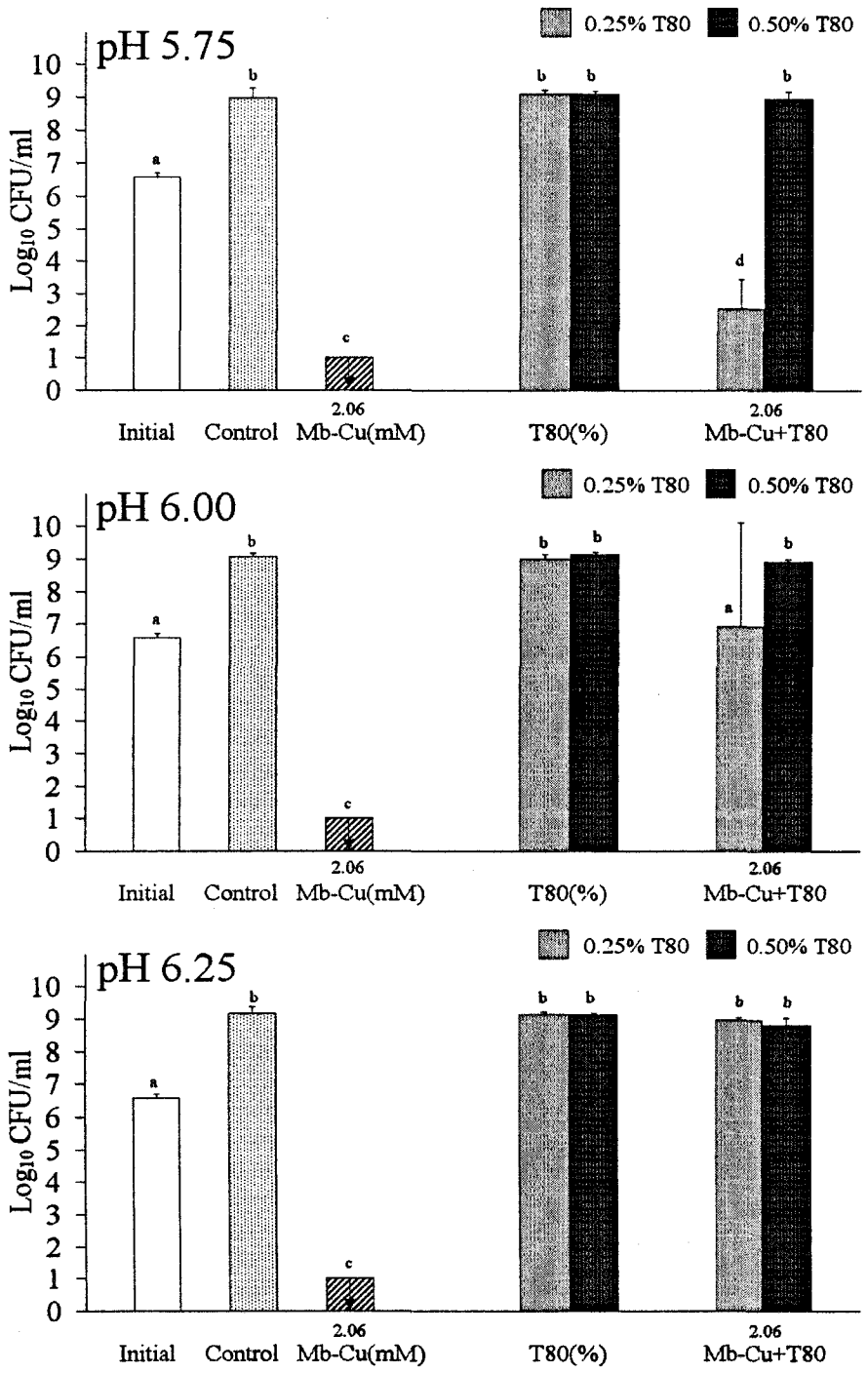

Figure 4. Viability of *L. monocytogenes* Scott A following exposure (24 h) to methanobactin, Tween 80, and their combination in different pH conditions, when enumerated on BHI agar. Values represent the means of at least three replicates ± SD (error bars). Letters indicate significant differences ($P<0.05$) between treatment means within a given pH value.

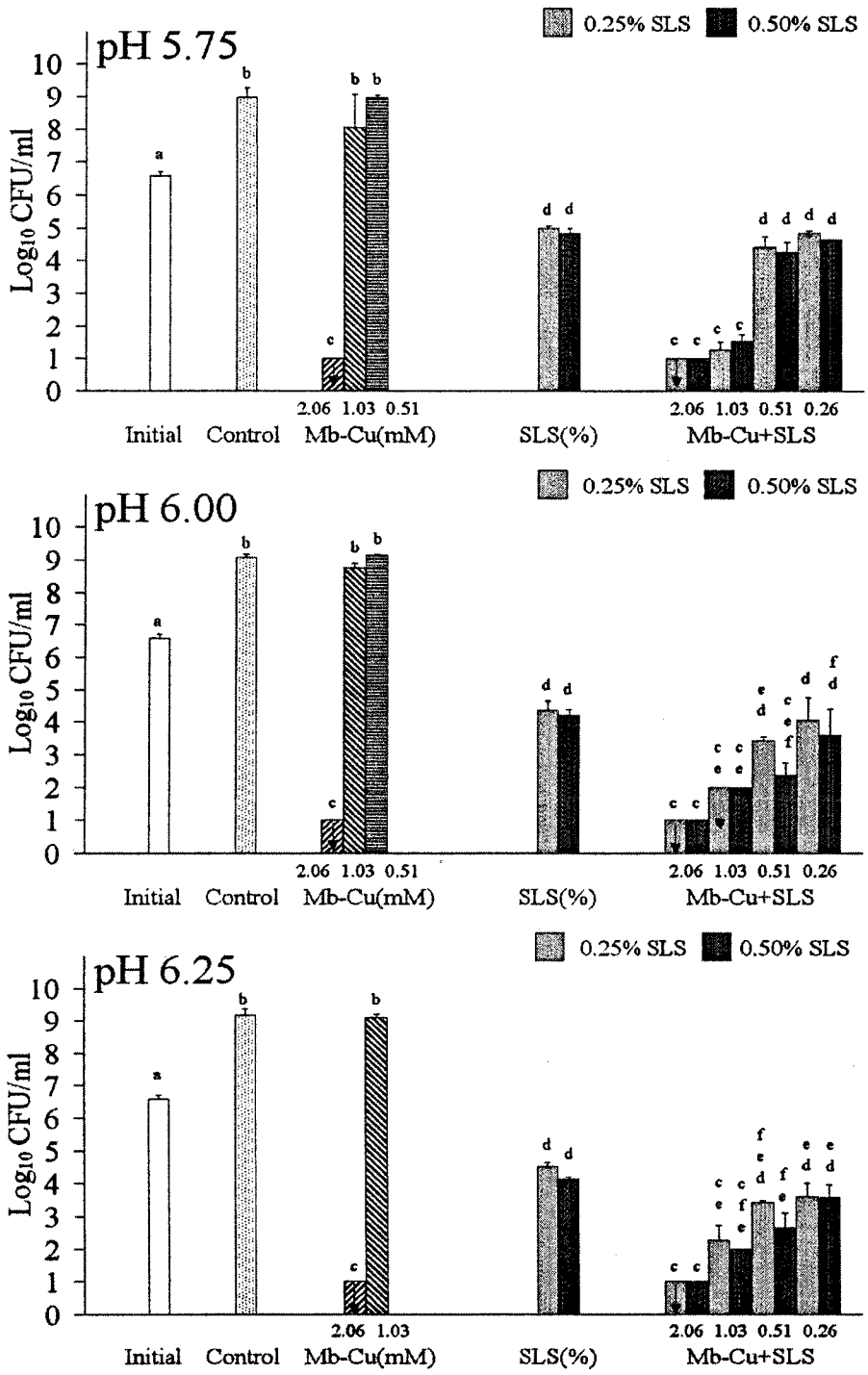

Figure 5. Viability of *L. monocytogenes* Scott A following exposure (24 h) to methanobactin, sodium lauryl sulfate, and their combination in different pH conditions, when enumerated on BHI agar. Values represent the means of at least three replicates ± SD (error bars). Letters indicate significant differences ($P<0.05$) between treatment means within a given pH value.

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CHAPTER 5. EFFICACY OF METHANOBACTIN ALONE OR COMBINED WITH SODIUM LAURYL SULFATE AS A SURFACE TREATMENT FOR THE CONTROL OF *LISTERIA MONOCYTOGENES* ON FRANKFURTERS MADE WITH OR WITHOUT SODIUM LACTATE

A paper to be submitted to the Journal of Food Protection

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ABSTRACT

Methanobactin, a "chalkophore" produced by methanotrophic bacteria has been previously shown to possess listericidal properties *in vitro*. The objectives of this study were to determine the effect of copper-bound methanobactin (Mb-Cu; 10 mM), sodium lauryl sulfate (SLS; 1%), and their combination on the survival of a 5-strain *Listeria monocytogenes* cocktail (~6.30 log CFU/frankfurter) and spoilage organisms, on vacuum-packaged frankfurters formulated with or without 2% sodium lactate (NaL). Frankfurter samples (1 frankfurter/bag) were uniformly surface-treated, inoculated, vacuum-packaged, and stored at 4 and 10°C, for 84 and 56 days, respectively. Periodically, each sample was rinsed using 0.1% peptone (20 ml). The rinse solution was serially-diluted, and

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surface-plated onto Modified Oxford (MOX; *L. monocytogenes*), tryptic soy agar supplemented with 0.6% yeast extract (TSAYE; total counts), and acidified Lactobacillus MRS agar (MRS; lactic acid bacteria). After 24-h storage, 10 mM Mb-Cu, 1% SLS, and 1% SLS + 10 mM Mb-Cu treatments reduced initial *L. monocytogenes* populations by 0.92-1.11, 0.93-1.70, and 1.91-2.66 log-cycles, respectively, with slightly greater reductions occurring on NaL-containing frankfurters. Surface treatments had little effect on lactic acid bacteria but extended the lag of the pathogen in the presence of NaL ($P<0.05$). Complete inhibition of the pathogen was not achieved for the length of storage at either temperature. On frankfurters formulated with NaL, final *L. monocytogenes* counts were not statistically higher ($P<0.05$) than initial populations using any treatment at 4°C. Based on these results, a combination of Mb-Cu (10 mM) and 1% SLS exhibits moderate inhibitory activity against *L. monocytogenes* on frankfurters. Further research is needed to optimize the use of Mb-Cu for control of *L. monocytogenes* in RTE meats.

INTRODUCTION

Consumption of ready-to-eat (RTE) meats, including poultry deli meats and frankfurters have been implicated in major multi-state listeriosis outbreaks, resulting in a significant number of deaths (~20% case fatality rate). Due to the nature of these meat products, they are at a particular high risk for contamination by *Listeria monocytogenes*. Normal frankfurter processing conditions have been shown to be sufficient to eliminate the pathogen from the finished product (*61*); however, there is no consumer safety intervention guarantee because frankfurters may be consumed without cooking or reheating. This risk is

imminent because post-processing contamination can likely occur during peeling of the casings and before packaging of the product (*44*). *L. monocytogenes* is a particular problem in these cured RTE meat products because, in general, it can grow to high numbers despite refrigerated storage, the presence of sodium chloride and nitrite salts, and the absence of atmospheric oxygen when vacuum-packaged (*37*). Also, the pH of these meat products (~5.8 to 6.2) is not sufficiently low enough to suppress growth despite the presence of these other aforementioned hurdles, nor has the natural spoilage microflora shown to play a major role in retarding the growth of *L. monocytogenes* (*48*).

In addition to this hardy organism's ability to proliferate on a number of foods, its persistence in the processing environment is most troubling due to its widespread distribution in nature (*56*). This, along with the uncertain infective dose and high fatality associated with listeriosis, led the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) to establish a "zero-tolerance" policy for its presence in RTE meat products. In 2003, the USDA-FSIS established an interim final rule (*21*), mandating food processors to choose from three alternatives, which has encouraged food processors to implement a postlethality treatment(s) to reduce or eliminate *L. monocytogenes*, and/or antimicrobial agents or processes to limit or suppress the growth of the pathogen throughout the shelf life of the product.

The salts of a variety of organic acids, namely lactates and diacetates, and their combinations have been studied extensively for use in batch formulations of frankfurters and other RTE meats, to control the growth of *L. monocytogenes* in the event of post-process contamination (*2, 3, 25, 43, 46, 51, 52*). A number of studies have also shown

potential effectiveness of post-process antimicrobial dipping or spraying solutions composed of organic acids or their salts to prevent the growth of the pathogen as well, although this practice is not commonly used in industrial food processing (*2, 23, 25, 38, 43, 44*). Most research indicate that in general there are minimal negative sensory changes associated with the formulation or surface treatment with these antimicrobials when used at levels to help control *L. monocytogenes* in frankfurters (*2, 7, 23, 39*). Although many organic acids are produced by natural means, there are a number of other less commonly investigated compounds that are produced naturally by plants, animals, and microorganisms, that may have good potential for controlling *L. monocytogenes* in RTE meat products. The consuming public has shown a demand favoring foods that are minimally processed or that are produced to include more natural food safety measures. This has been followed by increased research interest in a variety of natural compounds that can be used as surface treatments of meats by way of package incorporation, films, dips, and sprays, among others.

Spices (*59*) and their essential oils (*22, 62*), sorbates (*40, 49*), and other extracts (*1, 36, 41*) are among the most frequently studied plant-derived antimicrobials for use in/on meats. Antimicrobial compounds from animal sources have included lactoferrin (*42*), lysozyme (*24, 47*), chitosan (*62*), and components of the lactoperoxidase system (*18*). Natural antimicrobials derived from microorganisms, for use in/on meat products have been studied extensively as well. These include protective cultures (*7, 28*), bacteriocins (*9, 40, 58*) other compounds (*35, 54, 57*), and less commonly siderophores (*10*). Many of these antimicrobials may possess suitable activity for the incorporation into surface treatment –

food preservations systems for RTE meats and may have an advantage of bearing a gentler name, as opposed to "acid".

Hurdle technology is a well-established concept used by combining different antimicrobials at lower levels to improve overall performance of food preservation systems. These may include the utilization of antimicrobial adjuvants for improving the efficacy, delivery, and distribution of the antimicrobials when applied as surface treatments on meat products, or for preventing the attachment of microorganisms on their surfaces. Some of these include surfactants and emulsifiers (*17, 53*), antioxidants and metal chelators (*14, 50*), and fatty acids (*16, 27*).

Previous work in our laboratory has demonstrated that methanobactin (Mb-Cu), a novel chromopeptide naturally-produced by the methane-oxidizing bacterium *Methylosinus trichosporium* OB3b has listericidal properties, *in vitro*. This organism is important for global carbon cycling and has been studied for its use in single-cell protein (SCP) production. In addition, studies in our laboratory have shown that sodium lauryl sulfate (SLS) has good potential to help control the growth of *L. monocytogenes* on frankfurters. SLS is approved for food use as: an emulsifier for eggs, whipping agent in marshmallows, surfactant in fumarate-acidulated beverages, and wetting agent in edible fats and oils (*15*). Recently we observed *in vitro* synergistic, bactericidal activity when SLS and Mb-Cu are used in combination against *L. monocytogenes*, at pH values typical of RTE meats. Therefore the objectives of this study were: (1) Determine the initial reductions of *L. monocytogenes* on frankfurters (formulated with or without 2% sodium lactate) using surface treatments composed of SLS, Mb-Cu, or their combination; (2) Monitor the

behavior of *L. monocytogenes* and normal spoilage flora, on treated, vacuum-packaged frankfurters during extended storage at 4 or 10°C; and (3) Determine the impact of frankfurter formulation on the effectiveness of the surface treatments. Biophysical and structure characterization of this biopreservative has been recently performed (*33, 34*), and this is the first report of Mb-Cu efficacy against *L. monocytogenes* in a food system.

MATERIALS AND METHODS

Bacterial strains and inoculum preparation. A 5-strain composite of *L. monocytogenes* was used in this study that included Scott A (serotype 4b, human isolate), 101M (serotype 4b, beef and pork sausage isolate), 108M (1/2b, hard salami isolate), F6854 (serotype 1/2a, frankfurter isolate), and H7776 (serotype 4b, frankfurter isolate). With the exception of *L. monocytogenes* Scott A, which was obtained from the National Animal Disease Center, Agricultural Research Service (United States Department of Agriculture, Ames, IA), all strains were obtained from the Illinois Institute of Technology (Chicago, IL). Prior to the experiments, *L. monocytogenes* cultures were confirmed by streaking onto Modified Oxford (MOX) agar, followed by gram-staining, oxidase and catalase tests, and API test kit verification. Cultures were maintained as frozen (-20°C) stock in tryptic soy broth (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.6% (wt/vol) yeast extract (Difco; TSBYE) and 10% (vol/vol) glycerol. Prior to each experiment, the stock cultures were transferred twice in 10 ml of TSBYE and incubated at 32°C for 18-24 h. Overnight cultures were combined by transfer (6-ml aliquots) into a sterile centrifuge tube, then harvested by centrifugation (10,000 × *g*, 10 min) in a

refrigerated (4°C) centrifuge (Sorvall Super T21; DuPont Instruments, Wilmington, DE). The cells were washed once in 0.85% (wt/vol) NaCl, harvested by centrifugation, and resuspended in fresh saline. The washed cell suspension was then diluted 100-fold in order to obtain ~10^7 CFU/ml, which served as the experimental inoculum (~6.30 log CFU/frankfurter). The inoculum population was determined by surface-plating onto tryptic soy agar (Difco) supplemented with 0.6% (wt/vol) yeast extract (Difco; TSAYE) and enumerationed after incubation at 32°C for 48h.

Frankfurter preparation. Frankfurters were prepared with pork fat trimmings (40/60 lean/fat ratio) and lean beef trimmings (80/20 lean/fat ratio) at the Iowa State University Meat Laboratory. The meat was ground through a 0.79-cm grinder plate, and divided into two batches. One batch received sodium lactate (NaL), as a powder (Purac Inc., Lincolnshire, IL), added along with salt, sodium erythrobate, sodium nitrite, seasoning and water with ice during emulsification in a vacuum chopper to achieve a final concentration of 2% in the product (Kutter Supplies, Inc., Randolph, MA.). The other batch served as a control, and was prepared the same way as the first one, without the incorporation of NaL. The meat batters were extruded through a meat stuffer (Risco® Model RS 4003-165; Stoughton, MA) into 22-mm peelable cellulose casings (Devro Teepack™ Wiene-Pack®; Coastal Corrugated Inc., N. Charleston, SC). The encased meat batters were linked at 14.0-cm lengths by 2.2 cm in diameter, using a poly-clip system (GmbH and Co. KG, Frankfurt, Germany).

The linked products were hung on racks and cooked for 90 minutes using the conventional cooking-smoking cycle in a humidity-controlled smoke house (Alkar, DEC Intl. Inc., Lodi, WI) to an internal temperature of 71.1°C. Natural smoke (hardwood sawdust; Frantz Co., Milwaukee, WI) was applied during the cooking cycle. At the end of the cycle, frankfurters were showered with cool water, and then held in a walk-in refrigerator at 4.0°C for 18-19 hours. The following day, frankfurters were peeled (2600 High Speed Peeler; Townsend, Des Moines, IA), sealed in vacuum bags (10/package), and stored at -20°C until experimental use. Finished weight of the frankfurters was 34.8 ± 2.1 g per link.

Methanobactin preparation. Lyophilized copper-bound methanobactin (Mb-Cu) samples (referred to as methanobactin throughout this paper) were prepared using a modified protocol previously described by Choi *et al.* (*12*). Mb-Cu samples (12 mg) were transferred to 1.5-ml polystyrene microcentrifuge tubes (Eppendorf, Westbury, NY), placed in Seward stomacher bags (Seward Ltd., London, England), and held on ice for one hour prior to irradiation. Samples were sterilized via electron-beam irradiation at the Iowa State University Linear Accelerator Facility, which has a MeV CIRCE III Linear Electron Accelerator (MeV Industrie S. A., Jouy-en-Josas, France). Samples were irradiated at ≥30 kGy in the electron beam mode at an energy level of 10 MeV and an average dose rate of 58 kGy/min. Absorbed radiation doses were determined by the use of 5 (diameter) by 5 mm (length) dosimeter alanine pellets (Bruker Analytische Messtechnik, Rheinstetten, Germany) placed on the top and bottom surfaces of one stomacher bag containing Mb-Cu

samples. Immediately after irradiation, the pellets were placed in a Bruker EMS 104 EPR Analyzer to measure absorbed doses by electron paramagnetic resonance. The average absorbed dose was obtained from the arithmetic average of the top and bottom surface readings. Sterile samples were stored -20°C and held for no longer than 2 months.

Application of treatments. Prior to inoculation, frankfurters were treated as follows: (i) no treatment (control), (ii) 10 mM Mb-Cu, (iii) 1% (wt/vol) SLS (Sigma, St. Louis, MO), or (iv) 1% SLS + 10 mM Mb-Cu.

Frozen frankfurters of both formulations were thawed overnight at 4°C in a walk-in refrigerator. After thawing, each frankfurter was aseptically removed from the bulk packages and placed into a vacuum bag (1 frank per bag; Cryovac B-2540, Cryovac Sealed Air Corp., Duncan, SC; water vapor transmission=0.5-0.6g at 100°F, 100% RH/100 in^2/24h; oxygen transmission rate=36 CC at 40°F/m^2/24h/0% RH). The packages were separated according to formulation, held at 4°C until treatment addition, and randomly assigned to treatment. Two portions of Mb-Cu were aseptically weighed out and dissolved in either sterile water or 1% SLS to yield 10 mM concentrations for treatment use. Preliminary studies indicated that when frankfurters were dipped for 2 minutes, the average pickup was 0.42 ± 0.03 g per frank, or approximately 0.012% total pickup of the compounds or their combinations by the frankfurters. Based on the established pickup, 0.4 ml of the treatments were spot-inoculated onto the frankfurters, using a pipette. After the treatment application, the frankfurters were massaged manually from outside of the bag for 5-10 seconds to uniformly distribute the treatment over the surface. Control frankfurters

did not receive the addition of any treatment. Inoculation of the treated and control frankfurters followed within 30 minutes.

Inoculation and frankfurter packaging. After the addition of the treatments, each frankfurter was spot-inoculated with 0.2 ml of the 5-strain cocktail of *L. monocytogenes* using a pipette, then massaged manually from the outside of the bag for 5-10 seconds to spread the inoculum over the surface. Inoculated samples were vacuum-packaged using a Multivac A 300/51/52 vacuum-packaging machine (Multivac Sepp Haggenmuller GmbH & Co. KG, Wolfertschwenden, Germany). One half of the prepared samples were stored at 4°C and the other half were stored at 10°C.

Frankfurter pH measurement. The frankfurters were measured for pH prior to treatment and inoculation, after treatment and prior to inoculation, and on the final day of storage for each storage temperature. A model 410Aplus (Thermo Orion; Thermo Electron Corp., Beverly, MA) benchtop pH/temperature meter equipped with a flat surface combination pH electrode was used by applying the electrode to the surface near one end of the frankfurter (*29*).

Microbiological analyses. Vacuum-packaged frankfurter samples were opened aseptically and rinsed with 20 ml of 0.1% sterile peptone water (Difco), then vigorously massaged by hand from outside of the package for 30 seconds. Ten-fold serial dilutions of the rinsate were prepared in 0.1% peptone water and aliquots of appropriate dilutions were

surface-plated (in duplicate) onto MOX agar, TSAYE, and Lactobacillus MRS agar (pH 5.2) acidified with 0.16% (vol/vol) acetic acid. MOX agar was used to enumerate *L. monocytogenes*, TSAYE was used to estimate the total microbial population on the frankfurters, and acidified MRS (MRS) was used to enumerate lactic acid bacteria (LAB), the predominate spoilage flora of this meat product. Colonies were counted after incubation for 72 h at 32°C (MOX) or 30°C (TSAYE and MRS).

The initial microbial load of the products were determined using the aforementioned media. Vacuum-packaged frankfurters stored at 10°C were sampled on days 1, 7, 14, 28, and 56, while frankfurters at 4°C were sampled after 1, 7, 14, 28, and 84 days of storage.

Data analysis. Four surface treatments (control, 10 mM Mb-Cu, 1% SLS, or 1% SLS + 10 mM Mb-Cu), two frankfurter formulations (0 and 2% NaL), two storage temperatures (4 and 10°C), and six sampling times (0, 1, 7, 14, 28, and 56 or 84 d) were used in the present study. A randomized complete-block experimental design was used, all experiments were repeated at least twice, and the microbiological counts ($\log_{10}$ CFU/frank) and pH measurements are reported as means. Analysis of variance was used to determine significant differences among mean values following the Tukey-Kramer adjustment. Differences were considered statistically significant when the associated P-value was less than 0.05.

RESULTS

Initial microbial load of frankfurters and *L. monocytogenes* recovery. *L. monocytogenes* was not detected (<1.30 log CFU/frank) on frankfurters of either formulation, as determined by directly plating the rinsate onto MOX agar. Furthermore, the overall microbiological quality of the finished products was very high and no difference in the initial microbial load was found between the two frankfurter formulations. Populations did not exceed 1.40 and 1.30 log CFU/frank on TSAYE and MRS, respectively. The recovery method used for the enumeration of *L. monocytogenes* on frankfurters was very similar to the United States Department of Agriculture – Agricultural Research Service (USDA – ARS) package rinse method. It proved to be very reliable (Figure 1) where average loss of the inoculum (6.24 log CFU/frank) was -0.07 and 0.12 log CFU/frank for 0 and 2% NaL franks on MOX agar, and -0.04 and 0.23 log CFU/frank for 0 and 2% NaL franks on TSAYE, respectively. Recovery loss was only found on frankfurters formulated with 2% NaL. Losses did not exceed 0.36 log CFU/frank in any instance.

Effect of antimicrobial surface treatments on initial *L. monocytogenes* populations. As mentioned, the initial flora on TSAYE and MRS were very low, thus the counts obtained for initial reductions on the former media were entirely composed of *L. monocytogenes* colonies, while LAB populations being below the detection limit prevented quanititation on the latter. The initial 24-h reductions of *L. monocytogenes* on frankfurters is depicted in Figure 1. Counts were slightly lower on MOX than TSAYE when derived from frankfurters formulated with 2% NaL, while this was not true for the other

formulation. Although consistent, differences between media were considered negligible. Temperature was not found to be a major factor during the first 24 h of storage either, despite slightly larger reductions of *L. monocytogenes* at 10 than 4°C, on frankfurters formulated with 2% NaL.

Counts on MOX revealed that 24-h reductions of *L. monocytogenes* on frankfurters formulated without NaL and stored at 4°C, were 0.92-, 0.99-, and 1.99-log CFU/frankfurter treated with 10 mM Mb-Cu, 1% SLS, and 1% SLS + 10 mM Mb-Cu, respectively. Reductions by these treatments after storage at 10°C were 0.99, 0.93, and 1.91 log-cycles, respectively. Thus the two antimicrobials, when used alone, gave similar reductions ($P>0.05$) for this formulation, while a combination of the two antimicrobials resulted in a greater, additive effect ($P<0.05$). Counts on TSAYE were similar and all three treatments were significantly different ($P<0.05$) from the control, at both temperatures, when determined on either media. Reductions (MOX) of *L. monocytogenes* on frankfurters formulated with 2% NaL at 4°C were 1.11, 1.45, and 2.66 log-cycles for 10 mM Mb-Cu, 1% SLS, and 1% SLS + 10 mM Mb-Cu, respectively. At 10°C, these treatments gave 1.10, 1.70, and 2.63 log reductions, respectively. Counts on TSAYE were similar, and all treatments significantly reduced ($P<0.05$) *L. monocytogenes* populations (MOX).

Reductions were consistently higher, on frankfurters formulated with 2% NaL than without, regardless of storage temperature or media type, however these differences were not statistically significant ($P>0.05$). In contrast to the reductions on frankfurters formulated without NaL, 1% SLS resulted in a higher reductions than 10 mM Mb-Cu when used alone on 2% NaL –frankfurters. However, this observation was also not statistically

significant (P>0.05). This was observed for both storage temperatures, with the antimicrobial combination being the best treatment (P<0.05) displaying an additive effect. Despite the lack of significance, collectively, these results may demonstrate that the treatments exhibit slightly greater listericidal activity in the presence of 2% NaL, however this appears to be only an additive effect similar to combining the two single surface treatment antimicrobials. Overall, the combinational antimicrobial surface treatment, along with NaL in the formulation and stored at 10°C, gave the best initial reductions.

Effect of antimicrobial formulation and surface treatments on the survival and growth of *L. monocytogenes* and background flora during storage. When determined by plating on MOX, *L. monocytogenes* grew well on untreated frankfurters formulated without NaL during storage at 4°C (Figure 2). The lag phase was less than 7 days and outgrowth (or maximum population) occurred within 28 days, whereas 2% NaL extended the lag phase to around 14 days (P<0.05) resulting in a final *L. monocytogenes* population (day 84) that was 1.13 log CFU/frank less than on frankfurters without NaL. On frankfurters without NaL, the lag phase and growth rates seemed to be unaffected by the surface treatments. In contrast, the lag phase of *L. monocytogenes* was extended by at least 14 days (day 28) with any of the three surface treatments compared to the control on frankfurters formulated with 2% NaL (P<0.05). The order of increasing effectiveness for initial lethality (Control < 10 mM Mb-Cu < 1% SLS < 1% SLS + 10 mM Mb-Cu) remained consistent during early storage, however, at the end of the storage period at 4°C, surface-treated, 2% NaL-frankfurters had *L. monocytogenes* counts between 6.08 and 6.59 log

CFU/frank. Although the counts for these treatments were not statistically different ($P>0.05$) than the initial inoculum population, *L. monocytogenes* grew between 1.24 and 2.50 logs depending on treatment, following the initial reductions. Interestingly, 10 mM Mb-Cu alone on 2%-NaL frankfurters (4°C) seemed to inhibit *L. monocytogenes* better than 1% SLS alone ($P<0.05$), which is the reverse for that of the initial lethality, however this could be misleading since additional sampling times between days 28 and 84 would be needed to confirm this.

Total counts on TSAYE from frankfurter samples stored at 4°C were almost entirely *L. monocytogenes* until day 14 (Figure 3) when compared to the MOX data, however on day 14, TSAYE counts additively resembled those on MOX and MRS. TSAYE counts beyond day 14 resembled those on MRS (Figure 4) and thus were primarily LAB, the predominant spoilage organism of this meat product. Overall, the treatment effects when determined by plating on TSAYE were similar to the MOX data during early storage but eventually resembled the MRS data. Figure 4 shows that although MRS counts were typically lower (0.75-1.5 logs) on surface-treated frankfurters (without NaL) than the control, LAB grew rapidly and final populations were similar when stored at 4°C. LAB were not able to grow as rapidly on frankfurters formulated with 2% NaL. The lag phase was almost 7 days for the control group and MRS counts on frankfurters formulated with 2% NaL were nearly 2 logs lower than on frankfurters without NaL, at 28 days of storage; however populations were similar at the end of storage. Treatment order of inhibition effectiveness on LAB was the same between frankfurter formulations. On average LAB populations on 2% NaL-containing treated samples were 0.5 to almost 3 logs lower than on

control frankfurters through day 28. The combinational treatment on these frankfurters seemed to extend the shelf life of the product by approximately 1 week (through extrapolation of the curves) and counts were significantly different ($P<0.05$) than the control at day 14.

During storage at 10°C (Figure 5), *L. monocytogenes* (MOX) grew very rapidly on untreated frankfurters formulated without NaL, while growth was slowed somewhat by the incorporation of NaL into the formulation ($P<0.05$). Outgrowth occurred on samples of either formulation within 14 days, however, populations on frankfurters formulated with 2% NaL were always at least 1.5 logs lower than those on frankfurters formulated without NaL ($P>0.05$). Treatment effects on the inhibition of *L. monocytogenes* at 10°C were similar to the results at 4°C-storage. On frankfurters formulated with 2% NaL, initial reductions caused by 1% SLS alone were greater than using 10 mM Mb-Cu alone. However, although not significant ($P>0.05$), 10 mM Mb-Cu and the combinational treatment seemed to extend the lag phase at least 7 days longer (until day 14) than 1% SLS alone. This trend was more pronounced at this storage temperature than at 4°C. At 10°C, populations were similar at the end of storage even though the surface-treated 2%-NaL frankfurters generally had lower *L. monocytogenes* counts. On frankfurters without NaL, growth rates were unaffected and populations at the end of storage (56 days) were similar regardless of treatment.

Trends in microbial growth on TSAYE (Figure 6) were similar to those observed on MOX for all treatments, especially through 7 days of storage at 10°C, suggesting counts were almost entirely *L. monocytogenes*. The higher counts obtained on TSAYE, following

early storage reflect LAB populations as determined by plating on MRS. On frankfurters formulated without NaL LAB populations (Figure 7) grew very rapidly at 10°C despite the application of the antimicrobial surface treatments. After 14 days of storage and depending on treatment, populations were between 8.19 and 8.65 log CFU/frank, while counts were between 5.66 and 7.06 log CFU/frank when 2% NaL was included in the formulation. It appears that 2% NaL slightly increased the shelf life of the frankfurters at this temperature, especially when 1% SLS + 10 mM Mb-Cu was used. Overall, LAB grew much faster at this storage temperature compared to 4°C-storage, regardless of surface treatment.

Effect of treatments on the initial and final product pH. Figure 8 displays the surface pH values of the initial products, after surface treatments, and following storage at 4°C for 84 days or 10°C for 56 days. The initial pH of frankfurters formulated with 2% NaL (pH 5.94) and without NaL (pH 5.95) were nearly identical, and the surface treatments had negligible effects ($P>0.05$) on these values. Overall, pH values were higher on frankfurters formulated with 2% NaL than without, and lower storage temperature gave higher pH values than the higher storage temperature. There were no significant differences between final product pH of control and surface-treated frankfurters at either storage temperature or formulation.

DISCUSSION

The results of this study demonstrate that when used alone, Mb-Cu (10 mM) provides low bactericidal (~1 log reduction) and residual bacteriostatic activity against *L.*

monocytogenes on frankfurters when applied as a surface treatment. Previous Mb-Cu *in vitro* work within our laboratory demonstrated a concentration of 2.06 mM Mb-Cu in brain heart infusion (BHI) broth could generate a >5.00 log reduction, while as low as 125 uM Mb-Cu, in buffer, could reduce populations of *L. monocytogenes* Scott A by 4 log-cycles within 2 hours. The latter result involved a concentration 80 times less than that used in this study demonstrating that there is likely severe loss of Mb-Cu activity in this food product. In broth media, the bactericidal activity of Mb-Cu was considered to be an "all-or-nothing" event; however, death kinetics in buffer revealed dose-dependent behavior over a very narrow range. This result is difficult to explain in relation to the initial reductions of the present study. The obvious conclusion would be that loss in activity could be explained by non-specific binding of Mb-Cu to the food product, thus rendering it unavailable for activity against the pathogen. This, in theory, would lower the concentration of the antimicrobial to which *L. monocytogenes* is actually exposed. However, this previous work indicates that no reduction would be possible at concentrations below the minimum inhibitory concentration (MIC), unless, dose-dependent killing kinetics were not detected (as they were in buffer) because the range at which it is displayed is so narrow. In other words, a small reduction as in this study (1 log-cycle) could not be demonstrated *in vitro*, regardless of Mb-Cu concentration. Incompatibility of Mb-Cu on this food product may be further explained by our preliminary studies on frankfurters. Mb-Cu concentrations up to 50 mM did not provide additional lethality of *L. monocytogenes*, as well as this concentration being well beyond the solubility of this compound in water (data not shown). This RTE meat product was selected because optimal Mb-Cu activity fell within the pH

range of this product; however, there are a number of intrinsic factors and food constituents that may affect the activity of antimicrobials, particularly ones that are peptides or proteins.

Food lipids may reduce activity of an antimicrobial if it is hydrophobic or possesses hydrophobic components. Nisin, for example, is known to partition into the lipid phase of a food product, thereby reducing its activity. Studies in fluid milk revealed that as the fat content increased, higher concentrations of nisin were needed for effectiveness (*5, 31*). The structure of Mb-Cu (*34*) may indicate some hydrophobic character when copper – bound, especially with its modest solubility in water. The presence of NaCl (1.75% in our products) has been shown to antagonize the activity of lactoferricin (*60*) and nisin (*4, 8*); however there have been reports of it enhancing nisin activity (*45, 55*). Chloride and phosphate salts have also been shown to drastically reduce adsorption of Pediocin AcH onto the cell surface of target cells *in vitro* (*6*). With the vast distribution of the overall charge of the proteinaceous lantibiotic bacteriocins alone, ranging from highly positive to slightly negative (*30*), and the uncertainty of the net charge of copper-containing methanobactin in solution, it is not known whether this could be a major factor of Mb-Cu antimicrobial activity. The role of methanobactin in methanotrophic bacteria is likely that of extracellular copper – sequestration and/or detoxification, copper delivery, and interaction with respiratory enzymes (*11, 13, 34*). The proteinaceous nature of enzymes may indicate a natural tendency for Mb-Cu to non – specifically bind to proteins within the food product, thereby reducing antimicrobial efficacy. The presence of four other *L. monocytogenes* strains used in this study could also significantly alter the effectiveness of this antimicrobial since *L. monocytogenes* Scott A was the only strain selected for previous

in vitro studies. It is well established for many physical processing methods and antimicrobials, also including bacteriocins (*19, 32*), that *L. monocytogenes* strains can vary greatly in susceptibility. Product storage temperature, atmospheric conditions, and the high inoculum concentration (compared to likely contamination levels during processing) used in this study may also negate favorable antimicrobial action against *L. monocytogenes*.

Inclusion of SLS into the surface treatment additively improved reductions of *L. monocytogenes* by ~1 to 1.5 log-cycles, when combined with Mb-Cu. This is consistent with many studies where the use of SLS has improved initial reductions of pathogens on the surfaces of meats. A patent issued to Hill and Ivey in 1988 involves the use of poultry carcass surface washes where the solutions contained SLS for improved effectiveness. Tamblyn and Conner (*53*) found similar results. Delivery of essential oils (*22*), nisin (*5, 31*), and other bacteriocins (*20*) can be improved by encapsulation with nonionic surfactants. SLS, when used on this food product, does not seem to have the benefit of encapsulating the antimicrobial for improved delivery to target cells because initial reductions caused by SLS alone only adds to the reductions caused by Mb-Cu alone, when the two are combined. This is contrary to *in vitro* work showing more synergistic behavior.

Slightly larger initial reductions were accomplished on frankfurters formulated with 2% NaL than without. This was most apparent when the combinational treatment was used, but SLS showed improved activity against *L. monocytogenes* compared with Mb-Cu on 2%-NaL frankfurters or SLS on frankfurters formulated without NaL. This may simply be a result of adding yet another hurdle for the pathogen to overcome in order to survive on the product. In this study formulating frankfurters with 2% NaL could not completely suppress

the growth of *L. monocytogenes* at either temperature, regardless of surface treatment, for the length of the recommended commercial shelf life of ~90 days. This is in agreement with most literature, which indicates that >2% lactate alone is needed to reliably inhibit the growth of this pathogen on frankfurters for this length of time (*3, 25, 43, 51, 52*). It is worth noting that some of these studies show inhibition using 2% lactate for a major portion of the 3-month storage periods. This may indicate that this concentration borders on the threshold of complete inhibitory action because some studies have shown this formulation concentration to be sufficient (*46,* unpublished work in our lab). The implementation of this antimicrobial system would be sufficient to qualify for Alternative 1 as stipulated by USDA-FSIS (*21*), because 2% NaL will "limit" the growth of the pathogen.

Consistent with previous findings (*52*), formulating frankfurters with 2% NaL had little affect on the growth of LAB, thus the shelf-life of the product could not be extended from a quality standpoint by these means. Decrease in surface pH of the products was likely due to the growth of LAB. While some treatments resulted in higher pH values than others, this could not necessarily be attributed to final LAB populations on the product, but may be due to surface treatment- or NaL-induced extended lag of LAB, possibly lowering final acid concentrations on the product. Porto *et al.* (*46*) showed similar findings.

Collectively, the results of this study demonstrated that this anti-listerial system for use on frankfurters may be suitable on the basis of realistic contamination levels of frankfurters during processing. However, due to high concentrations of Mb-Cu needed to have a modest effect on *L. monocytogenes*, it seems evident that this product is not likely the best application for this system, possibly due to interference with food constituents

found in cured RTE meat products. However, determining an improved delivery system for this product (i.e. screening other surfactants) is worth considering. Susceptibility of other foodborne pathogens (such as gram-negatives) to Mb-Cu with the use of outer-membrane destabilizing agents, needs to be determined. Establishing the *in vitro* effects of selected fatty acids, salts, proteins, and other food components on Mb-Cu activity against *L. monocytogenes* may also help elucidate a more appropriate food product for its use, such as cheese and dairy products, vegetables or other products that have been associated with *L. monocytogenes* contamination. Finally, studies are needed to determine the overall safety of Mb-Cu, as well. With the vast amount of work remaining to answer the aforementioned questions, the use of Mb-Cu in foods to control the presence of *L. monocytogenes* shows promise.

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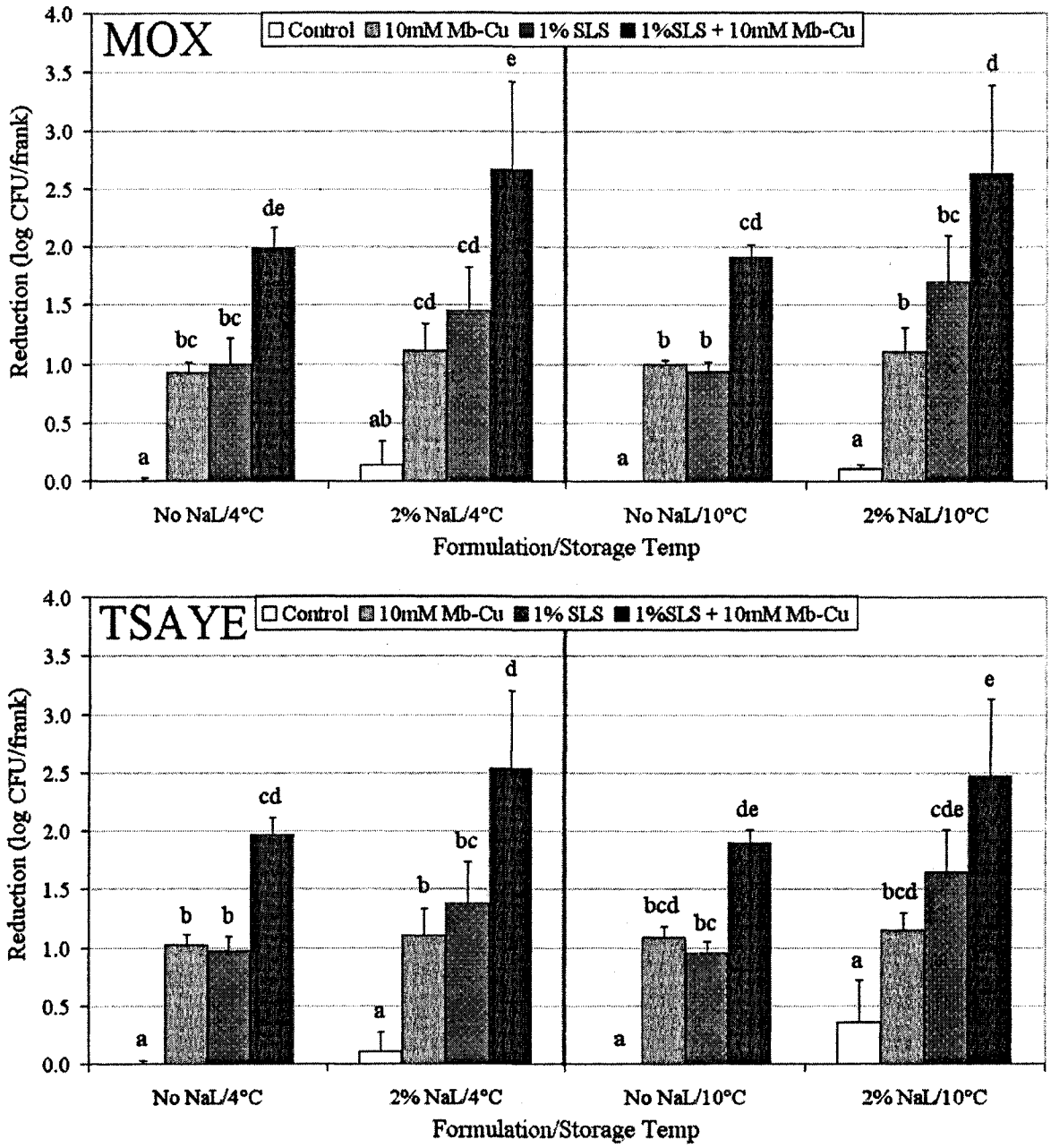

Figure 1. The effect of surface treatments on the initial reduction of *L. monocytogenes* on frankfurters formulated with and without 2% NaL, after storage for 24 h at 4 and 10°C. Values represent the means of at least three replicates ± SD (error bars) as determined by plating onto MOX and TSAYE. Letters indicate significant differences ($P<0.05$) between treatment means within a given storage temperature grouping.

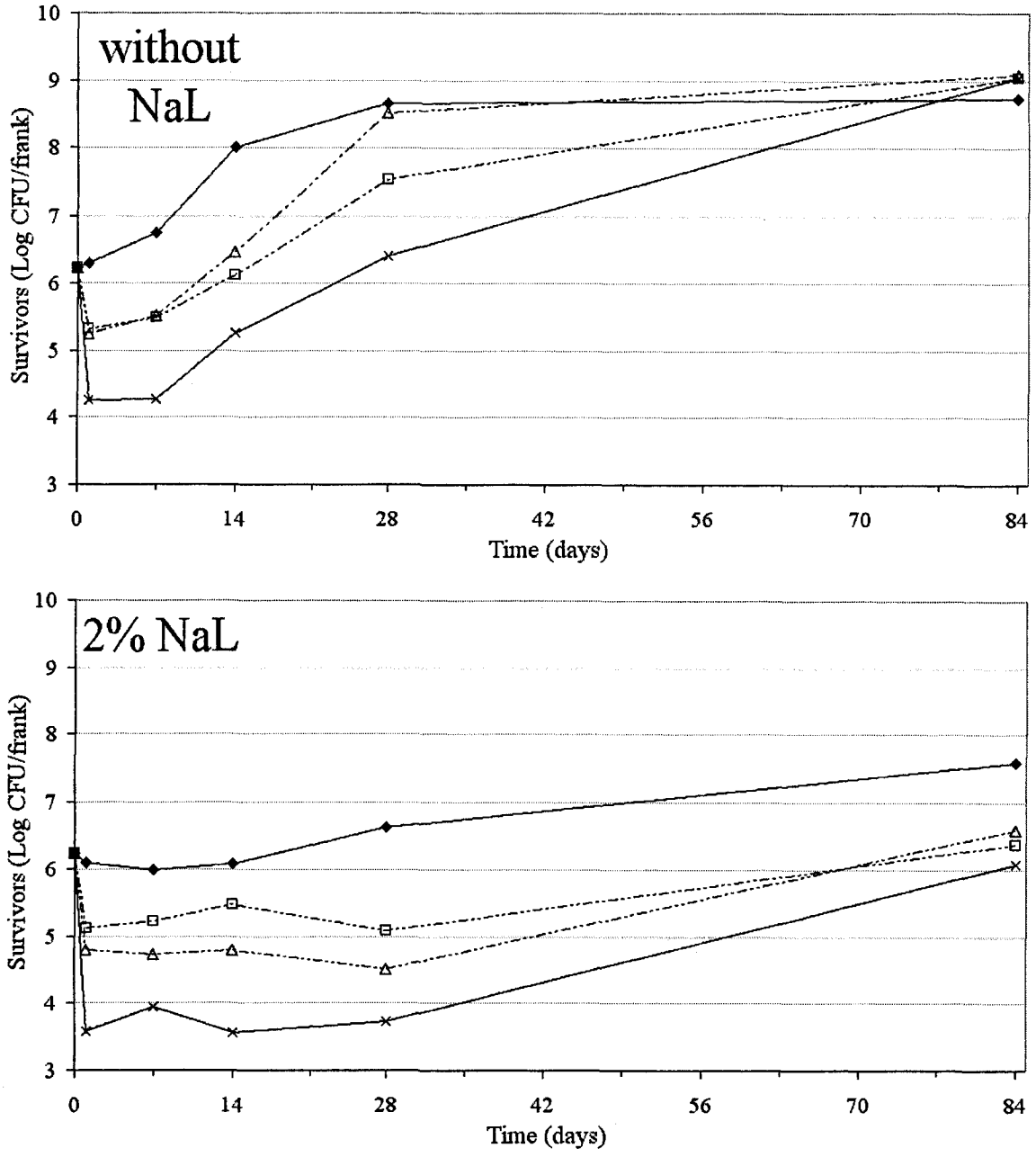

Figure 2. Mean populations of *L. monocytogenes* (MOX) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 4°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

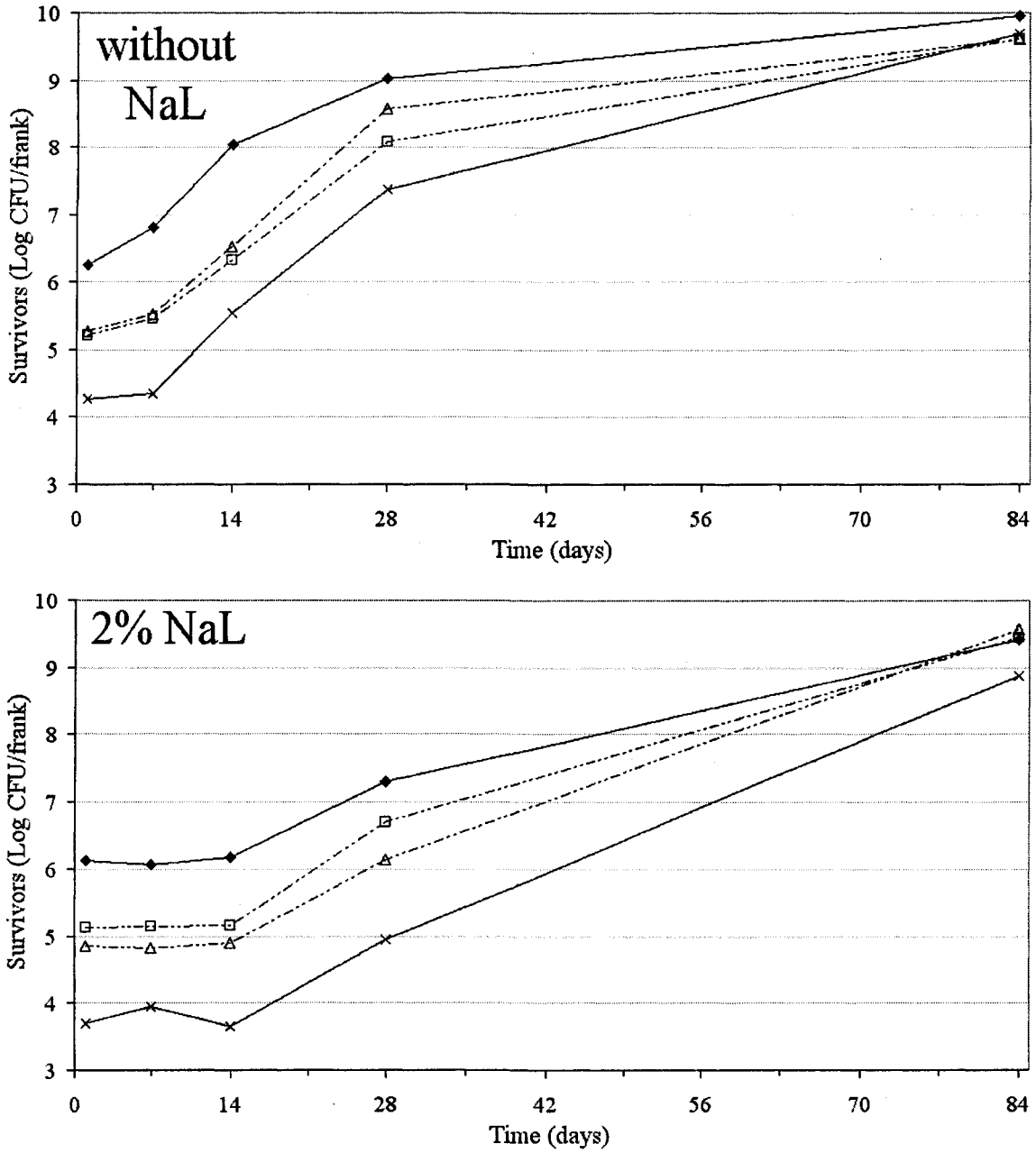

Figure 3. Mean total microbial populations (TSAYE) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 4°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

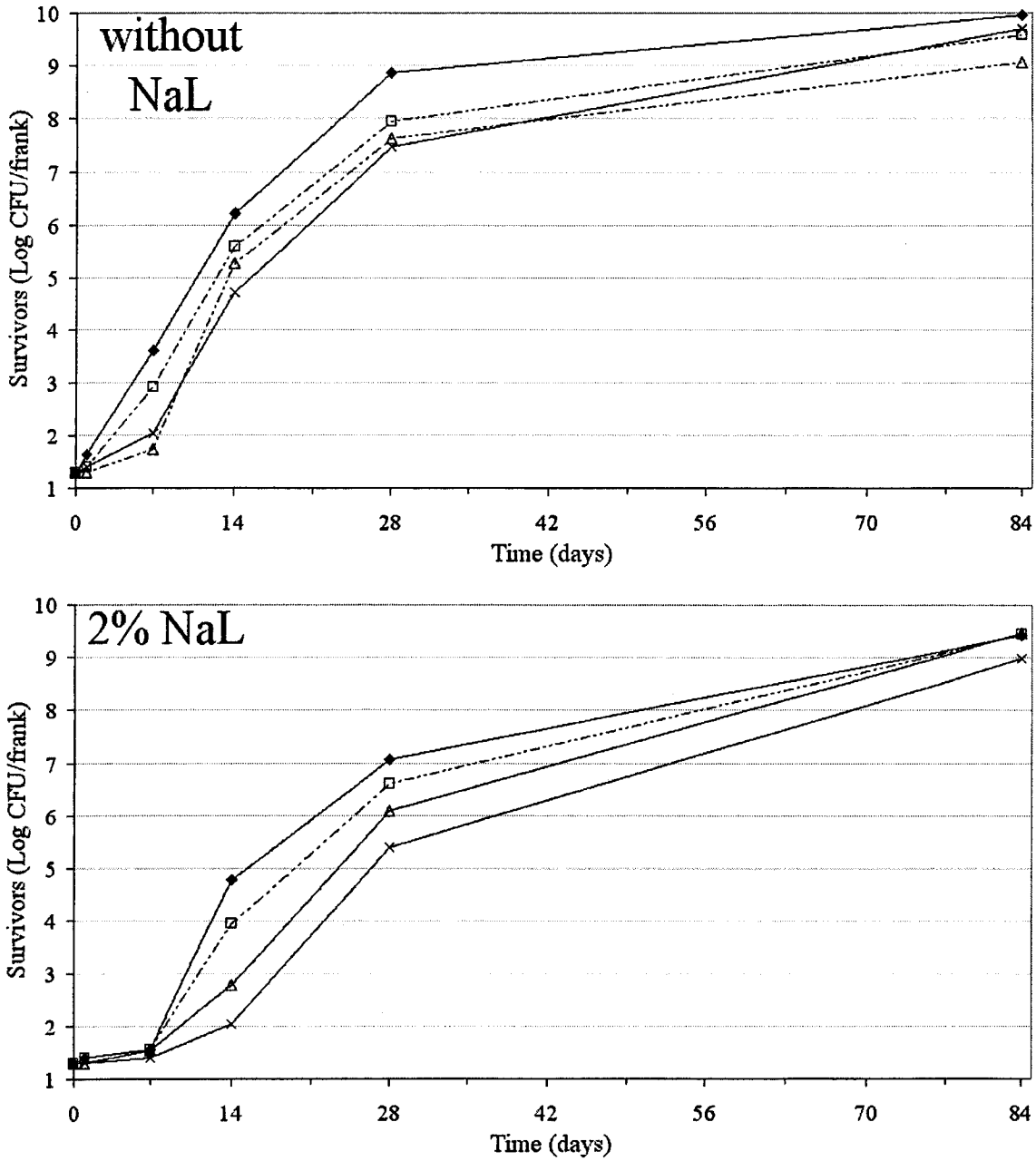

Figure 4. Mean LAB populations (MRS) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 4°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

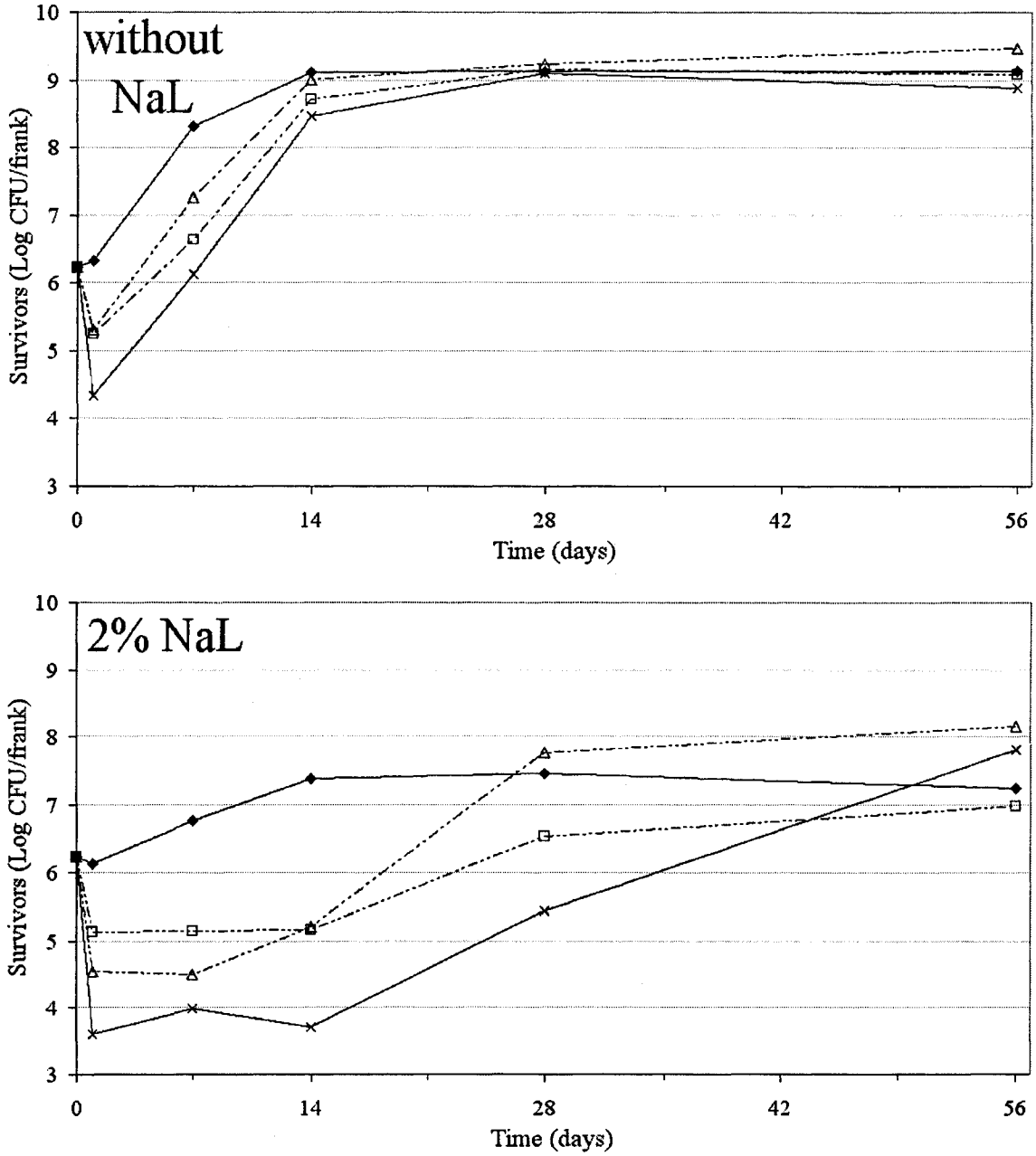

Figure 5. Mean populations of *L. monocytogenes* (MOX) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 10°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

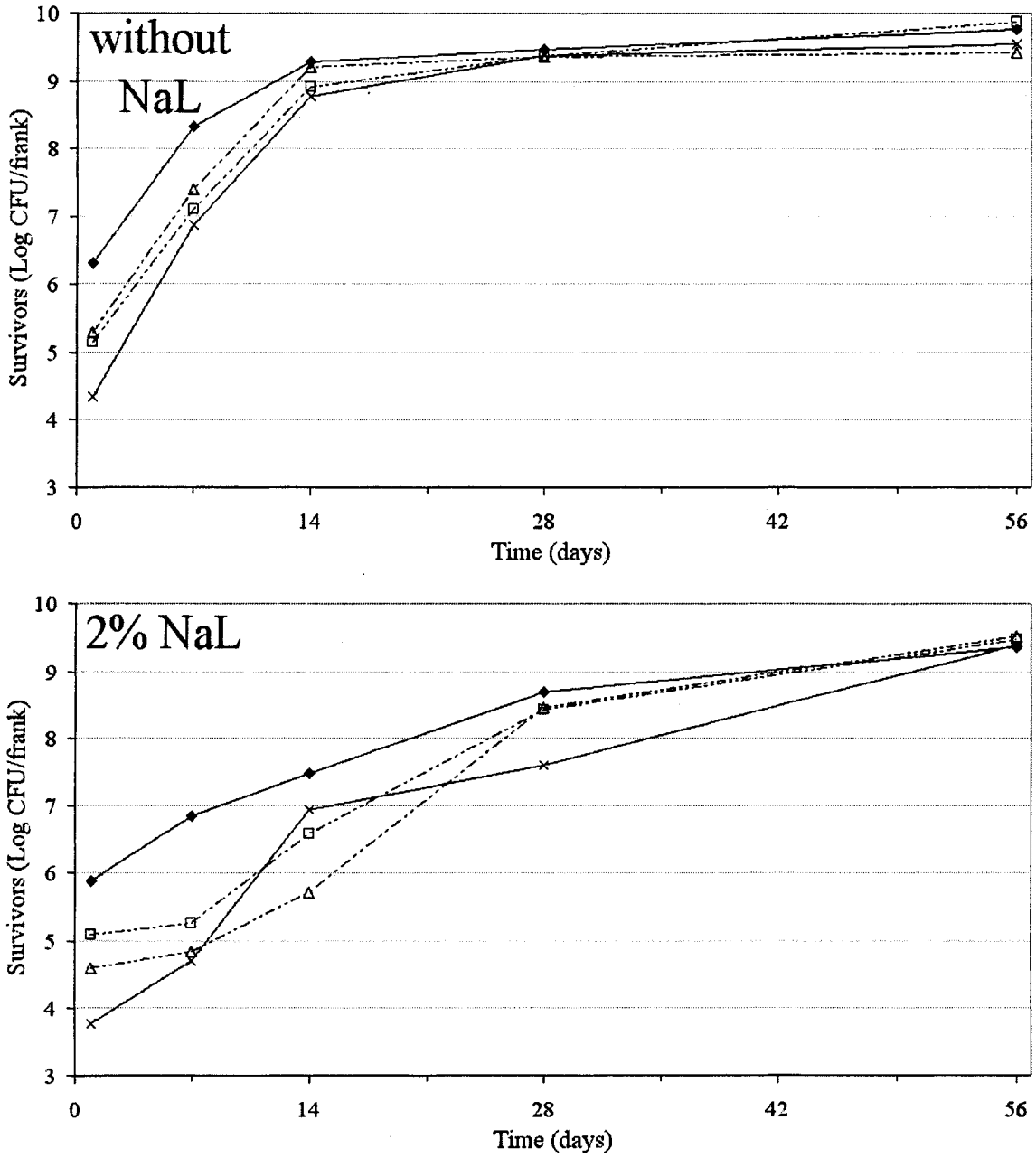

Figure 6. Mean total microbial populations (TSAYE) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 10°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

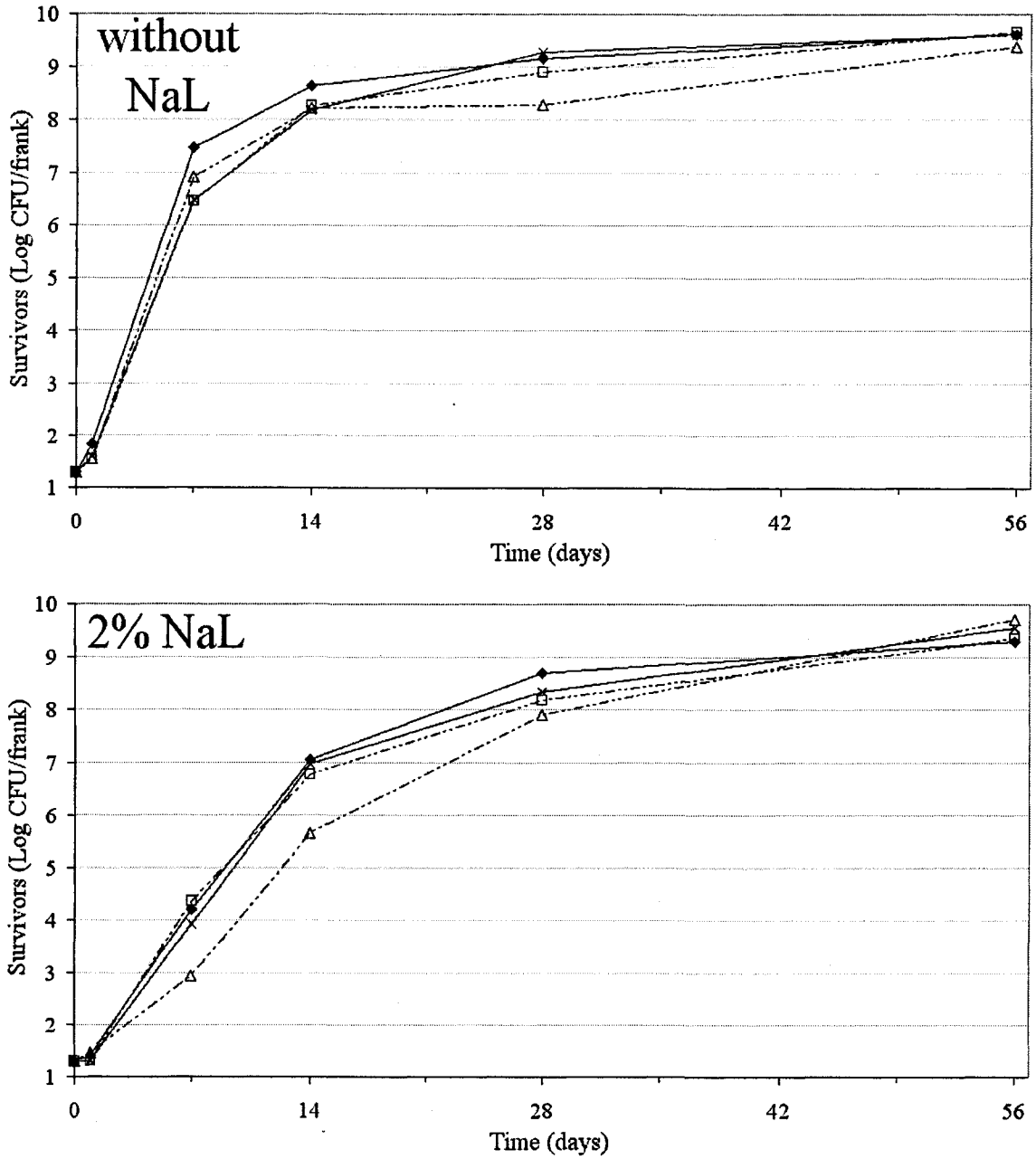

Figure 7. Mean LAB populations (MRS) on the surface of frankfurters without or with 2% sodium lactate in the formulation, during storage at 10°C. Samples were left untreated (◆) or were surface-treated with 10 mM Mb-Cu (□), 1% SLS (△), or 1% SLS + 10 mM Mb-Cu (×), inoculated with *L. monocytogenes* (~6.3 log CFU/frank), then vacuum-packaged.

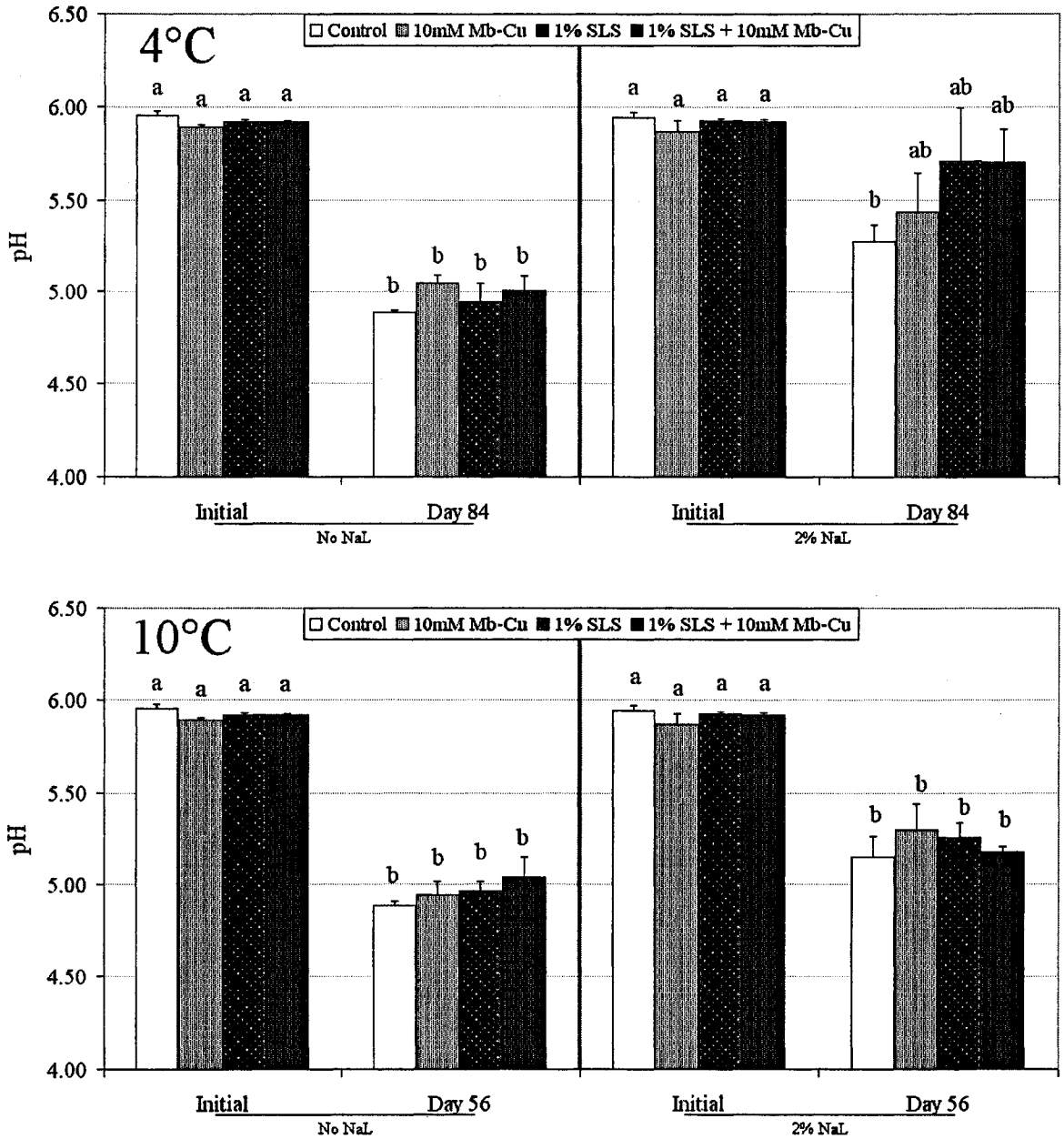

Figure 8. Mean pH values of untreated frankfurters, after surface treatment, and following storage at 4°C for 84 days and 10°C for 56 days. Values represent the means of at least three surface measurements ± SD (error bars). Letters indicate significant differences ($P<0.05$) between means within a given formulation grouping.

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CHAPTER 6. THE ANTIMICROBIAL MODE OF ACTION OF METHANOBACTIN AGAINST *LISTERIA MONOCYTOGENES* SCOTT A INVOLVES INHIBITION OF RESPIRATORY FUNCTION

A paper to be submitted to Applied and Environmental Microbiology

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ABSTRACT

Methanobactin, a novel chromopeptide appears to fulfill multiple roles including copper sequestration and/or detoxification, delivery, and induction of pMMO activity possibly by increasing electron flow in the producer-organism *Methylosinus trichosporium* OB3b. Recently, studies revealed that copper-bound methanobactin (Mb-Cu) is bactericidal to the foodborne pathogen *L. monocytogenes*. The objectives of this study were to gather preliminary evidence regarding the potential mode of action of Mb-Cu against this pathogen. The influence of Mb-Cu on the time-kill kinetics, cell lysis, leakage of UV-absorbing material, and respiration of *L. monocytogenes* were all determined in MES buffer (pH 6.0). Mb-Cu displayed dose-dependent lethality against *L. monocytogenes* Scott A.

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After 2 h, populations were reduced by 3.92, 5.97, and 6.59 log-cycles for 125, 500, and 2000 μM, respectively; when determined on Modified Oxford (MOX) agar. Counts were similar on tryptic soy agar plus 0.6% yeast extract (TSAYE), indicating there was little to no injury. No lysis was observed by monitoring the absorbance (595 nm) of bacterial suspensions, but for all the aforementioned concentrations, at least some leakage of 260 and 280 nm-absorbing material was detected in culture filtrates. In addition, respiration was inhibited in a dose-dependent manner, at Mb-Cu concentrations as low as 7.81 μM, with almost complete loss at 1000 μM. Loss in *L. monocytogenes* respiratory function was rapid and could be detected at the lowest Mb-Cu concentration tested. Collectively, these results indicate that the cell membrane is a likely candidate for the biological target of Mb-Cu against *L. monocytogenes*. More studies are needed to determine the precise binding requirement and/or receptor on cell surfaces, as well as the effect of Mb-Cu on the bioenergetics and leakage of other cellular constituents of *L. monocytogenes*.

INTRODUCTION

It has been established for many years that lactic acid bacteria (LAB), actinomycetes (i.e. *Streptomyces* spp.), and plant-dwelling bacteria (i.e. *Pseudomonas* spp.) are major groups of organisms known for their production of distinctly different classes of antimicrobial compounds. LAB produce a wide variety of antagonistic agents, namely organic acids, low MW compounds (i.e. reuterin and methylhydantoin), and bacteriocins (8, 30). The latter group of compounds is a large and fairly diverse set of peptides or proteins that are typically active against only closely-related organisms, with the exception of

lantibiotics. Actinomycetes are free-living widely distributed bacteria that are noteworthy as antibiotic producers (7). Pseudomonads are well characterized in their production of siderophores, extracellular iron-chelating molecules that play a major role in their complex iron metabolism. Within several major classes, there is wide variety of known proteinaceous siderophores produced by these organisms (17). Cyclic lipopeptides (29) and mainly siderophores, produced by *Pseudomonas* spp., have been shown to inhibit the growth of other bacteria (5, 9, 37).

Another important group of environmental bacteria that may also produce a distinct class of antimicrobial are methanotrophs. This group of organisms plays an integral role in global carbon cycling, where they are characterized by their ability to oxidize methane as a sole carbon and energy source via soluble methane monooxygenase (sMMO) and particulate methane monooxygenase, or pMMO (23). It is well established that the regulation between these two enzymes is tightly regulated by copper concentration (34), where under low copper-to-biomass ratios methane oxidation occurs through sMMO, but at high copper-to-biomass ratios sMMO activity is suppressed and pMMO is active. Studies on *Methylosinus trichosporium* OB3b constitutive sMMO mutants revealed the presence of extracellular copper solubilization even when pMMO could not be used in copper-containing media. It was suggested that the organism may excrete a copper-complexing agent similar to siderophores (18). This compound was originally thought to be a cofactor for pMMO in *Methylococcus capsulatus* Bath (42), and further studies on the previously mentioned sMMO^{c} mutant indicated that uptake or association of these so-called copper-binding ligands was prevented by this phenotype (38). DiSpirito *et al.* (15) demonstrated

that so-called copper-binding compounds produced by this organism had the highest concentrations present in the spent media when cells expressed pMMO and were stressed for copper, at the switch-over point between the two MMOs. Both of these studies isolated break-down components of what is now known as methanobactin.

Improvements in the isolation and purification of copper bound-methanobactin (Mb-Cu; 1217 Da) revealed the structure (26, 28) of what is now called a chalkophore, analogous to iron-binding siderophores (27). It is composed of a tetrapeptide, a tripeptide, and several unusual moieties, including a thionylhydroxyimidazolate, hydroxythionylimidazolate, pyrrolidine, and N-terminal isopropylester group. The role of this molecule is thought to be involved in copper sequestration and/or detoxification, handling, and uptake in methanotrophic bacteria due to the physiological importance of copper in these organisms. Recent studies on methanobactin indicate that it: may be synthesized by a nonribosomal peptide synthetase (NRPS; 39), possesses strong superoxide dismutase-like activity (12), chelates various metals (Choi *et al.*, submitted for publication), and stimulates pMMO activity possibly by increasing electron flow to the enzyme's type II Cu(II) centre(s) (10). Apo-methanobactin (Mb) binds Cu (I) or (II) where the former is found in the holo-form (10, 11, 21, 42). Finally, a model for copper binding by methanobactin has been proposed (11) which is based heavily on the potential of this chromopeptide being a "moonlighting protein", or protein which can serve multiple functions depending on conditions.

Methanobactin is considered analogous to the siderophore, and likely has a number of roles in methanotroph physiology. Investigations of copper-bound methanobactin (Mb-

Cu) antagonistic activity against the problematic foodborne pathogen, *Listeria monocytogenes* were conducted previously in our laboratory. Studies revealed that Mb-Cu is highly bactericidal to the pathogen and activity was optimum at pH 6.0. In broth media the minimum inhibitory concentration (MIC) was 4.11 mM, and at this concentration large reductions in bacterial populations occurred. Activity appeared to be an "all-or-nothing" event and such drastic cell death indicated that the bacterial cell membrane could be a likely target, possibly involving increased permeability or lysis. We hypothesized that this chromopeptide may function against *L. monocytogenes* in a similar fashion to previously described common mode of action for many antimicrobial peptides. Therefore, the objectives of this study were: (i) Determine if death kinetics behave in a dose-dependent manner; (ii) Determine if Mb-Cu induces gross cell leakage and/or lysis; and (iii) Examine Mb-Cu effects on respiration.

MATERIALS AND METHODS

Microorganism and inoculum preparation. *L. monocytogenes* Scott A NADC 2045 serotype 4b (human isolate from a 1983 milk outbreak) was obtained from the National Animal Disease Center, Agricultural Research Service (United States Department of Agriculture, Ames, IA), and used throughout the study. Prior to the experiments, *L. monocytogenes* cultures were confirmed by streaking onto Modified Oxford (MOX) agar, followed by gram-staining, oxidase and catalase tests, and API test kit verification. The culture was maintained as frozen (-20°C) stock in tryptic soy broth (Difco, Becton Dickinson and Co., Franklin Lakes, NJ) supplemented with 0.6% (wt/vol) yeast extract

(Difco; TSBYE) and 10% (vol/vol) glycerol. Prior to each experiment, the stock culture was transferred twice in 10 ml of brain heart infusion (BHI; Difco) broth and incubated at 32°C for 18 h. The inoculum (≥30 ml) was then harvested by centrifugation (10,000 × *g*, 10 min) in a refrigerated (4°C) centrifuge (Sorvall Super T21; DuPont Instruments, Wilmington, DE), and cells were washed twice in 0.02M 2-morpholinoethanesulfonic acid (MES) buffer (pH 6.0; Sigma, St. Louis, MO). The inoculum was resuspended in 0.1M MES buffer (pH 6.0) and depending on experiment, volumes differed. Inocula used for the time-kill and leakage experiment were not adjusted (~10^9 CFU/ml; assay concentration was ~10^8/ml) while the inocula used for monitoring lysis and respiration were concentrated (~6.3 × 10^9 and 1.6 × 10^{10} CFU/ml, respectively). A final cell concentration of ~10^9/ml was used for each assay.

Methanobactin preparation. Lyophilized Mb-Cu samples (referred to as methanobactin throughout this paper) were prepared using a modified protocol previously described by Choi *et al.* (12). Mb-Cu samples were transferred to sterile 125-mm screw-capped test tubes, placed in Seward stomacher bags (Seward Ltd., London, England), and held on ice for one hour prior to irradiation. Samples were sterilized via electron-beam irradiation at the Iowa State University Linear Accelerator Facility, which has a MeV CIRCE III Linear Electron Accelerator (MeV Industrie S. A., Jouy-en-Josas, France). Samples were sterilized by irradiation at ≥30 kGy in the electron beam mode at an energy level of 10 MeV and an average dose rate of 58 kGy/min. Absorbed radiation doses were determined by the use of 5 (diameter) by 5 mm (length) dosimeter alanine pellets (Bruker

Analytische Messtechnik, Rheinstetten, Germany) placed on the top and bottom surfaces of one stomacher bag containing Mb-Cu samples. Immediately after irradiation, the pellets were placed in a Bruker EMS 104 EPR Analyzer to measure absorbed doses by electron paramagnetic resonance. The average absorbed dose was obtained from the arithmetic average of the top and bottom surface readings. Sterile samples were stored at -20°C and held for no longer than 2 months.

Time-kill studies. A 2.2 mM Mb-Cu stock solution was prepared aseptically in 0.1M MES buffer (pH 6.0) and appropriate dilutions were made in 125-ml Erlenmeyer flasks to give 125, 500, and 2000 μM final assay concentrations. Preliminary studies indicated these three concentrations represent the middle and extreme ends of bactericidal activity in buffer, but were quite lower than MICs determined in BHI broth. Treatment and control flasks were placed inside a gyratory shaker water bath (Model G76; New Brunswick Scientific Co. Inc., Edison, NJ) set at 32°C. After pre-warming, the washed *L. monocytogenes* inoculum (3-ml aliquots) prepared as previously described, was added to the flasks and incubated for 2 h while shaking at 150 rpm. At 10-min intervals samples were pulled from the treatment and control flasks, serially-diluted in 0.1% peptone water (Difco), and surface-plated, in duplicate, onto tryptic soy agar (Difco) supplemented with 0.6% (wt/vol) yeast extract (Difco; TSAYE) and MOX agar. Initial counts were determined by plating the washed inoculum onto TSAYE and MOX agar. All inoculated plates were incubated aerobically at 32°C and bacterial colonies were counted at 72 h.

During this experiment, samples were taken from the flasks to measure leakage of UV-absorbing material.

Measurement of UV-absorbing material leakage. These experiments were used as indicators of the loss of cell membrane integrity of *L. monocytogenes* (16). Samples for these experiments were taken in parallel with time-kill studies and thus, were collected from the same flasks (control, 125, 500, and 2000 μM Mb-Cu). At selected time intervals, up until 60 minutes, samples were removed and filtered through sterile 0.2-μm, 25-mm polyethersulphone (PES) syringe filters (Whatman Inc., Florham Park, NJ) into clean, sterile vials. Samples of filtrate (2-ml aliquots) were immediately frozen at -20°C and stored for <1 week before analysis. Non-inoculated control and treatment samples were also prepared for use as blanks. Filtrates were thawed to room temperature (~30 min), then transferred (100-μl aliquots except for 2000 μM) to 96-well, round-bottom, polystyrene microtiter plates (Becton Dickinson, Franklin Lakes, NJ). Mb-Cu concentrations at 2000 μM were found to absorb outside the limit of detection for the instrument, thus these samples, including blanks, were diluted 10-fold in buffer prior to taking readings. Absorbance of the samples were measured at 260 (nucleic acids) and 280 nm (nucleic acids + proteins) using a SpectraMax M5/M5^{e} microplate reader (Molecular Devices, Chicago, IL; wavelength range ± accuracy: 200-1000 ± 2.0 nm; photometric range ± accuracy: 0-4.0 < ± 0.006 OD ± 1.0%, 0-2 OD) equipped SoftMax Pro Software v5.0 (Molecular Devices). Each replicate of the experiment was performed in triplicate and raw data was corrected using $Abs_{260\ or\ 280}$ values associated with the blanks.

Determination of cell lysis. A 4.8 mM Mb-Cu stock solution was prepared aseptically in 0.1M MES buffer (pH 6.0) and 2-fold dilutions were made in small culture tubes to give assay concentrations ranging from 4000 to 7.81 μM. Samples were transferred (100-μl aliquots) to the microtiter plates described above and followed by inoculation (20 μl) with *L. monocytogenes* culture, prepared as described earlier. The plates were incubated statically at 32°C and absorbance readings (595 nm) were taken periodically for up to 24 h using a Model 550 microplate reader (Bio-Rad Laboratories, Hercules, CA), after 10-sec agitation. Each control and treatment replicate was performed in triplicate, and raw Abs_{595} values were corrected using blanks prepared by the addition of buffer instead of cell suspension to the samples.

Measurement of oxygen consumption. Experiments were conducted to determine the effect of Mb-Cu on the respiration of *L. monocytogenes*. A 12 mM Mb-Cu stock solution was prepared aseptically in 0.1M MES buffer (pH 6.0) and 2-fold dilutions were prepared in small culture tubes to give assay concentrations ranging from 4000 to 7.81 μM. Oxygen uptake was measured at 23.5 ± 0.5°C with a Clark-type oxygen probe (Biological Oxygen Monitor, Model 5300; YSI Co., Yellow Springs, OH) immersed in a magnetically stirred sample chamber containing 0.1M MES buffer (pH 6.0). The inoculum was injected into the chamber (~10^9 CFU/ml assay concentration) followed by addition of Mb-Cu. After 5 minutes, glucose (10 mM assay concentration; Sigma) was added as substrate, and oxygen consumption was monitored under constant stirring.

Data analysis. All experiments were repeated at least twice; microbiological counts (log_{10} CFU/ml), corrected absorbance readings (260, 280, and 595 nm), and oxygen consumption rates ($\%O_2$ min^{-1}) are reported as means. Analysis of variance was used to determine significant differences among mean values following the Tukey-Kramer adjustment. Differences were considered statistically significant when the associated P-value was less than 0.05.

RESULTS

Methanobactin time-kill effects on *L. monocytogenes*. The results of the time-kill studies are shown in Figure 1. As expected, MES buffer had no effect on the viability of *L. monocytogenes*. Exposure to Mb-Cu for 2 h resulted in 3.92, 5.97, and 6.59 log-reductions (MOX) for 125, 500, and 2000 μM, respectively; while on TSAYE these reductions were 3.60, 6.58, and 6.73 log-cycles. This shows there was little difference in total reductions between the 2 higher Mb-Cu concentrations ($P>0.05$) or between media type. However, there were some differences in recovery between the two media types at earlier sampling times. Overall, *L. monocytogenes* counts were higher on MOX agar than TSAYE, where this was especially noticeable for both the 125 μM treatment and at early sampling times (up to ~2 log difference). As Mb-Cu concentration increased, higher counts on TSAYE than MOX were observed at earlier time intervals. For example, at 500 μM, counts on TSAYE did not exceed those on MOX until 40 min, but at 2000 μM this occurred at 20 minutes. In general, kill curves determined by plating on TSAYE were more linear than those derived from using MOX (based on R^2 values; data not shown) with a slight "dip" at

intermediate times. MOX kill curves were slightly sigmoidal-shaped, where more rapid killing occurred at earlier times as Mb-Cu concentration increased. The rate of *L. monocytogenes* death was most rapid at <20 min for MOX and <10 min for TSAYE, especially at 2000 μM of Mb-Cu ($P<0.05$), followed by fairly similar kinetics among Mb-Cu concentrations for the duration of incubation. Curves tended to "tail off" slightly for 125 μM of Mb-Cu though. Results show that Mb-Cu does displayed dose-dependent effects on the viability of *L. monocytogenes*; differences were mainly seen early during incubation, and higher Mb-Cu concentrations tended to generate larger death rates sooner. In addition, there did not seem to be a major benefit of using 2000 of μM Mb-Cu compared to 500 μM against *L. monocytogenes* in buffer.

Effect of methanobactin on leakage of UV-absorbing material and cell lysis. In general, leakage of 260 and 280 nm-absorbing material from *L. monocytogenes* cells increased over time in the presence of Mb-Cu (Figure 2), however, only increases from exposure of cells to 125 of μM Mb-Cu were significant ($P<0.05$). The highest level of 260 nm-absorbing material was 0.022, 0.025, and 0.044 absorbance units for 125, 500, and 2000 μM Mb-Cu, respectively. At 280 nm, these were 0.083, 0.048, and 0.122 absorbance units. Significant differences ($P<0.05$) among Mb-Cu treatments at a given sampling time were only found at 60 minutes for 260 nm-absorbing material, and 45 minutes for 280 nm-absorbing material. Compared to the control, Abs_{260} and Abs_{280} were largest for 2000 μM of Mb-Cu at 60 minutes, while 125 μM of Mb-Cu Abs_{280} were largest at 45 minutes. Results to this experiment showed high variability overall, which limits its value in making

inferences related to Mb-Cu's mode of action. In addition, correlations between UV-absorbing material leakage rates to that of cell death or cell death rates were modest ranging from ~0.5 to 0.7 (data not shown). Although very little statistical significance was found amongst these data, the increases in UV-absorbing material may be of biological significance. For example, at 60 minutes, samples containing 125, 500, and 2000 μM of Mb-Cu had 11, 12.5, and 22 times the amount of 260 nm-absorbing material, respectively, compared to the control. Similar behavior was seen with 280 nm-absorbing material leakage, however when leakage is evaluated this way, leakage amounts of 260 nm-absorbing material was about 7-8 times that of 280 nm-absorbing material.

Contrary to preliminary indications in broth media, monitoring changes in cell suspension Abs_{595} (~0.3), in buffer, did not demonstrate any detectable cell lysis (data not shown) by these methods. Changes were not larger at any Mb-Cu concentration from that of the control for a 24-h incubation period.

Effect of methanobactin on respiration. Figure 3 displays the effect of varying concentrations of Mb-Cu on O_2 consumption and consumption rates by *L. monocytogenes*. The addition of glucose to the system is indicated by the arrow (Figure 3A), occurring after a 5-minute incubation period of Mb-Cu with the pathogen. Calculated rates (Figure 3B) clearly demonstrate dose-dependent effects of Mb-Cu on O_2 consumption, or respiration rate, of *L. monocytogenes*. At a concentration as little as 7.81 μM, the respiration rate decreased due to Mb-Cu activity ($P>0.05$). Significant decreases in respiration rate were only observed at Mb-Cu concentrations of ≥125 μM ($P<0.05$). Almost complete inhibition

of respiratory activity occurred with a treatment of 1000 to 4000 μM of Mb-Cu ($P<0.05$). Respiration rate as a function of Mb-Cu concentration generated a near perfect linear fit as shown in Figure 3. Simply put, for every doubling in Mb-Cu concentration, the respiration rate decreased by 0.42% O_2 per minute. Good correlations were found between respiration rate and cell viability ($R^2>0.98$), and respiration rate and death rates ($R^2>0.90$), while the relationship between UV-absorbing material leakage rate and respiration rate were modest (data not shown). At any rate, there was a demonstrated Mb-Cu-induced loss in respiratory activity when Mb-Cu was incubated with *L. monocytogenes* for 5 minutes prior to substrate addition. In contrast, viability loss could not be detected until after 10 minutes and significant leakage of UV-absorbing material even later, when exposed to 125 μM of Mb-Cu.

DISCUSSION

The preliminary evidence presented here may help lay the ground work for further elucidation of the antimicrobial mode of action of Mb-Cu against *L. monocytogenes* Scott A. Concentrations used in the present study were far lower than those used in earlier work that determined the MICs in broth media (data not shown). For example, in BHI (pH 6.0), 4.11 mM of Mb-Cu was determined as the MIC, while in a later study 2.06 mM was considered the MIC when buffer was included in the growth media. Reductions in viable cell populations were nearly 5 log- and >5 log-cycles for these studies, respectively. This demonstrates that MICs may not necessarily be the appropriate concentrations to use when trying to determine the mode of action of antimicrobials. In the present study 125 μM of

Mb-Cu could generate a 4-log reduction in 2 hours while in broth media, a concentration of at least 32 times this was needed to reduce populations (unpublished data). It should also be noted that initial populations were 1.5 logs higher in this study than in previous work. It seems plausible that components of broth media may interfere with Mb-Cu activity.

Mb-Cu appears to inactivate *L. monocytogenes* in a dose-dependent manner where in buffer, this was found mainly between 62.5 (data not shown) and 500 μM. Although use of 2000 μM of Mb-Cu resulted in slightly higher death rates than 500 μM, these differences were mainly found during the first 10 minutes of incubation, and slopes appeared parallel thereafter. Reduction profiles caused by Mb-Cu are similar to reductions caused by other peptide antimicrobials such as bacteriocins. However, amounts are difficult to compare because bacteriocin or activity units (BU or AU) and arbitrary units (AU) are typically based on amounts needed to reduce a percentage of growth measured by absorbance, or create a zone of clearing on an agar plate. For instance, 10,000 BU/ml of carnocin UI49 (36), which is 10,000 times the amount needed to inhibit growth by 50%, reduced populations of LAB by 6 log-cycles in 4 hours, in buffer. Studies on Lactostrepcin 5 indicated at least 200 AUs calculated by these means were needed to reduce *Streptococcus cremoris* 202 by 3 log-cycles (43). Based on results of our study, a Mb-Cu concentration 32 times less than the MIC could reduce *L. monocytogenes* by 4 log-cycles, in 2 hours. From this standpoint, Mb-Cu is far more effective at killing susceptible cells than these bacteriocins. Nisin, on the other hand completely eliminated *L. monocytogenes* using 0.3 μg/ml (1), a far lower concentration than that of Mb-Cu used in the present study. Killing potential by Mb-Cu also seems to be better in comparison to other antimicrobial peptides as

well. Protamine concentrations >10,000 μg/ml were required to have any listericidal activity (22). Friedrich *et al.* (19) tested six cationic peptides against *Staphylococcus* spp. and found that at 10 times the MIC, none had exceeded 4-log reductions of the indicator strains. However, it is worth mentioning that the MICs found in the latter study were all lower than that of Mb-Cu on a molarity basis.

Prior to the onset of these experiments, we felt that Mb-Cu-induced cell lysis could be a probable mode of action since negative absorbance readings were found to occur at higher Mb-Cu concentrations and the cut-off between growth and death was a fine line. No direct evidence of cell lysis could be demonstrated in the present study based on monitoring absorbance at 595 nm. One major difference between the previous studies and the present is that an exogenous energy source was provided in the former (broth media), but it is not known whether this can be attributed to the discrepancy. Studies have shown that energized cells versus non-energized cells differ in susceptibility to some bacteriocins but differences tend to be opposite of that described here (33, 40, 43), thus this may not be a credible explanation. Methodology used to determine cell lysis varies by spectrophotometric determination (595 to 660 nm) and microscopy methods can also be used (6, 25). Possible improvements used in the present study would have been to follow the growth of *L. monocytogenes* in broth spectrophotometrically, and "spike" the culture with Mb-Cu at a designated time interval to view changes in absorbance (32). Although this may improve sensitivity, there is no indication of cell lysis occurring based on methods used in the present study.

The first sign of an increase in membrane permeability is usually provided by the leakage of UV-absorbing material and other metabolic pool materials (16). Absorbance increases were not found to be particularly significant; however, these increases could be of biologically significance. Leakage of 260 nm-absorbing material in the present study may be of lower magnitude (≤0.044 Abs units) since starvation of *Arthrobacter crystallopoietes* resulted in values in excess of 1.4 Abs_{260} units. However, Carson *et al.* (6) converted 260 nm-absorbing material leakage amounts to proportions, and attributed tea tree oil-induced *S. aureus* cell death, in part, to 260 nm-absorbing material leakage. Conversion of the data in the present study by these means would demonstrate far more leakage than their study. Additionally, the application of pulsed electric fields on *L. monocytogenes* (35) and *L. innocua* (2) generated very similar values to the present study. The most extreme treatments in those reports generated leakage amounts similar to 2000 μM Mb-Cu. Of more consequence, the lowest Mb-Cu concentration used (125 μM) also seemed to induce leakage. This may imply that because some leakage occurs at the lowest concentration used, and cell death occurred by this treatment, increases in membrane permeability may be a primary cause for Mb-Cu lethality. One major source of variability that probably contributed to the high variance in calculated UV-absorbing leakage amounts is that Mb-Cu absorbs readily in the UV range (27). In addition, copper-bound methanobactin in a 1:1 molar ratio (as used in the present study) produces a distinctive peak at 282 nm (11). Although the proper use of samples to correct raw data can eliminate this characteristic, it is highly dependent upon accuracy of sample volumetric measurement. Given that leakage of *L. monocytogenes* cells may be indicated by small increases in filtrate UV absorbance

values, especially in relation to the absorbance of Mb-Cu, it may be desirable to establish a method of eliminating the compound from the filtrate prior to measurement (16).

Mb-Cu-induced loss in respiratory activity of *L. monocytogenes* was clearly shown in the present study. Also, out of the other experiments conducted within this work, it appears that this cellular function was the most sensitive to Mb-Cu. A decrease in respiration rate was found in the presence of the lowest Mb-Cu concentration and this appeared after only 5 minutes of Mb-Cu exposure, where leakage and viability loss were shown later. This may indicate that Mb-Cu inhibits respiration by directly acting on a respiratory enzyme(s) or function(s), or permeabilizing the cytoplasmic membrane. Recent evidence has shown that Mb-Cu can increase the electron flow to the type II Cu (II) centre(s) of pMMO in *Methylococcus capsulatus* Bath (10). In addition, other redox-active extracellular siderophores have been found to shuttle electrons between reduced and oxidized compounds in association with respiration, and some virulence factors connections are being drawn (24). Siderophores exhibit structural and conformational specificities to fit into membrane receptors and/or transporters, which also may apply to Mb-Cu, to some extent (41). Based on these points, it is conceivable that Mb-Cu may in fact interact with the respiratory chain, at some point, thereby blocking its activity. However, this mechanism alone might not necessarily lead to cell death as *L. monocytogenes* can generate ATP by multiple fermentation pathways (20). In addition, loss in viability (only slight) could not be detected until the use of 62.5 μM Mb-Cu (data not shown), with more apparent death at 125 μM. Disruption of membrane organization and function may also lead to decreased respiratory activity, which has been shown for protamine against *L.*

monocytogenes and *Shewanella putrefaciens* (25). Also, insect defensin forms voltage-dependent channels in *Micrococcus luteus*, leading to inhibition of respiration (14). No stimulation of respiration was seen in these two studies, indicating they do not necessarily act as uncouplers (31). This was not observed in the present study either, but was shown with the small peptide linenscin OC2 against *L. innocua* (4).

In summary, the results of this study indicate that the cytoplasmic membrane is the likely biological target of Mb-Cu's inhibitory action against *L. monocytogenes*, without concurrent cell lysis. Overall, cell viability decreased fairly rapidly in buffer in a dose-dependent manner, at least some leakage of UV-absorbing material was observed, and respiratory activity was strongly inhibited. Little to no injury could be demonstrated using selective and non-selective media, a result that supports structural changes of the membrane, rather than physiological or metabolic damage (2). Further support of cell membrane alteration or damage is that we have not been able to demonstrate any Mb-Cu bacteriostatic activity, only bactericidal (3). It is tempting to speculate that Mb-Cu may interact with the cytoplasmic membrane by binding a respiratory enzyme, thereby causing inhibition. Binding of the H^+-translocating ATPase cannot be ruled out, nor binding of phospholipids within the membrane. Small pores may be formed or major disruption could be a likely cause of UV-absorbing material leakage, leading to the collapse of membrane potential, and subsequent loss in energy. Without an intact cytoplasmic membrane, *L. monocytogenes* would not be able to carry out essential cellular functions such as compartmentalization, transport, and energy production, among others. If small pores are indeed formed, it is likely prerequisite that Mb-Cu would have to be able to oligomerize or

"stack" up on itself, a behavior that has been suggested to occur with this compound, consistent with other moonlighting proteins (11). Further study is needed to conclusively determine the mode of action of Mb-Cu. The use of lipid vesicles (carboxyfluorescein filled) and protoplasts prepared from *L. monocytogenes* Scott A cells could help determine whether Mb-Cu binds with a component of peptidoglycan, cell membrane lipids, or cell membrane proteins. Also, cytoplasmic membrane vesicle preparations made from the pathogen and *in vitro* investigation on the activities of ATPase and respiratory enzymes could determine whether Mb-Cu directly inhibits respiration. Studies on the effects of Mb-Cu-induced leakage of other cellular constituents such as protons, K^+, amino acids, and ATP, as well as cellular bioenergetics, such as $\Delta\Psi$ and ΔpH, are warranted. Determination of the precise mode of action of this novel biopreservative will be useful in determining other suitable compounds to use along with Mb-Cu as synergists. This could lead to a more effective means for controlling *L. monocytogenes* in foods.

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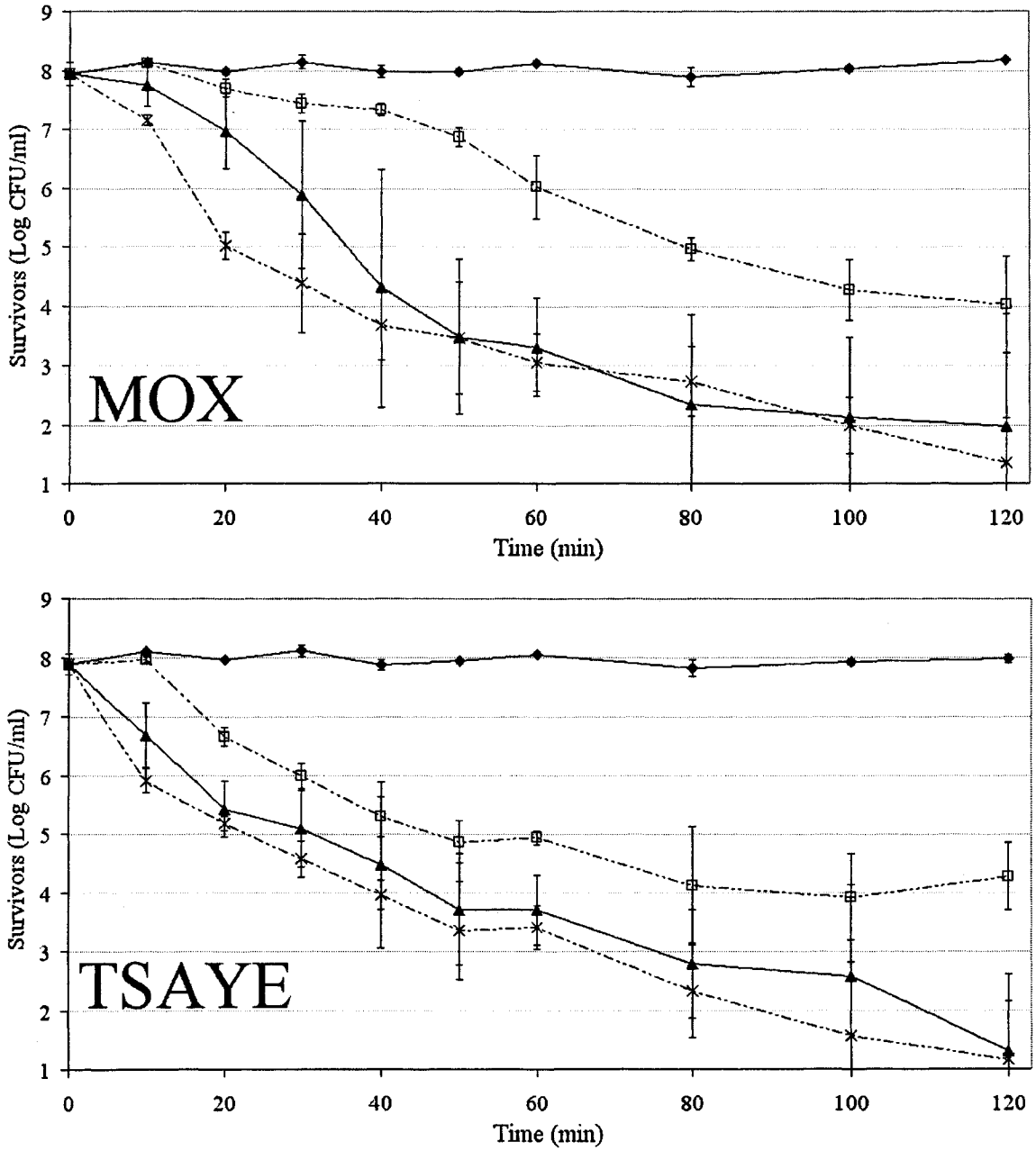

Figure 1. Mean survival curves of *L monocytogenes* Scott A determined by plating on MOX and TSAYE after exposure to Mb-Cu in 0.1M MES buffer (pH 6.0) at 32°C for 2 h. Treatments include: control (◆), 125 μM (□), 500 μM (▲), and 2000 μM (×) methanobactin (n=3).

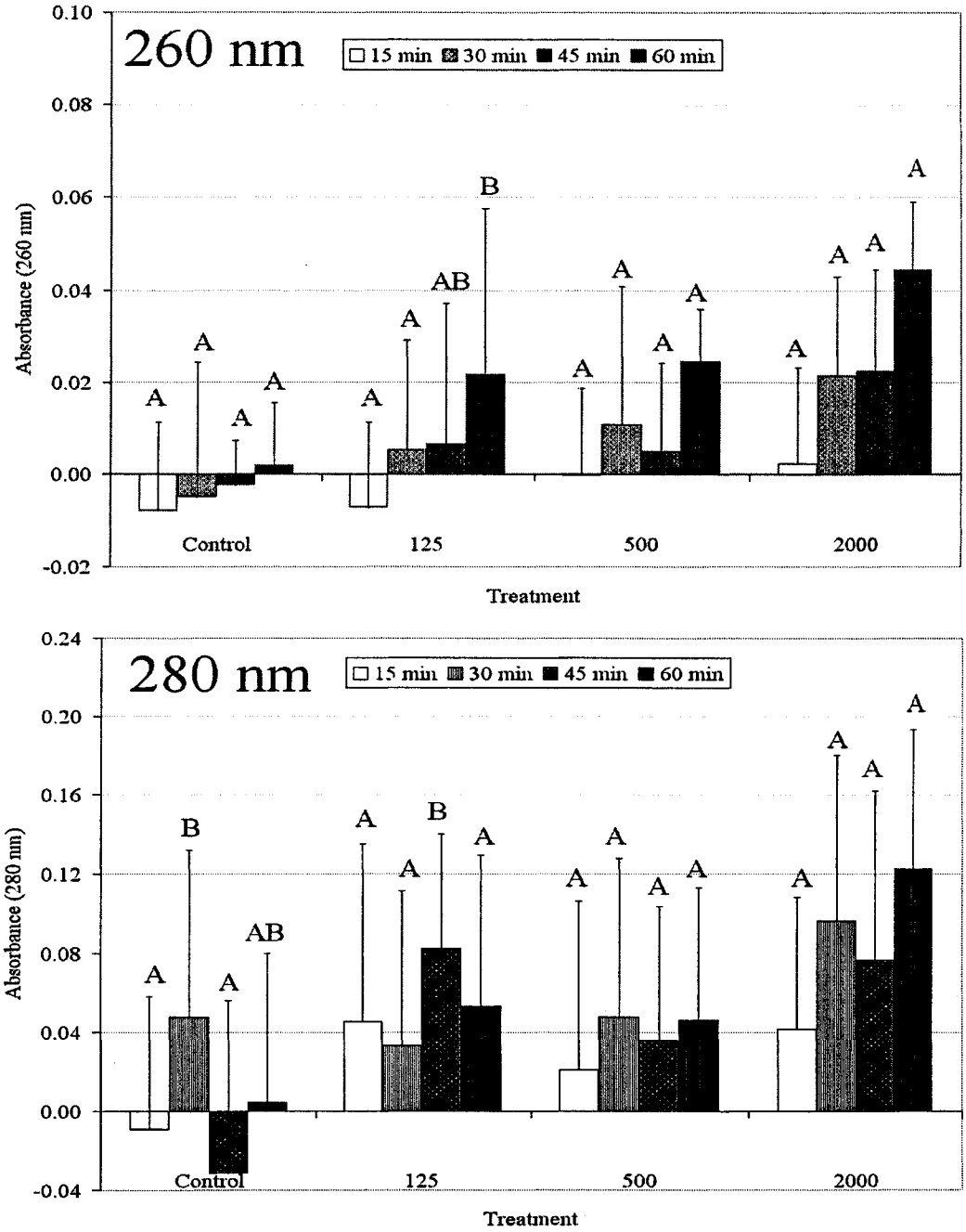

Figure 2. Appearance of 260 and 280 nm-absorbing material in the filtrates of *L. monocytogenes* Scott A (~10^8 CFU/ml) control suspensions and after treatment with 125, 500, or 2000 μM methanobactin in 0.1M MES buffer (pH 6.0). Values (corrected) represent the means of three replicates (each done in triplicate) ± SD (error bars) as determined by spectrophotometric measurement. Letters indicate significant differences ($P<0.05$) within a treatment grouping (n=3). Non-inoculated control and treatment samples were used as blanks

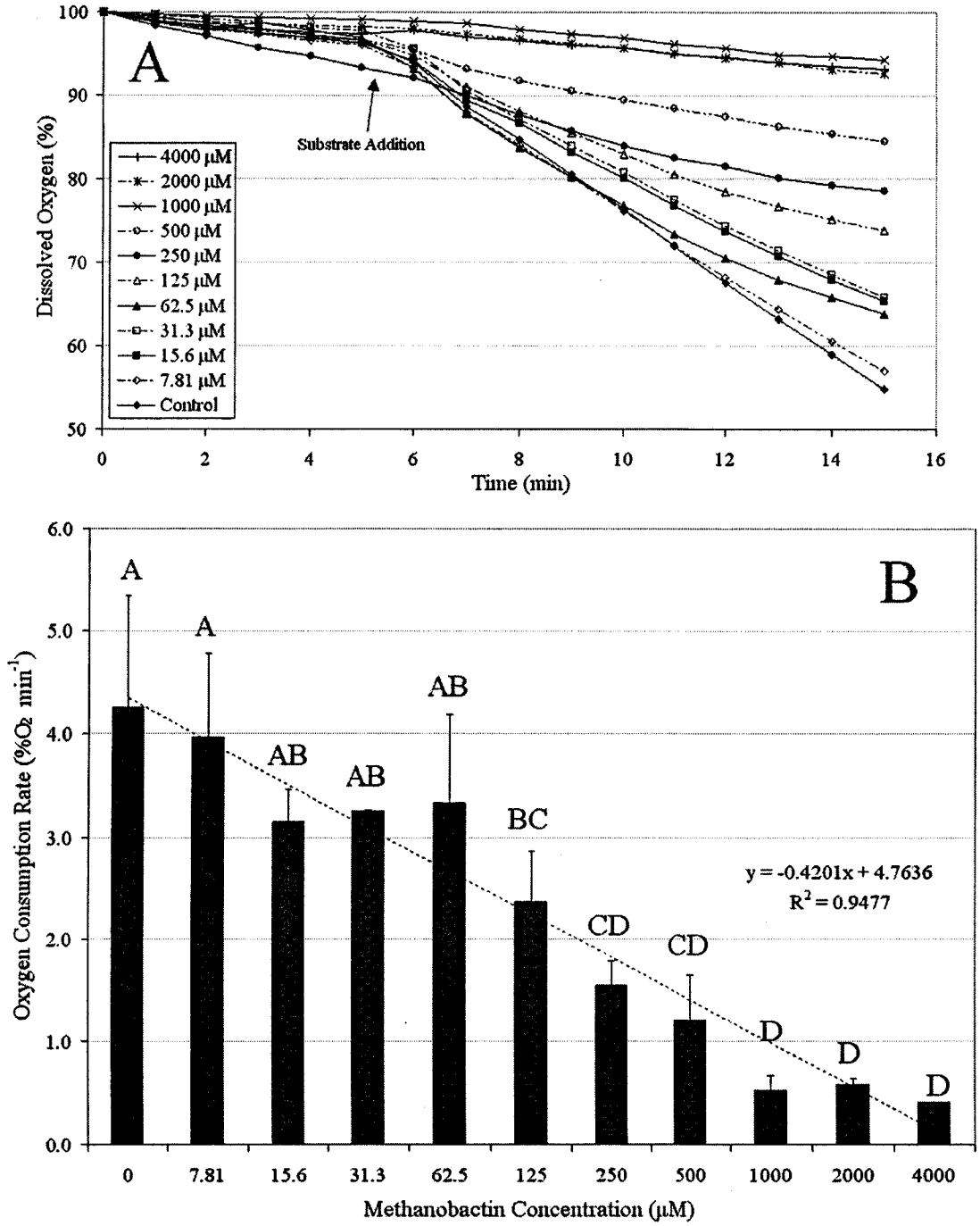

Figure 3. Effect of methanobactin on oxygen consumption curves (A) and rates (B) by *L. monocytogenes* Scott A (~10^9 CFU/ml) in 0.1M MES buffer (pH 6.0) at 23.5°C. Values represent the means of three replicates ± SD (error bars) as determined by electrode measurement. Letters indicate significant differences ($P<0.05$) between treatment means (n=3).

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CHAPTER 7. GENERAL CONCLUSIONS

CONCLUSION

It is clear that *Listeria monocytogenes* is a deadly foodborne pathogen that has gained a significant amount of attention due to its ubiquity in nature, resistance in the food processing environment, ability to grow at refrigeration temperatures, and implication in recent outbreaks associated with ready-to-eat (RTE) meat products. In response to changes in consumer preferences, many investigators have focused research efforts on the use of naturally-produced and novel antimicrobial systems for activity against the pathogen, and their potential use in RTE meats and other food products. As previously mentioned, methanobactin is a naturally occurring compound that is produced by the methanotrophic bacterium, *Methylosinus trichosporium* OB3b. Results described in this dissertation are the first to report the antimicrobial activity of methanobactin against a foodborne pathogen. Specifically, our studies on this compound for use against *L. monocytogenes* determined: minimum inhibitory concentrations (MICs) as influenced by pH, bactericidal versus bacteriostatic activity, its affect on growth, the influence of surfactants at two concentrations on activity, its use as a surface treatment in an antimicrobial system for frankfurters, and potential antimicrobial mode of action.

These studies indicate that methanobactin is highly bactericidal against *L. monocytogenes* displaying little to no bacteriostatic activity. Additionally, activity was highest at pH 6.0 where there was a non-linear relationship between the MIC of methanobactin and pH. The use of the non-ionic surfactants, Tween 20 and Tween 80 antagonized methanobactin activity, where this effect was more pronounced with increasing

pH and surfactant concentration. In contrast, sodium lauryl sulfate when used alone and in combination with methanobactin displayed high lethality, where the latter treatment was shown to be synergistic. This system allowed the use of lower methanobactin amounts to achieve the same desired effect as when it is used alone at a higher concentration. Frankfurters that were surface-treated with methanobactin, sodium lauryl sulfate, and their combination showed moderate initial reductions and growth inhibition of the pathogen during product storage. Formulating frankfurters with 2% sodium lactate did not completely inhibit the pathogen for the length of the study; however, counts were similar to initial numbers due to initial lethality caused by surface treatments. It was concluded that the system showed moderate activity on this cured RTE meat product against *L. monocytogenes*. Studies conducted to generate preliminary evidence on the mode of action of methanobactin against the pathogen demonstrated: Dose-dependent killing kinetics over a very narrow concentration range, dose-dependent loss in respiratory activity, and low leakage of UV-absorbing material without lysis. It was concluded that the cell membrane is a likely candidate for the biological target of methanobactin against *L. monocytogenes*.

Overall, methanobactin was shown to have good potential in controlling the presence of *L. monocytogenes* on foods, with many possibilities remaining for improving activity. Not only does methanobactin need to be further characterized, but its use on foods warrants further investigation.

RECOMMENDATIONS FOR FUTURE RESEARCH

Research remaining on methanobactin food-use is quite extensive. It is much more logical to determine whether a novel compound possesses antimicrobial activity in model and applied systems prior to determining safety, due to the length of time and subsequent costs the latter studies require. Because this compound has been shown to possess antimicrobial properties against *L. monocytogenes* both *in vitro* and on a food product, work evaluating its safety should be a main research topic. Of course, this antimicrobial must not be toxic to test animals and humans, based on several studies. It is also important that the antimicrobial be metabolized and excreted by the body. Finally, the compound or its breakdown products should also not result in buildup of residues in body tissues. Methanotrophic bacteria have been used for single-cell protein (SCP) production and very large quantities of Mb-Cu are produced by these organisms, thus safety concerns of Mb-Cu at this point seem negligible.

Although there is more work to be done in regards to methanobactin activity against *L. monocytogenes*, nothing is known about its activity against gram-negative bacteria. It may prove to be a very effective bacteriocide against gram-negative pathogens, despite the possibility of having to use chelators such as EDTA.

To improve delivery, other surfactants should be evaluated for compatibility with methanobactin, and concentrations need to be optimized. Coupling methanobactin with other antimicrobials such as organic acids, bacteriocins, or any other naturally-occurring substances is yet to be determined. In addition, it is not known whether methanobactin can

sensitize *L. monocytogenes* to other types of physical or chemical processing methods and vice versa. Coupling these treatments as hurdles also remains to be investigated.

Production of methanobactin on a larger scale would be critical to commercialize the product for use in antimicrobial hurdle systems for use on foods. The ability to do this seems very feasible at this point.

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