Iowa State University
Digital Repository

Retrospective Theses and Dissertations

Iowa State University Capstones, Theses and Dissertations

2007

Applications and mechanisms of colicin E1

Brenda Sue Patton
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/rtd

Part of the Agriculture Commons, Food Science Commons, and the Microbiology Commons

Recommended Citation
Patton, Brenda Sue, "Applications and mechanisms of colicin E1" (2007). *Retrospective Theses and Dissertations*. 15847.
https://lib.dr.iastate.edu/rtd/15847

This Dissertation is brought to you for free and open access by the Iowa State University Capstones, Theses and Dissertations at Iowa State University Digital Repository. It has been accepted for inclusion in Retrospective Theses and Dissertations by an authorized administrator of Iowa State University Digital Repository. For more information, please contact digirep@iastate.edu.

المنارة للاستشارات
www.manaraa.com

Applications and mechanisms of colicin E1

by

Brenda Sue Patton

A dissertation submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Major: Food Science and Technology

Program of Study Committee:
James S. Dickson, Major Professor
Byron Brehm-Stecher
Terri Boylston
Aubrey Mendonca
Nancy Cornick

Iowa State University

Ames, Iowa

2007

Copyright © Brenda Sue Patton, 2007. All rights reserved.

المنارة للاستشارات

www.manaraa.com

UMI Number: 3291056

UMI®

UMI Microform 3291056

Copyright 2008 by ProQuest Information and Learning Company.
All rights reserved. This microform edition is protected against
unauthorized copying under Title 17, United States Code.

ProQuest Information and Learning Company
300 North Zeeb Road
P.O. Box 1346
Ann Arbor, MI 48106-1346

المنارة للاستشارات

www.manaraa.com

TABLE OF CONTENTS

LIST OF FIGURES	**iv**
LIST OF TABLES	**v**
ABSTRACT	**vi**
CHAPTER 1. GENERAL INTRODUCTION	**1**
Introduction	**1**
Dissertation Organization	**2**
Literature Review	**3**
References	**37**
CHAPTER 2. APPLICATION OF COLICIN E1 AS A CARCASS WASH INTERVENTION STRATEGY	**61**
Abstract	**61**
Introduction	**62**
Materials and Methods	**63**
Results	**66**
Discussion	**67**
Conclusions	**70**
Acknowledgements	**70**
References	**70**
CHAPTER 3. INHIBITORY ACTIVITY OF COLICIN E1 AGAINST *LISTERIA MONOCYTOGENES*	**78**
Abstract	**78**
Introduction	**79**
Materials and Methods	**80**
Results	**84**
Discussion	**86**
Conclusions	**89**
Acknowledgements	**90**
References	**90**
CHAPTER 4. ON THE ROLE OF COLICIN E1 AGAINST *LISTERIA MONOCYTOGENES*	**102**
Abstract	**102**
Introduction	**103**
Materials and Methods	**104**
Results	**111**
Discussion	**114**
Conclusions	**118**
Acknowledgements	**119**
References	**119**

المنارة للاستشارات
www.manaraa.com

CHAPTER 5. GENERAL CONCLUSIONS **135**
  General Discussion **135**
  Recommendations for Future Research **136**
  References **137**

ACKNOWLEDGEMENTS **138**

www.manaraa.com

LIST OF FIGURES

CHAPTER 1

Figure 1. BtuB, Prokaryotic Vitamin B12 receptor. **59**

Figure 2. Colicin E1 translocation events. **60**

CHAPTER 2

Figure 1. Colicin E1 inhibits *E. coli* O157:H7 in broth culture. **76**

Figure 2. The effect of ColE1 against *E. coli* O157:H7 on beef carcass samples. **77**

CHAPTER 3

Figure 1. Purity of colicin E1 preparation. **98**

Figure 2. The effects of ColE1 on *Listera monocytogenes* in broth culture. **99**

Figure 3. Acquired resistance evaluation of *Listeria monocytogenes* to ColE1. **100**

Figure 4. The efficacy of ColE1 against *L. monocytogenes* in RTE products. **101**

CHAPTER 4

Figure 1. Biotinylation of colicin E1. **127**

Figure 2. Colicin E1 activity against *Escherichia coli* O157:H7. **128**

Figure 3. Colicin E1 activity against *Listeria monocytogenes*. **129**

Figure 4. Potassium leakage induced by colicin E1. **130**

Figure 5. Protein leakage induced by colicin E1. **131**

Figure 6. Colicin E1-treated *L. monocytogenes* cells over 60-min period. **132**

Figure 7. *L. monocytogenes* proteins with affinity to colicin E1. **133**

Figure 8. Pore-formation by colicin E1 in *Listeria monocytogenes*. **134**

المنارة للاستشارات

www.manaraa.com

LIST OF TABLES

CHAPTER 3

Table 1. Optical Density (600nm) of *Listeria monocytogenes* strains treated with ColE1 at 0, 3, and 6 hours. **97**

CHAPTER 4

Table 1. Identification of elution fraction protein fragments associated with colicin E1. **126**

المنارة للاستشارات

www.manaraa.com

ABSTRACT

To combat the rise of foodborne disease outbreaks in fresh and ready-to-eat (RTE) meats and to reduce further bacterial drug resistance, researchers are looking to natural and chemical alternatives to antibiotics. These alternatives include, but are not limited to, plant and herbal extracts, vaccines, and bacterial proteins. One alternative, bacteriocins, are being used in livestock production, food manufacturing and processing, and retail packaging and sanitization. Bacteriocins are proteinaceous compounds produced by and lethal to bacteria of species similar to the producing strain. Various bacteriocins—including nisin, colicin, pediocin, cloacin, and lactic acid bacterial peptides—have been evaluated for use in food products or animal production as antibacterial strategies. Colicins, specifically, are produced by certain strains of *Escherichia coli* and like species. Bacteriocins within the colicin family have been shown to have several different killing mechanisms including pore-formation, nuclease activity against the DNA and RNA of target cells, as well as inhibition of protein synthesis. Work presented in this dissertation involves the use of colicin E1, a pore-forming colicin, against *E. coli* O157:H7, as well as the first documented antimicrobial efficacy of colicin E1 against the gram-positive pathogen *Listeria monocytogenes*. Colicin E1 provided powerful reduction of *E. coli* O157:H7 as a beef carcass spray intervention. Further testing with this bacteriocin demonstrated its unexpected inhibitory activity against the gram-positive pathogen *Listeria monocytogenes*. The mechanism of action of colicin E1 against *L. monocytogenes* was investigated using flow cytometry, spectrophotmetric leakage assays, and both light and transmission electron microscopies. Colicin E1 had direct affinity to several *L. monocytogenes* proteins. These proteins were discovered using affinity chromatography and identified using MALDI-TOF. These colicin E1-binding proteins

www.manaraa.com

included DNA polymerase III, LepA, and a cell wall anchor family protein. At this point, there is no literature stating any colicin having interaction with these proteins, suggesting Colicin E1 exerts its antimicrobial activity against *L. monocytogenes* in an independent and novel fashion.

المنارة للاستشارات

www.manaraa.com

CHAPTER 1. GENERAL INTRODUCTION

Introduction

Acute gastroenteritis affects 250 to 350 million people in the United States annually, and an estimated 22 to 30% of these cases are attributed to foodborne disease (97). Although the causes of many foodborne outbreaks reported to the CDC are unknown, the leading known causes are viral and bacterial. A broad spectrum of microbial pathogens can contaminate human food and water supplies and cause illness after they or their toxins are consumed.

Initial interest in the use of antibiotics to combat the rise of foodborne diseases has been tempered by the recognition that antibiotic overuse has led to the development of bacterial resistance to these compounds (76, 144).

In an effort to reduce further bacterial drug resistance, researchers are looking to natural and chemical alternatives to antibiotics—termed antimicrobials—in livestock production, food manufacturing and processing, and in retail packaging systems (7, 143). A variety of treatments for prevention of food spoilage and pathogenic bacterial contamination have been investigated, including nonmicrobial enzymes or peptides such as lysozyme, lactoferrin and magainins; plant-derived products such as herbs and spices or essential oils derived from these; and microbial metabolites including hydrogen peroxide, organic acids, and bacteriocins (143). Bacteriocins are proteinaceous compounds produced by, and lethal to, bacteria of species similar to the producing strain. Such bacteriocins as nisin, colicin, pediocin, cloacin, and lactic acid bacterial peptides have been evaluated for use in food products or animal production as antibacterial strategies (123). Colicins, specifically, are produced by certain strains of *Escherichia coli* and like species. Colicins have several

www.manaraa.com

different killing mechanisms within their protein family including pore-formation, nuclease activity against the DNA and RNA of target cells, and inhibition of protein synthesis (27). Several colicins have shown to be effective against gram-negative pathogens, such as *E. coli* O157:H7 and *Salmonella enterica* Ser. Typhimurium (24, 39). Work presented within this dissertation provides insight into a specific colicin, colicin E1, and its ability to kill E. coli O157:H7, as well as the gram-positive pathogen *Listeria monocytogenes.* Bacteriocins such as colicins have previously been thought to have an extremely narrow range of killing ability and seldom are active against differing groups of bacteria (27). Therefore, the mechanism of action for colicin against gram-positive bacteria such as *Listeria* is of great interest.

This dissertation reports the use of bacteriocin antimicrobials in animal production and meat and poultry products for the control of foodborne pathogens. Specifically, the use of colicins and colicin E1 in different livestock, meat, and poultry processing applications were evaluated.

Dissertation Organization

This dissertation was compiled following an alternative journal paper format. Contents include general introduction and review of the literature, followed by three separate chapters in which journal articles already published in or submitted to peer-reviewed scientific journals will be presented. A brief conclusion is included at the end of the dissertation.

المنارة للاستشارات
www.manaraa.com

Literature Review

Part 1. An Introduction to E. coli Species and Listeria monocytogenes

Escherichia Coli

Escherichia coli (*E. coli*) are gram-negative bacteria belonging to the family *Enterobacteriaceae*. Bacteria belonging to this family are commonly referred to as "enterics" as they are inhabitants of human and animal gastrointestinal tracts. Approximately 0.1% of the total bacteria within an adult's intestinal tract are represented by *E. coli* (141). Although limited in overall number, *E. coli* are the predominant facultative anaerobe bacteria found in this environment. The colonization of the large intestine by nonpathogenic *E. coli*, as well by as other types of *enterobacteria,* promotes intestinal health. These bacteria synthesize compounds such as K and B-complex vitamins which are then absorbed by the body. *E. coli* strains are mostly benign and rarely cause adverse effects. Some *E. coli* strains, however, have acquired the ability to cause infections ranging from intestinal disruption to meningitis and sepsis.

Given the mortality associated with the disease-causing strains, the primary research focus was placed on distinguishing benign and pathogenic strains of *E. coli*. There are over 700 different serovars of known *E. coli* that differ by distinguishing somatic (O) antigens and the flagellar (H) antigens. The O antigen is a lipopolysaccharide which identifies the serogroup of the strain, and the H antigen identifies its serotype. These antigenic determinants function as epidemiological detection tools. There are seven total groupings of pathogenic *E. coli*, called virotypes, including opportunistic *E. coli*, enteropathogenic *E. coli* (EPEC), enteroaggregative *E. coli* (EAEC), enterohaemorrhagic or shiga-toxin-producing

المنارة للاستشارات

www.manaraa.com

E. coli (EHEC/STEC), enteroinvasive *E. coli* (EIEC), diffusely adhering *E. coli* (DAEC), and enterotoxigenic *E. coli* (ETEC) (110). The virotyping scheme used groups of *E. coli* strains based on virulence characteristics such as toxin production and invasiveness; each virotype has a different pathogenesis and comprises a different set of O:H serotypes. The seven basic virotypes of pathogenic *E. coli* are described in further detail below.

Opportunistic E. coli. Opportunistic *Escherichia coli* are nonpathogenic unless an environmental change within the gastrointestinal tract promotes overpopulation and growth of the bacteria. Opportunistic behavior is typical in postsurgical wounds, on surgical implants (49), urinary tract infections, and peritonitis (12).

Enteropathogenic E. coli. EPEC strains are classically associated with diarrhea in young children (110). The most prevalent association of EPEC is with infants and young children in developing countries with high mortality rates of between 10 and 40% (35). Outbreaks of EPEC occur where children are in close contact such as in nurseries and daycare centers (154). The first stage in EPEC pathogenesis involves the initial adherence of bacteria to epithelial cells. This adherence is initiated in part by the bundle-forming-pilus proteins (BFP) secreted by EPEC. EPEC strains typically infect intestines via "attaching-and-effacing" (110). Using this virulence strategy, EPEC induces substantial cytoskeletal alterations in the intestine, which disrupts the brush border cytoskeleton and leads to a proliferation of filamentous actin beneath adherent bacteria. Effacement of the intestinal microvilli and intimate adherence between the bacterium and the epithelial cell membrane are also associated with EPEC infection (142). Other virulence factors include EPEC's use of a type III secretion apparatus. This system is utilized by many enteric pathogens, and it acts as a macromolecular syringe to inject effector proteins directly into host cells. The

المنارة للاستشارات
www.manaraa.com

effector proteins secreted are associated with the previously mentioned attachment and effacing mechanisms on epithelial cells (35). The underlying intereactions of other processes with EPEC-related diarrhea resulting from EPEC infection is still under investigation; however, the dramatic loss of microvilli and the subsequent malabsorption due to brush border enzyme deficiency certainly drive this resulting effect.

Enteroaggregative E. coli. Nataro and Kaper (110) were the first to describe EAEC in culture. Using a common adhesion assay using HEp-2 cells, these researchers found that certain strains of *E. coli* exhibit characterized by a "stacked-brick" formation of bacterial cells attached to the HEp-2 cells. This "aggregative" characteristic sets these strains apart from other virotypes of *E. coli*. Typical illness due to EAEC infection is characterized by watery, mucoid, and secretory diarrhea with low-grade fever and little to no vomiting (13). The basic strategy of EAEC is to comprise colonization of the intestinal mucosa—predominantly that of the colon—followed by secretion of enterotoxins and cytotoxins (110). EAEC strains characteristically enhance mucus secretion from the mucosa, with trapping of the bacteria in a bacterium-mucus biofilm (110). This heavy biofilm is related to the EAEC's ability to cause persistent colonization and diarrhea (73). In addition to forming a mucous biofilm, many EAEC strains induce cytotoxic effects on the intestinal mucosa including microvilli shortening and necrosis. Virulence factors associated with EAEC are a heat-stable toxin and several aggregative adherence fimbriae (128, 109).

Enterohemorrhagic E. coli. EHEC can be found in the fecal flora of a wide variety of animals, but the most important animal specie in terms of human infection is cattle. Colonization in beef cattle leads to an abnormally high prevalence of EHEC in cattle transport and lairage, which can contaminate carcasses during slaughter and subsequently

المنارة للاستشارات
www.manaraa.com

onto retail product. The pathogenesis involves establishment of EHEC in the gastrointestinal tract where it has to compete for space and nutrients with other microorganisms of the normal intestinal microflora. The most important virulence characteristic of the organism, once established, is its ability to produce one or more phage encoded verocytotoxins or Shiga toxins. It is generally believed that after intestinal infection with EHEC, Stxs cross the intestinal barrier and bind to endothelial cells. At this point they presumably injure the host cell by inhibition of protein synthesis, stimulation of prothrombotic messages, or induction of apoptosis. The actions of these toxins on intestinal tissues cause the development of bloody diarrhea. Hemolytic Uremic Syndrome (HUS) is the microvascular disease resulting from toxin entrance in the blood stream. Once circulating, the toxins can bind to receptors on endothelial cells associated with the kidneys and brain (86). This association leads to renal failure and hemolytic anemia.

The most important EHEC serotype implicated worldwide is O157:H7. *Escherichia coli* serotype 0157:H7 is a known cause of hemorrhagic colitis and HUS. Hemorrhagic colitis is acute disease caused by *E. coli* O157:H7 and is characterized by severe abdominal cramping and diarrhea. Consumption of raw milk and ground beef has been linked epidemiologically with several outbreaks of disease caused by *E. coli* 0157:H7 (31, 87, 152). Further outbreaks from consumption of unpastuerized apple juice (75), cheeses (29), lettuce (74), bagged spinach (30), and other vegetative products have all been reported. Increasing reported cases of human *Escherichia coli* O157:H7 illnesses have also been related to contact with animals or to water supplies contaminated by runoff from cattle farms. *E. coli* O157:H7 was first recognized as a cause of illness in 1982 during an outbreak traced to contaminated

www.manaraa.com

hamburgers. Since then more infections in the United States have been caused by eating undercooked ground beef than by any other food (32).

Enteroinvasive E. coli. EIEC are primarily involved in causing Shigellosis, a self-limiting infectious disease such as dysentery in humans. The burden of this disease was estimated to 150 million cases and one million deaths per year in developing countries (89). Shigellosis is characterized by the destruction of the colonic epithelium provoked by the inflammatory response that is induced upon invasion of the mucosa by bacteria. The overall pathogenic schema of EIEC and Shigella species is to invade epithelial cells and induce an inflammatory response, leading to the construction of the epithelium. EIEC invasion is mediated by a large plasmid (140 MDa) coding for the production of several outer membrane proteins involved in invasiveness. Similarly to EPEC, EIEC produce a type III secretion system key to the invasiveness and virulence against target cells. By use of this system, as well as other secreted virulence proteins, intracellular bacteria move within the cytoplasm of infected cells without being exposed to the external environment (66).

Diffusely-adhering E. coli. Similar to the findings of Nataro and Kaper (110) in EAEC, DAEC were identified from their diffuse adherence (DA) pattern on cultured epithelial HEp-2 as well as HeLa cells (108). These *E. coli* strains have been found to be associated with urinary tract infections (UTIs) (pyelonephritis, cystitis, and asymptomatic bacteria) and with various enteric infections such as chronic watery diarrhea in young children (110). A key virulence factor in DAEC is the production of adhesins. Three adhesins, Afa, Dr, and F1845, are fimbrial adhesins allowing for the diffuse cell adherence to epithelial tissues. Afa and Dr are commonly expressed together and are greatly associated with urinary tract infection (134). Epidemiological studies show that DAEC strains that

المنارة للاستشارات
www.manaraa.com

express adhesins of the Afa/Dr family are involved in 25 to 50% of cases of cystitis in children and 30% of cases of pyelonephritis in pregnant women (3, 58). *E. coli* expressing Dr adhesin has been shown to be associated with a twofold increase in the risk of a second UTI, suggesting its possible association with recurrent or chronic UTI (58). Following adhesion to cell surfaces, these adhesins also double as invasins, allowing for entry into the intestinal cell lumen as well as macrophages (134). Another prominent virulence factor in DAEC is the flagella. It has recently been reported that motile Dr and F1845-positive *E. coli* strains were more able to induce an inflammatory response than nonmotile strains (4). Destruction and breakdown of the brush-border and severe intestinal inflammation follow invasion, and certain DAEC induce apoptosis in invaded cells (57).

Enterotoxigenic E. coli. ETEC is the most common cause of *E. coli*-mediated human diarrhea worldwide (61). Human ETEC infections are contracted by consumption or use of contaminated food and water and are most commonly associated with travel to underdeveloped countries. In addition, ETEC is a major pathogen of animals, being responsible for scours in cattle and neonatal and postweaning diarrhea in pigs (126, 155). Immediate symptoms include a sudden onset of secretory diarrhea which can lead to dehydration due to loss of fluid and electrolytes (118). ETEC strains follow a specific strategy when invading host cells. First, the bacteria adhere to host cells and multiply. At this time, ETEC strains need to evade host immune defenses. Strategies for ETEC fitness against immune defenses often lead to host damage, such as intestinal lining deterioriation (99). These steps are usually mediated by a number of proteinaceous virulence factors called colonization factors. These factors allow for the direct adhesion to intestinal cells in the human and animal digestive tracts (107). Also, ETEC strains produce one or more

www.manaraa.com

plasmid-encoded and heat-stabile enterotoxins that induce a secretory diarrhea. These toxins reduce absorption and increase fluid and electrolyte secretion of small intestinal epithelial cells, resulting in severe diarrhea (147).

Prevalence and Control of E. coli O157:H7 in Meat Products

As mentioned previously, cattle are the most common reservoir for EHEC O157:H7. The main sources of *E. coli* infection of cattle during production are contaminated drinking water and feed and the immediate environment of the animal. Risk factors that have been identified for infection of animals with *E. coli* O157 include age, weaning practices, movement of the animals, season, feed composition, and the ability of the bacteria to persist in the environment (55). Carcass and meat contamination of *E. coli* O157:H7 can occur from animal contact, as well as unsanitary conditions during transport, lairage, and slaughter. A critical point for controlling the contamination of meat with *E. coli* O157:H7 is proper and hygienic removal of cattle hides. A recent review by Arthur et al. (5) found that due to cattle transport conditions, the prevalence of *E. coli* O157:H7 on hides increased from 50.3 to 94.4% between the time cattle were loaded onto tractor trailers at the feedlot and the time hides were removed in the processing plant. In that study, carcasses were swabbed prior to transport and lairage as well as directly prior to slaughter. *E. coli* isolates were typed using pulse-field gel electrophoresis to evaluate differences in strain prevalence over time. Interestingly, only 29% of the *E. coli* O157:H7 isolates collected post-harvest matched those isolates collected before transport. These data indicated that a significant increase in O157 prevalence accompanied an increase in strain differentiation and abundance. Combating populations of various O157:H7 strains with differing fitness and/or antimicrobial resistance capabilities could be extremely difficult. It is therefore crucial to minimize the amount of *E.*

المنارة للاستشارات

www.manaraa.com

coli O157: H7 on cattle hides before slaughter. Cattle can shed *E. coli* O157:H7 in their feces at concentrations as high as 10^6 CFU/g (124), which can then cross-contaminate adjacent carcasses and product surface areas.

In the United States during 1992 and 1993, outbreaks of *Escherichia coli* O157:H7 infection associated with human consumption of ground beef caused hundreds of illnesses and four deaths (148). As a result of these and like outbreaks, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) mandated the Cattle Clean Meat Program and zero-tolerance standard regarding the complete removal of fecal material, ingesta, and udder fluids from beef carcasses, as well as detectable growth of *E. coli* 0157:H7 (149). Although these standards have long been in place, outbreaks continue to occur in comminuted products such as ground beef as well as in marinated steaks (92, 152). *E. coli* O157:H7 contamination occurring during slaughter carries over into fabrication and retail products. *E. coli* O157 can be attenuated at temperatures between 55–70°C (84); however, product remains uncooked during fresh meat production. Continuous addition of antimicrobials throughout the processing facility would presumably inhibit *E. coli* O157 contamination; however, this would be expensive and time-consuming. Also, a recent survey by Srinivasan et al. (138) analyzed ~130 isolated *E. coli* O157 strains from various animal reservoirs. All *E. coli* isolates exhibited resistance to five or more antimicrobial agents, and the majority of isolates carried one or more target antimicrobial resistance genes in different combinations. Even in combination, antimicrobials would not fully prevent further growth in fresh meat products.

Although not widespread, *E. coli* O157:H7 has also been associated with swine and pork products. Booher et al. (14) reported that in pigs experimentally dosed with *E. coli*

المنارة للاستشارات
www.manaraa.com

O157:H7, these strains persisted in the alimentary tracts for up to two months postinoculation. The authors concluded that the data indicated that swine were potential reservoirs for *E. coli* O157:H7. Supporting that conclusion, Doane et al. (45) stated that 8.9% of swine rectal samples taken for a large survey study were positive for *E. coli* O157:H7. No major outbreaks with retail pork product have been reported; however, a recent family outbreak of *Escherichia coli* O157 infection was microbiologically associated with consumption of dry-fermented salami made with pork meat only (38).

It seems logical to target on-farm contamination in cattle to reduce further contamination. This strategy has proven extremely difficult since *E. coli* O157:H7 infection may not provide clearly visible symptoms or signs to alert the producer or caretaker. Callaway et al. (23) stated that fecal shedding can be very sporadic with an animal testing positive for EHEC one day but not again for several days or weeks. An ideal, though technologically and physically unfeasible, solution would be to somehow effectively identify infected cattle and treat them away from the rest of the herd. Instead, researchers are focusing on the following antimicrobial intervention strategies that can be applied in a whole-herd scenario and be effective throughout movement from production to slaughter.

Probiotics—competitive exclusion. Fuller (60) described competitive exclusion (CE) as "the addition of exogenous bacteria to the intestinal tract of the animal in order to reduce colonization or decrease existing populations of pathogenic bacteria in the gastrointestinal tract" (p. 367). Historically, CE was not considered a viable technique for cattle because of the size and environment of the rumen and the length of feeding time associated with cattle production. However, recent research has demonstrated that CE and other probiotics could be effectively used to reduce *E. coli* O157:H7 and other bacteria in cattle (163, 164).

المنارة للاستشارات
www.manaraa.com

Commonly referred to as "direct-fed microbials," probiotics such as *Lactobacillus acidophilus* (117) and *Propionibacterium freudenreichii* (51) have been shown to directly reduce *E. coli* O157:H7 prevalence when fed to cattle. When feeding *L. acidophilis* at a level of 10^9 CFU/steer, *E. coli* O157:H7 was reduced by 30% compared to untreated controls (156). Along with maintenance of intestinal homeostasis, production of antimicrobial compounds, promotion of gut barrier function, and immune modulation, competitively excluding and controlling *E. coli* O157:H7 can be added to the attributes of direct-feeding probiotics.

A similar approach has been taken by direct-feeding strains of bacteria that produce bacteriocins. Bacteriocins are proteins produced by and effective against the producing strain of bacteria and like species. Certain *E. coli* species produce bacteriocins called "colicins." Schamberger et al. (130) described the use of a colicinogenic strain of *E. coli* fed to beef cattle to control *E. coli* O157:H7 shedding during production. Cattle were first given a feed additive containing 8 strains of colicinogenic *E. coli* at a level of 10^7 CFU/gram of feed. The cattle were orally inoculated with nalidixic acid-resistant *E. coli* O157:H7 strains seven days after the treatment started. Over a feeding period of 200 days, the colicinogenic *E. coli* additive was continuously fed at a level of 10^8 CFU/gram of feed. During the last 30 days on test, calves again were challenged with *E. coli* O157:H7. Microbiological assays on fecal samples indicated a 25% decrease in *E. coli* O157:H7 shedding when cattle were fed a colicinogenic "probiotic." Similar results were reported by Zhao et al. (164) and in weanling calves by Tkalcic et al. (145).

المنارة للاستشارات
www.manaraa.com

Bacteriocins and bacteriophage. Although no work has been reported on the use of these cultures on retail product or carcasses, bacteriocins themselves are being evaluated for these purposes. Zhang and Mustapha (162) compared the use of the *Lactococcus*-produced bacteriocin nisin, either alone or with EDTA, on *E. coli* O157:H7 in vacuum-packaged cubed beef. The beef cubes were inoculated with 7 log CFU/mL of *E. coli* O157:H7 and dipped in control solutions of 350µg nisin or 350µg nisin with EDTA. After 30 days at 4°C, only marginal amounts (<1 log CFU/mL) of *E. coli* were inhibited. A more convincing experiment analyzed bacteriocins of *Pediococcus acidilactici* K10 combined with organic acids against *E. coli* O157 in broth culture as well as in ground beef. When combined with 0.35% lactic acid, this bacteriocin inhibited *E. coli* O157 growth by 2.8 log CFU/g of sample (102). Several reports have shown that certain colicins can be effective against *E. coli* O157 in-vitro (24, 131); however, limited work has been done with these bacteriocins on beef products (1).

Bacteriophage are viruses that specifically infect bacteria and are common members of the intestinal microbial flora of food animals. The ability of certain bacteriophages to infect and kill or inhibit *E. coli* O157:H7 has been well documented (6, 91, 136). The ability of bacteriophages to kill *E. coli* O157 in vivo is a different matter; bacteriophages specific against *E. coli* O157:H7 have been repeatedly applied to both sheep (22) and beef cattle (6), minimally reducing concentrations of *E. coli* O157. Although much work needs to be done in the in vivo efficacy of bacteriophages as an intervention strategy, factors such as their history of safe use, host specificity, and host-replication strategies provide a solid foundation against *E. coli* O157 colonization (79).

المنارة للاستشارات
www.manaraa.com

Organic acids. The most widely researched area concerning beef cattle and microbiological control is at the carcass spray and wash stage of processing. Various treatments have been designed to decontaminate beef carcasses including the use of sanitizing agents such as hot water or organic acid sprays. Today, the most common carcass intervention is the use of organic acid sprays in which several acids are combined and sprayed at several points throughout hot processing. With the use of multiple or "hurdle" antimicrobials, carcasses have an average overall reduction in surface contamination by 30% and up to two log cycles (16). Organic acids are commonly used in the slaughter environment to reduce pathogen loads on carcasses but are not commonly used in fabrication or ground beef facilities. An interesting experiment by Harris et al. (70) validated the use of organic acids and acidified sodium chlorite (ASC) to reduce *E. coli* O157:H7 in beef trim and ground beef in a simulated processing environment. The data indicated that all antimicrobial interventions reduced the level of pathogens to a nondetectable level when challenged with a low-level inoculum but were not efficient at levels over 1 x 10^4 CFU/g. They also reported that organic acids applied at the 2% level or ASC applied at 1,200 ppm could be effective interventions for ground beef processors, although these levels were negatively associated with sensory acceptance.

Prior to carcass washing, organic acids are being proposed as on-farm strategies as well. Contaminated drinking water is an important area of control. Research targeting water intake reservoirs as a preharvest intervention strategy has been analyzed. The use of different chemical treatments including lactic acid, acidic calcium sulfate, chlorine, chlorine dioxide, hydrogen peroxide, caprylic acid, ozone, butyric acid, and sodium benzoate were

المنارة للاستشارات
www.manaraa.com

effective at reducing populations up to five log cycles when applied at 100ppm of water fed to cattle (165).

Listeria Monocytogenes

Listeria monocytogenes is a facultative, intracellular, gram-positive pathogen that causes the human disease listeriosis. *L. monocytogenes* is one of the six species of *Listeria* currently recognized, which also include *L. innocua, L. seeligeri, L. welshimeri, L. ivanovii,* and *L. grayi.* Although ubiquitous in the environment, *Listeria* infection is relatively uncommon. It is estimated that *L. monocytogenes* causes approximately 1,600 listeriosis cases annually resulting in 400 to 500 deaths (63). *L. monocytogenes* can cause a variety of diseases including meningitis and septicemia (56, 94). *L. monocytogenes* has been found in raw milk, soft cheese, fresh and frozen meat, poultry, and seafood products, as well as in fruits and vegetables (32). One reason for *L. monocytogenes'* wide range of habitat is that this organism also inhabits wild animals and birds, insects, soil and waste water, as well as decaying vegetation (120). The soil and water habitation directly relates to its existence in foraging livestock such as cattle and sheep. Therefore, humans, as well as animals, can contract listeriosis by foodborne contamination.

Listeria infection begins by intestinal colonization. From the intestinal tract, the organism invades tissues such as female placenta and liver. *L. monocytogenes* is an intracellular pathogen, meaning it must first enter susceptible cells and be able to replicate. The bacteria produce a cholesterol-dependent cytolysin called Listerolysin O (LLO), which allows for escape from phago-lysozomal membranes and bacteria growth in the cytoplasm of infected cells including macrophages. Other virulence proteins function as invasins and internalins, specific for entry into epithelial cells. *L. monocytogenes* actively multiplies and

المنارة للاستشارات
www.manaraa.com

moves from cell to cell throughout bodily tissues by a highly orchestrated mechanism. Once inside the cytosol, an *L. monocytogenes* surface protein ActA propels the organism towards the cytoplasmic membrane by facilitating the polymerization of actin. ActaA spans both the bacterial membrane and the peptidoglycan cell wall and aids in the formation of actin tails that "push" the bacterial cell towards the outside of the cell (119). The bacteria travel from cell to cell by the formation of a filopodium, which is absorbed by an adjacent cell, thus repeating the process (80).

The ability of *L. monocytogenes* to survive and grow in a wide variety of cell types as well as travel through the circulatory system is the critical characteristic allowing for listeriosis to ensue. Highly susceptible individuals to listeriosis are pregnant or postpartum women, fetuses and newborn children, and elderly and/or immunocompromised individuals. Symptoms include fever, muscle aches, and sometimes gastrointestinal symptoms such as nausea or diarrhea. *Listeria monocytogenes* infection can spread to the central nervous system, and those symptoms would include headache, stiff neck, confusion, loss of balance, or convulsions (33). *Listeria* are readily killed by pasteurization and cooking; however, in certain RTE foods such as hot dogs and deli meats, contamination may occur after cooking but before packaging. As a precaution, the Centers for Disease Control (CDC) suggest that high-risk individuals heat all RTE foods (e.g., deli meats, frankfurters, etc.) prior to eating and avoid eating soft cheeses made from unpasteurized milk and refrigerated meat spreads given the prevalence of *L. monocytogenes* in these products (33).

Prevalence and Control of L. Monocytogenes in Meat Products

Even though the statistics show that the incidence of listeriosis has declined, outbreaks and contaminated product recalls continue to occur (32). A multi-state outbreak of

المنارة للاستشارات
www.manaraa.com

L. monocytogenes occurred in the United States in 2002. Consumption of contaminated turkey deli meat resulted in 46 culture-confirmed cases, seven deaths and three fetal deaths in eight states (67). *L. monocytogenes* can grow over a temperature range of 1–45°C and a pH range of 4.1–9.6. Also, these pathogens are remarkably stable against acid and osmotic shifts (62). Given these characteristics, RTE foods and manufacturing facilities are a prime reservoir for *L. monocytogenes* colonization. These products are typically high in salt with a low pH, and most are kept at refrigeration temperatures, all conditions which are appealing to psychrotropic *L. monocytogenes.* The ability to persist in food-processing environments and multiply under refrigeration temperatures makes *L. monocytogenes* a significant threat to public health.

Currently, FDA has a zero-tolerance policy in place for *L. monocytogenes* in RTE foods. Based on this policy, the presence of any level of *L. monocytogenes* in the food constitutes adulteration. Although no single infectious dosage has been reported, several murine studies have shown that minimal concentrations (1 log CFU/mL) of *L. monocytogenes* corresponded to susceptibility in immunocompromised subjects (65). A wide variety of intervention strategies are being introduced and evaluated in the manufacture of RTE foods including organic acids, irradiation, bacteriocins, and plant extracts.

Competitive exclusion. As in *E. coli* O157 intervention, the use of probiotic bacterial cultures has recently been shown to support anti-listerial strategies in RTE meats. Amézquita and Brashears (2) analyzed *Pediococcus acidilactici, Lactobacillus casei*, and *Lactobacillus paracasei* on their anti-listerial effectiveness in frankfurters and cooked ham. Bacteriostatic activity was observed in cooked ham, whereas bactericidal activity was observed in frankfurters. Numbers of *L. monocytogenes* were 4.2 to 4.7 $\log_{10}$ and 2.6 $\log_{10}$ cycles lower

المنارة للاستشارات
www.manaraa.com

than controls in frankfurters and cooked ham, respectively, after the 28-day refrigerated storage at 4°C. It is important to note that due to the zero-tolerance policy, an inhibition of at least one log cycle could be efficient at controlling *L. monocytogenes* contamination. In other studies where lactic acid bacteria was used to competitively exclude *L. monocytogenes*, sensory data indicated no difference in preference of treated cooked ham compared to untreated control slices (151). One aspect to these CE experiments was that all probiotic cultures were lactic acid bacteria, and several were isolates obtained from RTE products and recultured.

Organic acids and other chemical antimicrobials. The largest area of *L. monocytogenes* control has been placed on organic acids and salts (10, 64). The most critical area of contamination in RTE meats is during post-processing procedures such as peeling, slicing, dicing, and packaging. Many meat processors are currently adding sodium or potassium lactate (up to 2%) in combination with sodium diacetate (0.05–0.15%) to product formulations (146). Studies have also shown that antimicrobials—such as other organic acids or their salts—applied as immersion or spraying solutions alone, in combination, or sequentially can also potentially control *L. monocytogenes* contamination on RTE meat products during storage (8, 127) Sodium, potassium, or other salts of lactic, acetic, and other organic acids have had significant antimicrobial activity against *L. monocytogenes* in broth and in meat products (10, 21, 50). Although organic acids have been effective anti-*Listeria* agents, the concentrations required for this activity are extremely high. In several studies, *L. monocytogenes*on RTE meat products survived in refrigerated storage despite the presence of organic acids that have bacteriostatic activity against *L. monocytogenes* (10, 95). Further limitations to the use of organic acids are their variable efficacy and objectionable sensory

المنارة للاستشارات
www.manaraa.com

attributes. The antimicrobial activity of these acids is dramatically influenced by the presence of other antimicrobials or other ingredients commonly used in RTE meat manufacture, usually by lowering efficacy (8, 10, 36), and studies have indicated that RTE meat products formulated or treated with organic acids had lower overall consumer acceptability compared with untreated products.

Bacteriocins. In the hopes of overcoming the limitations of organic acids, bacteriocins are being examined as potential interventions for *L. monocytogenes* control on RTE food products (37, 81). One such bacteriocin, nisin, inhibited growth of *L. monocytogenes*on the surface of bologna by 2.4 to 3.8 log CFU/cm^2 at a concentration of 125 µg/ml (64). Enterocin, another gram-positive bacteriocin of the pediocin family, has reduced *L. monocytogenes* in broth culture by 3 log CFU/ml when added at 4 µg/ml (53). Other pediocins have had variable antilisterial activity, ranging from 0.5- to 4-log reductions when added at concentrations greater than 500 µg/ml to RTE deli products (10). Bacteriocins, being proteinaceous, could lose efficacy over the manufacturing process, such as during thermal processing and cooling. A possible solution to this would be simply to only use bacteriocins as post-processing strategies. A film-based bacteriocin application was demonstrated by Ercolini et al. (54). These researchers formed active polythene films, coated with a solution of bacteriocin 32Y from *Lactobacillus curvatus*, and tested the capacity in which the film could inhibit growth of *L. monocytogenes*. The results showed that the direct contact between active film surface and *L. monocytogenes* cells was effective for a fast and irreversible inactivation of the bacterial population and could be used to package RTE meats.

المنارة للاستشارات
www.manaraa.com

Part 2. Bacteriocins

Introduction to Bacteriocins

Bacteriocins are peptides produced and/or secreted by bacteria that possess antimicrobial properties towards the producing bacteria, as well as like species. Bacteriocins differ from most therapeutic antibiotics in being proteinaceous and generally possessing a narrow specificity of action against strains of the same or closely related species (140). Bacteria express, produce, and utilize these proteins for survival and proliferation of an organism in a mixed population (47). Bacteriocins may serve as anticompetitors enabling the invasion of a strain into an established microbial community. They may also play a defensive role and act to prohibit the invasion of other strains or species into an occupied niche or limit the advance of neighboring cells. An additional role has recently been proposed for gram-positive bacteriocins in which they mediate quorum sensing (98). Riley (122) explained that thorough examination of bacteriocins in natural settings, such as *Lactobacillus plantarum* in green olive fermentations, *Escherichia coli* in guinea pig conjunctivae, and *Streptococcus mutans* in the human oral cavity, have indicated that the competitive advantage is substantially increased for bacteriocin-producing cells against bacteriocin-sensitive bacteria in the same environments.

Bacteriocins were originally defined as bacteriocidal proteins characterized by having producer-cell lethal biosynthesis, a very narrow range of activity, and adsorption to specific cell envelope receptors (82). These observations were solely based on *E. coli*-derived bacteriocins, coined “colicins.” Later, the recognized association of bacteriocin biosynthesis with plasmids was added to the description. The definition has since been modified to incorporate the properties of bacteriocins produced by gram-positive bacteria (140).

المنارة للاستشارات

www.manaraa.com

Bacteriocins from gram-positive bacteria commonly do not possess a specific receptor for adsorption, although exceptions exist and are most frequently of lower molecular weight and overall mass than bacteriocins from gram-negative bacteria, such as the colicins (27, 42, 123).

Gram-negative bacteriocins. All gram-negative bacteriocins are large proteins usually comprising 449 to 629 amino acids. Gram-negative bacteriocins generally have three "modes of action," which include DNAase or RNAase activity or pore formation in the cell membrane (123). Colicins, a large bacteriocins family produced by *E. coli* species, will be discussed in length in part three of this review.

One common event found in gram-negative bacteriocin production is the lethality effect of the producing cell. The bacteriocin is generally produced and released, which causes cell death. These three events are all directed by the bacteriocin lysis protein, also expressed and produced by the host cell (27, 85).

Regardless of expression locale, bacteriocins isolated from gram-negative bacteria appear to have been created by recombination between existing bacteriocins (19, 125). Such frequent recombination is facilitated by the domain structure of bacteriocin proteins. All gram-negative bacteriocins have the same three-domain structure in which: (a) a central domain is involved in the recognition of specific cell surface receptors, (b) the N-terminal domain is responsible for translocation of the protein into the target cell, and (c) the third domain of the protein houses the killing domain and the immunity region, which is a short sequence involved in immunity protein binding.

Gram-negative bacteriocins such as colicin E1 (115), Colicin N (24), Microcin 24 (59), and PsVP-10, isolated from *Pseudomonas* species (113), have all been shown to be

المنارة للاستشارات
www.manaraa.com

active across bacterial groups in a wide variety of applications. Gram-negative bacteriocins show particular affinity for gram-negative bacteria associated with raw and processed meat. Due to the large population of enteric bacteria in the gastrointestinal tract of humans and animals, these bacteriocins have also been used as dietary additives.

Gram-positive bacteriocins. To date, many more gram-positive bacteriocins have been identified than gram-negative. They differ from gram-negative bacteriocins in two fundamental ways. First, bacteriocin production is not necessarily the lethal event it is for gram-negative bacteria. Also, gram-positive bacteriocins have evolved bacteriocin-specific regulation, whereas bacteriocins of gram-negative bacteria rely solely on host regulatory networks (123). The most researched gram-positive bacteriocin family is that of the lactic acid bacteria. There are several classes of these bacteria, all differing in amino acid composition, structural composition, and mode of killing. Bacteriocins are classified into separate groups such as the lantibiotics or "lanthionine-containing peptide antibiotics" (Class I); the small (<10 kDa) heat-stable post-translationally unmodified non-lantibiotics (Class II)—which is further subdivided in the pediocin-like and anti-*Listeria* bacteriocins (subclass IIa); the two-peptide bacteriocins (subclass Ilb); and the sec-dependent bacteriocins (subclass IIc); and the large (>30 kDa) beat-labile non-lantibiotics (Class III) (88).

Production of bacteriocins in gram-positive bacteria is generally associated with the shift from log phase to stationary phase, as well as a direct result of bacterial SOS response initiated by environmental stress. Nisin production begins during mid-log phase and increases to a maximum as the cells enter stationary phase (20). The regulation of expression is not cell cycle-dependent per se, but rather culture density-dependent. It

المنارة للاستشارات
www.manaraa.com

has been demonstrated that nisin acts as a protein pheromone in regulating its own expression, which is controlled by a two-component signal transduction system typical of many quorum-sensing systems (46).

It was first documented that gram-positive bacteriocins exerted their antimicrobial effect via membrane disruption. Recent work suggest that several different mechanisms work in concert to either create pores in membrane walls, unravel the peptidoglycan synthesis pathway, as well as dissipate the bacterial cell's protein motive force (72). Hasper et al. (71) visualized the effect of nisin on giant unilamellar vesicles (GUVs) doped with fluorescently labeled lipid II. Lipid II is a biosynthesis molecule that it is essential for the transport of cell wall subunits across the bacterial cytoplasmic membrane of bacteria. These images and further research concluded that nisin was actually displacing this essential molecule from its functional location in gram-positive bacteria. Nisin binds to the pyrophosphate of lipid II, and their effect depends on the combination of the length of the peptide and the thickness of the lipid bilayer. Previously, nisin was thought to be a pore-forming bacteriocin; this detailed experiment proved a different strategy used by nisin against target bacteria.

Gram-positive bacteriocins such as nisin, pediocin (34), and enterocin (111) have all been shown to have extremely broad killing effects against target bacteria. The conventional wisdom about the lethality of gram-positive bacteriocins is that they are restricted to killing other gram-positive bacteria. The lethality can vary significantly from relatively narrow as in the case of lactococcins A, B, and M, which have been found to kill only *Lactococcus,* to extraordinarily broad. For instance, some lantibiotics such as nisin and mutacin B-Ny266 have been shown to kill a wide range of organisms including *Actinomyces, Bacillus,*

المنارة للاستشارات
www.manaraa.com

Clostridium, Corynebacterium, Enterococcus, Gardnerella, Lactococcus, Listeria, Micrococcus, Mycobacterium, Propionibacterium, Streptococcus, and *Staphylococcus* (104).

Part 3. Colicins

Introduction to Colicins

Colicins are bacteriocins produced by and effective against certain strains of *Escherichia coli* and like species. Colicins are the most well studied of all the bacteriocins and are commonly used as a model for bacteriocin evolutionary studies. Among the colicins, there are two main evolutionary lineages which also distinguish the two primary modes of killing: pore formation and nuclease activity (122). The first colicin was identified in 1932 as a "heat-labile product present in cultures of *E. coli* V that was toxic against *E. coli* φ" (27). In part three of this literature review, a discussion of varying types of colicins will be discussed. The primary focus will be placed on the pore-forming colicins including the expression, release, and mechanistic approach to pore-formation and target cell death of these colicins. Colicin E1, the focus of this dissertation, is a pore-forming colicin.

Variations among colicins: expression, mechanisms, and activity against target bacteria. Colicins comprise the most diverse and abundant microbial defense system (123). To date, there are five groups of colicins, differing in specific mechanisms against target bacterial cells. These include pore-formation, DNAase, RNAase, protein synthesis inhibition, and murein biosynthesis inhibition. Despite differences in killing mechanisms, the activities of these colicins are mediated by specific binding to receptors on target cells. The proteins are then translocated across the membrane of the bacteria and inserted into the cytoplasm to exert their toxic effect (25). All colicins exhibit the same tripartite structural organization with each of three domains responsible for specific activities. The central

المنارة للاستشارات
www.manaraa.com

regions of each molecule are the receptor binding domain, which forms unique structures that bind to outer membrane receptor proteins (44). The amino-terminus acts in "unknown" ways during the transport of the toxin through the outer membrane of gram-negative bacteria (17, 26). Current hypotheses on these "unknown" translocation events involve the associated with a system of proteins called Tol or TonB. These proteins have been shown to be required for colicin import (25). The Tol system is composed of five proteins that form a multi-protein complex in the cell envelope of most gram-negative bacteria. The TonB system consists of an outer-membrane transporter and three known inter-membrane proteins: TonB, ExbB, and ExbD. Based on their interactions with these protein systems, colicins are divided into two groups, group A and group B. Group A colicins (A, E1 to E9, K, L, N, and cloacin DF13) use the Tol system (41), whereas group B colicins (B, D, Ia, Ib, M, V, 5, and 10) use the TonB system (43). A recent literature review by Cao and Klebba (25) tried to elucidate if colicins utilize these protein systems solely or bi-functionally with other unknown "machinery" for proper colicin translocation. Cao and Klebba (25) concluded that:

> Our inability to solve the physiological riddles of TonB and Tol now stands as the principal stumbling block to the delineation of colicin uptake processes. Unfortunately, the exact functions that TonB and the Tol proteins perform in the cell envelope and the mechanisms by which they accomplish them remain elusive . . . (p. 410)

The carbon-terminus contain the toxic activities of colicins (9) that either (a) create voltage-gated pores in the cytoplasmic membrane (A, E1, Ia, N, B), (b) digest target cell DNA or RNA (E2-E9, D), or (c) degrade peptidoglycan (M), or nuclease activity-mediated

المنارة للاستشارات
www.manaraa.com

inhibition of protein synthesis (E3, D). This area is under investigation as well since this terminus seems to be the most unbiased in terms of target bacteria.

All types of colicins are produced by strains of *Escherichia coli* that harbor a colicinogenic plasmid, pCol. There are two main classes of pCol, Type 1 and Type 2. Type 1 is a small plasmid that can be amplified and is mobilizable in the presence of a conjugative plasmid. The Type 1 plasmid encodes mainly colicins of group A. Type 2 plasmids are slightly larger, conjugative, and can transfer other components of the plasmid including the colicin operon and other mobilizable plasmids at the time of horizontal transfer (69). Type 2 plasmids also generally encode colicins of group B. Exceptions to the type of plasmid and colicin produced have been found where group B colicins are carried by Type 1 pCol plasmids (77). A recent experiment by Schamberger and Diez-Gonzalez (132) provided insight into the non-specific nature of colicinogeny exhibited by *E. coli*. In that study, 14 different colicinogenic *E. coli* strains were individually tested for colicin type using polymerase chain reaction (PCR) and specific primers for known colicins. Each colicin strain produced one to five different colicins, and colicins were generally from different groups. For instance, colicinogenic *E. coli* strain B23 was isolated from cattle, and data indicated the presence of colicins B, Ia, Ib and M.

Colicins of both group A (Tol-using) and group B (TonB-using) target *E. coli* cells by interacting with specific outer membrane proteins. In group A, colicins A, E1, and E9 target the vitamin B12 transporter BtuB (27). Several other colicin-target combinations have been identified including colicin K to Tsx; U to OmpA; B and D to FepA; and M to FhuA. Both FepA and FhuA are involved in iron transport.

المنارة للاستشارات
www.manaraa.com

Although colicin translocation across the outer membrane is still poorly understood, it is clear that Tol-dependent (group A) colicin translocation likely occurs by a mechanism distinct from that of Ton-dependent (group B) colicins. The main differences between the two systems are that the Tol-dependent colicins utilize many proteins to translocate across the membrane of target bacteria, while TonB colicins appear to use a single outer membrane protein for binding and transport. Most pore-forming colicins are housed in group A and are therefore Tol-dependent, whereas group B is extremely diverse and includes colicins having a variety of toxic mechanisms. It is unclear whether or not pore-formation or effectiveness of colicins is increased or aided by use of the Tol system; recent work, however, has shown that colicin binding to OmpF (outer membrane protein F), a gram-negative porin, is essential for death of the target cell (78). OmpF is closely associated with the Tol system; therefore, it is likely that pore-forming colicins should reside within group A instead of group B.

Upon their transport into the periplasm, colicins exert their cytotoxic effects in a variety of ways. Three cytotoxic activities have thus far been found among group A colicins. These include a pore-forming ion channel that depolarizes the inner membrane (52), an H–N–H endonuclease activity that degrades chromosomal DNA (129), and a ribonuclease activity that specifically cleaves either16S ribosomal RNA (15) or specific tRNAs (112). For pore-forming colicins, their cytotoxic roles all take place within the periplasm and are dependent on the curvature of the lipid bilayer (137). The pore-forming group permeabilizes the cytoplasmic membrane, thereby destroying the cell's membrane potential. From studies carried out on artificial membranes, it is clear that these colicins form well-defined voltage-gated ion channels in target membranes (116).

المنارة للاستشارات

www.manaraa.com

In contrast, once a nuclease toxin has been translocated across the periplasmic space, the task faced by the cytotoxic domain is quite different to that of a pore-forming colicin, since the entire enzymatic domain has to cross the cytoplasmic membrane (83).

Recent evidence has shown that the nuclease colicins, specifically DNAases, actually form channels with their cytotoxic domains that allows for transport into the cytoplasm (103). Nuclease colicins target essential components of a bacterial cell. In the case of certain nuclease colicins such as colicin E3, the target is a single phosphodiester bond in 16S ribosomal RNA that results in the inhibition of translation (161), whereas for colicin E9, it is thought that non-specific degradation of the chromosome is responsible for bacterial cell death (83).

It is interesting to observe the differences in activity the types of colicins exert over the same target bacteria. Bradley et al. (18) evaluated the sensitivity of 20 *E. coli* O157:H7 strains to colicins A, E2, K, B, D, Ia, V, G, and H. Colicin G and H inhibited all 20 strains, while Colicin E2 and V inhibited 12 and 18 of the strains, respectively. The remaining colicins tested had no effect. Colicin G and H are now referred to as "microcins" and are no longer considered part of the colicin family. Microcins are gene-encoded antimicrobial polypeptides secreted by certain *Enterobacteriaceae*, but not specifically *E. coli* (27). The other two active colicins, E2 and V, function as a DNAase and a pore-former, respectively. Data from that study also indicated that colicins belonging to group B were not effective against *E. coli* O157:H7. Complementing these results was a study by Murinda et al. (105) that evaluated 24 colicinogenic *E. coli* strains producing standard for inhibitory activity against 27 diarrheagenic *E. coli* strains of various serotypes. In that study, all B-group colicins were ineffective at reducing *E. coli* O157:H7 populations, and colicin V only

المنارة للاستشارات
www.manaraa.com

reduced the populations of two out of the 27 strains tested. In both of these studies, colicin inhibition was employed using an overlay method of *E. coli* O157:H7 by colicin-producing *E. coli* strains. A typical colicin inducing agent used in the proliferation and purification of colicins is mitomycin C. In these experiments, mitomycin C was supplied in the overlay agar. Callaway et al. (24) demonstrated that colicin E1, N, and A purified at 98% were lethal against *E. coli* O157:H7 strains in-vitro. That study indicated that colicin E1 was more efficient at killing *E. coli* O157:H7 than N or A at a level of 0.1µg/mL. It seems reasonable that colicins could be more effective against target bacteria if they are applied in a pure, strain-released form. A more critical evaluation needs to be employed when comparing experimental results of colicins when different purities, specific activities, and preparatory strategies were undertaken.

Colicin E1. Colicin E1 is a group A, pore-forming colicin produced by and effective against *Escherichia coli* and like bacteria. Colicin E1 is a product of the gene "cea" whose induction is part of the bacterial SOS response (48). Colicin is produced in large quantities as a result of the SOS response, mimicked in laboratory techniques by the use of such agents as mitomycin C.

Colicin E1 gene clusters contain a third gene which codes for a protein required for the secretion of the bacteriocins, the so-called bacteriocin release protein "kil." The "kil" protein is also referred to as the bacteriocin release protein (BRP) and is a small lipoprotein composed of 27 to 35 amino acids. The main function of the BRP is to promote colicin release; concomitantly, they provoke (a) quasilysis, modifications of the structure of the cell envelope, (b) activation of OmpLA, the outer membrane phospholipase A, (c) and death of the producing cell.

www.manaraa.com

Upon colicin E1 expression and production, the protein accumulates in the cytoplasm of the producing cell. How the colicin BRP allows colicin release has not been fully described. Current research indicates the release protein aids in the formation of trans-envelope pores. The molecule would use these pores to transverse to the outside of the cell (96) .

While colicin E1 is being expressed and produced, a lysis protein CelA is concurrently expressed and produced. The result of this timely expression results in the death of the host cell upon colicin release. The killing process of CelA against its host is unknown. It seems to be responsible for the shut-off of chromosomal protein synthesis reported during colicin induction (28). As a result, producing cells will lyse upon release of the colicin to the extracellular environment

In addition to the bacteriocin, the lysis protein, and the BRP, colicinogenic cells synthesize an immunity protein which protects the producing cells against the homologous bacteriocin. In the case of pore-forming bacteriocins such as E1, this protein is actually conserved on the cytoplasmic membrane of the producer cells. Instead of providing immunity for the cell itself, it instead functions as a protectant against bacteriocins that may be targeting the producer cell (150).

The first “lethality” step after colicin E1 is released from the producer cell is its binding to its target receptor. Colicin E1 and like pore-forming colicins bind to the *E. coli* outer membrane cobalamin transporter BtuB. The structure of BtuB in Figure 1 (160) consists of a 22-stranded β-barrel with an amino-terminal hatch domain. At the time of this writing, colicin E3, a nuclease colicin that also utilizes BtuB, is the only

www.manaraa.com

colicin-binding protein complex to have been fully crystallized (101). The initial binding of colicin to the membrane is nearly irreversible under some conditions. The proposed explanation for this binding characteristic is that colicin E1 forms an umbrella-like structure in which its hydrophobic segment would be inserted into the membrane where it may interact with the hydrophobic core of the bilayer, while the other domains, many of which are amphipathic, splay out onto the surface while retaining their secondary structure (114).

Once in its membrane-bound state, colicin E1 must go through a series of orchestrated events that result in pore-formation. One of the distinguishing characteristics of colicin E1 compared to other pore-forming colicins is the “two receptor” theory. Zakharov and Cramer (159) showed that BtuB functions as an initial receptor to bind colicin, and one of the Tol system proteins, TolC, functions to support uptake by the membrane. Artificial membrane work in this experiment showed that without interaction with TolC, colicin import did not occur. These authors propose that TolC is positioned on the membrane surface and acts as a passage-support, similar to the rung of a ladder, upon which the colicin cross into the membrane (160).

The initial events associated with the enormous conformational changes undergone by the insertion of colicin into membranes have been studied through the interaction of the 178-residue C-terminal channel domain of colicin E1 P178, with liposome membranes of defined lipid composition (158). In Figure 2, the pathway of colicin E1 P178 is followed from binding to insertion into the bilayer. It appears that when mimicking the environmental conditions within cells, colicin E1 binds to its target receptor, becomes irreversibly bound to the membrane, unfolds and elongates, and then is able to insert into the membrane to create a pore (158).

المنارة للاستشارات

www.manaraa.com

At this time, the events surrounding pore-formation are not understood. It is known that colicin E1 unfolds on the lipid membrane surface to form a surface-bound two-dimensional flexible helical array that is a precursor state to the inserted channel (157). The channel domain subsequently inserts into the membrane and forms ion-conducting pores that are voltage-dependent (133).

It has been documented that the use of purified forms of colicin E1 can be effective against pathogenic bacteria such as *E. coli* O157:H7 at minute concentrations (24, 130, 139). Recently, work with colicin E1 has described efficacy against gram-positive bacteria including *Listeria monocytogenes* (115). Given the necessity of an outer-membrane receptor, as well as gram-negative protein complexes such the Tol system, it is intriguing that this colicin E1 would be effective against gram-positive bacteria lacking these proteins.

When observing the activity colicin E1 has against pathogenic bacteria, it is interesting to explore the possibility for use as antimicrobial interventions. Due to the overwhelming emergence of antimicrobial resistance, the ability of these proteins to generate resistance is of great importance. Foodborne pathogens such as *Listeria monocytogenes* and *Staphylococcus aureus* have been reported to spontaneously mutate and acquire the ability to resist nisin (100).

Colicin resistance has been evaluated previously (132). *Escherichia coli* can resist a colicin if the cell is capable of producing the specific immunity protein that neutralizes the colicin effect. Resistance can also occur if an *E. coli* cell undergoes a mutation in a receptor or translocation system that a colicin may use to gain entry into a cell (121). Schamberger and Diez-Gonzalez (132) evaluated 14 colicinogenic *E. coli* strains against *E. coli* O157:H7, as well as the resistance potential of the treated *E. coli* O157:H7 strains to colicins. In that

المنارة للاستشارات
www.manaraa.com

study, the ability to develop colicin resistance against single colicin producing *E. coli* strains was observed, but rarely against multiple-colicinogenic strains. The authors concluded that when using unpurified, colicinogenic *E. coli* as a probiotic, use of more than one strain would limit the onset of colicin resistance. No studies have demonstrated resistance to purified colicin E1 added to culture.

The safety of this protein for human consumption is of particular importance for its use on food and food contact surfaces. Murinda et al. (106) compared the cytotoxicity of ColE1, nisin, and pediocin in mammalian cell culture and found that ColE1 was significantly less cytotoxic than were both nisin and pediocin. This finding and our evolutionary history of exposure to colicins produced by commensal organisms in the human gastrointestinal tract (123) suggest that there should be no concerns for the use of this protein as a biopreservative in products meant for human consumption.

Colicins are an extremely diverse, abundant group of antimicrobial proteins. Bacterial responses to stress or an SOS stimulus abundantly provide *E. coli* strains with a selective advantage over other bacteria in mixed populations. The ability to exploit this survival mechanism of bacteria and subsequently produce and purify these antibacterial proteins for use in medical, agricultural, or food safety interventions could provide a tremendous breakthrough for antibiotic and antimicrobial research and application.

Part 4. Colicins and Food Safety

The use of antibacterial compounds in livestock, in and on foods, as well as applications with food packaging and contact surfaces is an extremely dynamic area of bacterial intervention. In the early 1940s, prominence was placed on the newly synthesized sulfanomides, penicillin, and chloramphenicol as bacterial "super killers." An inevitable side

المنارة للاستشارات
www.manaraa.com

effect of the use of antibiotics is the selection for, emergence, and spread of resistant bacteria including human pathogens. Not only have bacteria acquired the mechanisms necessary to withstand the effects of antibiotics, but to also "share" these acquired traits with other genera or species within the host microenvironment via plasmid, transposon, insertion sequence (IS), or cassette-mediated gene transfer (11, 135, 153). The effects of enhanced bacterial resistance are many. Firstly, antibacterial resistance is growing into a multi-drug phenomenon where bacteria are inherently resistant to several classes of antibacterial compounds (68). Multiple drug resistant bacteria limit the availability of efficient treatments to animals and humans suffering from a bacterial infection or disease with the potential to be life-threatening on a global scale (93). Also of great importance is the fact that conjugation and transfer plasmids can occur between bacterial strains of human, animal, and fish origins that are unrelated either evolutionarily or ecologically even in the absence of antibiotics (90). The phenomenon of antibiotic resistance, coupled with their persistence in clinical and food manufacturing settings, poses a giant risk to human and animal health.

The "ideal" antimicrobial would be a nontoxic, environmentally stable compound that is unable to promote resistance in target bacteria. This antimicrobial could withstand changes in environmental conditions including pH or osmotic shifts, respond to host changes in lipid or amino acid profile and concentration, and be effective over long periods of time. While this description seems unattainable, colicin E1 could potentially provide protection against bacteria while meeting several of these criteria.

Antibiotics were long used due to their lack of toxicity towards humans and animals when used at correct levels. While no direct comparison has been made to antibiotics and

المنارة للاستشارات
www.manaraa.com

colicin E1, the toxicity of colicin E1 was reported and discussed by Murinda et al. (106). In these experiments, the gram-positive bacteriocins nisin and pediocin—as well as colicins E1, E3, E6, E7, and K—were evaluated for cytotoxicity against cultured simian virus 40-transfected human colon and Vero monkey kidney cells. Nisin and pediocin were the most cytotoxic bacteriocins tested here, while colicins E1, E3, E7, and K demonstrated little toxicity at the levels tested. The authors concluded from these results that colicins are safe and have potential for use as food bio-preservatives. It seems logical that bacteriocins isolated from nonpathogenic bacteria specifically associated with the gastrointestinal tract would not be harmful to humans. Although this paper showed advanced cytotoxic activity with nisin, this is the only bacteriocin which has been approved by the World Health Organization (WHO) to be used as a preservative in the food industry.

With limited toxicity, the next requirement of colicins as food antimicrobials would be resistance. As mentioned in part three, Schamberger and Diez-Gonzalez (132) reported the ability of previously colicin-treated *E. coli* O157:H7 to become resistant to various colicins, including colicin E1, over time. An interesting dynamic of that research was that those colicins that *E. coli* O157:H7 became resistant to were not pore-forming colicins but rather nuclease colicins. No strains evaluated became resistant to colicin E1. Colicin E1 is a pore-forming colicin and has shown efficacy against various strains of *E. coli* O157:H7 (24, 139). More experimental data need to be collected on the resistance assessment of *E. coli* O157:H7, other *E. coli* species, and bacterial isolates of gram-negative and gram-positive groupings to fully elucidate if a resistance mechanism exists. The apparent lack of toxicity, as well as potential avoidance of resistance, builds a strong case for the use of colicin E1 as a food safety intervention strategy.

المنارة للاستشارات

www.manaraa.com

To show effectiveness over time as well as the ability to withstand environmental changes, it is reasonable to look at the effect of colicin E1 when applied in different settings. When applied as a feed additive, colicin E1 has shown to be effective and active over a long range of time. Cutler et al. (40) analyzed the effect of colicin E1 against post-weaning diarrhea (PWD) in pigs. Weaned pigs were fed corn-soy pelleted diets that contained either 0, 11, or 22 mg colicin E1/kg diet. After two days of being fed the colicin-containing diets, all pigs were orally inoculated with 9 log CFU of two *E. coli* F18 strains that were isolated from pigs with PWD. The animals were evaluated for diarrheal incidence and growth pattern over a five-day period. Control animals had persistent diarrhea that remained until the end of the five-day study. Only one pig in the 22mg group had any indication of diarrhea, and that was seen only on the last day of the study.

When applied as a feed additive, colicin E1 had to withstand the harsh environment of the gastrointestinal tract and be able to exert its bacteriocidal effects in the small and large intestines. Similar effectiveness was seen when colicin E1 was applied as a sanitizing dip in packaged deli ham slices. When evaluated over a fourteen-day period, 10μg colicin E1 was able to reduce *L. monocytogenes* populations by 5 $\log_{10}$ CFU/gm of product. These slices were kept at 4°C, which meant that colicin E1 had to be thermally stable in cold temperatures as well (115).

The ability to produce and purify this compound could be the factor that pushes this protein into the industry. Stahl et al. (139) has recently explained the use of ultra-filtration and ion exchange chromatography to obtain a purified (98%) quantity of colicin E1 at a reduced cost. Advances in this system, as well as commercially produced methods, could provide a cost effective approach at producing large amounts of colicin in a short period of

المنارة للاستشارات
www.manaraa.com

time. Keeping in mind the low doses required for pathogen reduction, being able to purchase large amounts of extremely pure colicin could be beneficial in terms of industry cost.

Further application research needs to be done marrying colicin E1 to use in foods and animals, but at this time the preliminary research provides a solid ground for continued development of this product. Thermal stability evaluation at minimal and maximal temperatures, food matrix-environment analyses, and optimizing the production and purification of a stable colicin E1 product are paramount in the advancement of colicin E1 for use in the food industry.

References

1. Abercrombie, J. G., M. J. B. Paynter, and S. S. Hayasaka. 2006. Ability of colicin V to control *Escherichia coli* O157:H7 in ground beef. *J. Food Saf*. 26:103–114.

2. Amézquita, A., and M. M. Brashears. 2002. Competitive inhibition of *Listeria monocytogenes* in ready-to-eat meat products by lactic acid bacteria. *J. Food Prot*. 65:316–325.

3. Archambaud, M., P. Courcoux, and A. Labigne-Roussel. 1988. Detection by molecular hybridization of pap, afa, and sfa adherence systems in *Escherichia coli* strains associated with urinary and enteral infections. *Ann. Inst. Pasteur Microbiol*. 139:575–588.

4. Arikawa, K., I. M. Meraz, Y. Nishikawa, J. Ogasawara, and A. Hase. 2005. Interleukin-8 secretion by epithelial cells infected with diffusely adherent *Escherichia coli* possessing Afa adhesion coding genes. *Microbiol. Immunol*. 49:493–503.

المنارة للاستشارات
www.manaraa.com

5. Arthur, T. M., J. M. Bosilevac, D. M. Brichta-Harhay, M. N. Guerini, N. Kalchayanand, S. D. Shackelford, T. L. Wheeler, and M. Koohmaraie. 2007. Transportation and lairage environment effects on prevalence, numbers, and diversity of *Escherichia coli* O157:H7 on hides and carcasses of beef cattle at processing. *J. Food. Prot*. 70:280–286.

6. Bach, S. J., T. A. McAllister, D. M. Veira, V. P. Gannon, and R. A. Holley. 2002. Evaluation of bacteriophage DC22 for control of *Escherichia coli* O157:H7. *J. Anim. Sci*. 80(Suppl. 1):263. (Abstr.)

7. Banks, J. G., R. G. Board, and N. H. Sparks. 1986. Natural antimicrobial systems and their potential in food preservation of the future. *Biotechnol Appl Biochem*. 8:103–147.

8. Barmpalia, I. M., I. Geornaras, K. E. Belk, J. A. Scanga, P. A. Kendall, G. C. Smith, and J. N. Sofos. 2004. Control of *Listeria monocytogenes* on frankfurters with antimicrobials in the formulation and by dipping in organic acid solutions. *J. Food Prot*. 67:2456–2464.

9. Baty, D., M. Frenette, R. Lloubes, V. Geli, S. P. Howard, F. Pattus, and C. Lazdunski. 1988. Functional domains of colicin A. *Mol. Microbiol*. 2:807–811.

10. Bedie, G. K. 2001. Antimicrobials in the formulation to control *Listeria monocytogenes* post-processing contamination on frankfurters stored at 4°C in vacuum packages. *J. Food Prot*. 64:1949–1955.

11. Benveniste, R., and J. Davies. 1973. Mechanisms of antibiotic resistance in bacteria. *Annu. Rev. Biochem*. 42:471–506.

12. Bergogne-Bérézin, E. 1999. Current guidelines for the treatment and prevention of nosocomial infections. *Drugs*. 58:51–67.

المنارة للاستشارات

www.manaraa.com

13. Bhan, M. K., P. Raj, M. M. Levine, J. B. Kaper, N. Bhandari, R. Srivastava, R. Kumar, and S. Sazawal. 1989. Enteroaggregative *Escherichia coli* associated with persistent diarrhea in a cohort of rural children in India. *J. Infect. Dis*. 159:1061–1064.

14. Booher, S. L., N. A. Cornick, and H. W. Moon. 2002. Persistence of *Escherichia coli* O157:H7 in experimentally infected swine. *Vet Microbiol*. 89:69–81.

15. Boon, T. 1971. Inactivation of ribosomes in vitro by colicin E3. *Proc. Natl. Acad. Sci.* 68:2421–2425.

16. Bosilevac, J. M., T. M. Arthur, T. L. Wheeler, S. D. Shackelford, M. Rossman, J. O. Reagan, and M. Koohmaraie. 2004. Prevalence of *Escherichia coli* O157 and levels of aerobic bacteria and Enterobacteriaceae are reduced when hides are washed and treated with cetylpyridinium chloride at a commercial beef processing plant. *J. Food Prot*. 67:646–650.

17. Bouveret, E., A. Rigal, C. Lazdunski, and H. Benedetti. 1998. Distinct regions of the colicin A translocation domain are involved in the interaction with TolA and TolB proteins upon import into *Escherichia coli*. *Mol. Microbiol*. 27:143–157.

18. Bradley, D. E., S. P. Howard, and H. Lior. 1991. Colicinogeny of O157:H7 enterohemorrhagic *Escherichia coli* and the shielding of colicin and phage receptors by their O-antigenic side chains. *Can. J. Microbiol*. 37:97–104.

19. Braun, V., H. Pilsl, and P. Gross. 1994. Colicins: structures, modes of actions, transfer through membranes, and evolution. *Arch. Microbiol*. 161:199–206.

20. Buchman, G., S. Banerjee, and J. Hansen. 1988. Structure, expression and evolution of gene encoding the precursor of nisin, a small protein antibiotic. *J. Biol. Chem.* 263:16260–16266.

المنارة للاستشارات
www.manaraa.com

21. Buncic, S., C. M. Fitzgerald, R. G. Bell, and J. A. Hudson. 1995. Individual and combined listericidal effects of sodium lactate, potassium sorbate, nisin and curing salts at refrigeration temperatures. *J. Food Saf.* 15:247–264.

22. Callaway, T. R., T. S. Edrington, R. C. Anderson, Y. S. Jung, K. J. Genovese, R. O. Elder, and D. J. Nisbet. 2003. Isolation of naturally-occurring bacteriophage from sheep that reduce populations of *E. coli* O157:H7 in vitro and in vivo. Pg. 25 in Proc. 5th Int. Symp. on Shiga Toxin-Producing *Escherichia coli* Infections, Edinburgh, U. K.

23. Callaway, T. R., R. C. Anderson, T. S. Edrington, K. J. Genovese, K. M. Bischoff, T. L. Poole, Y. S. Jung, R. B. Harvey, and D. J. Nisbet. 2004a. What are we doing about *Escherichia coli* O157:H7 in cattle? *J. Anim. Sci*. 82E-Suppl:E93–99.

24. Callaway, T. R., C. H. Stahl, T. S. Edrington, K. J. Genovese, L. M. Lincoln, R. C. Anderson, S. M. Lonergan, T. L. Poole, R. B. Harvey, and D. J. Nisbet. 2004b. Colicin concentrations inhibit growth of *Escherichia coli* O157:H7 in vitro. *J. Food Prot*. 67:2603–2607.

25. Cao, Z., and P. E. Klebba. 2002. Mechanisms of colicin binding and transport through outer membrane porins. *Biochimie.* 84:399–412.

26. Carr, S., C. N. Penfold, V. Bamford, R. James, A. M. Hemmings, J. K. Davies, and P. Reeves. 2000. The structure of TolB, an essential component of the tol-dependent translocation system, and its protein–protein interaction with the translocation domain of colicin E9. *Structure.* 8:57–66.

27. Cascales, E., S. K. Buchanan, D. Duche, C. Kleanthous, R. Lloubes, K. Postle, M. Riley, S. Slatin, and D. Cavard. 2007. Colicin biology. *Microbiol. Mol. Biol. Rev*. 71:158–229.

المنارة للاستشارات
www.manaraa.com

28. Cavard, D. 1991. Synthesis and functioning of the colicin-E1 lysis protein – comparison with the colicin-A lysis protein. *J. Bacteriol*. 173:191–196.

29. Centers for Disease Control and Prevention (CDC). 2000. Outbreak of *Escherichia coli* O157:H7 infection associated with eating fresh cheese curds--Wisconsin, June 1998. *MMWR Morb. Mortal. Wkly Rep*. 49:911–913.

30. Centers for Disease Control and Prevention (CDC). 2006. Ongoing multistate outbreak of *Escherichia coli* serotype O157:H7 infections associated with consumption of fresh spinach—United States, September 2006. *MMWR Morb. Mortal. Wkly Rep*. 55:1045–1046.

31. Centers for Disease Control and Prevention (CDC). 2007a. *Escherichia coli* O157:H7 infection associated with drinking raw milk--Washington and Oregon, November-December 2005. *MMWR Morb Mortal Wkly Rep*. 56:165–167.

32. Centers for Disease Control and Prevention. 2007b. Preliminary FoodNet data on the incidence of infection with pathogens transmitted commonly through food--10 states, 2006. *Morb Mortal Wkly Rep*. 14:336–339.

33. Centers for Disease Control and Prevention. 2007c. General information: Listeriosis. On Centers for Disease Cntrol and Prevention, Disease Listing website. Accessed 24 June 2007 at http://www.cdc.gov/ncidod/dbmd/diseaseinfo/listeriosis_g.htm.

34. Chen, C. M., J. G. Sebranek, J. S. Dickson, and A. F. Mendonca. 2004. Combining pediocin with postpackaging irradiation for control of *Listeria monocytogenes* on frankfurters. *J. Food Prot*. 67:1866–1875.

35. Chen, H. D., and G. Frankel. 2005. Enteropathogenic *Escherichia coli*: unravelling pathogenesis. *FEMS Microbiol. Rev*. 29:83–98.

المنارة للاستشارات
www.manaraa.com

36. Chen, N., and L. A. Shelef. 1992. Relationship between water activity, salts of lactic acid and growth of *Listeria monocytogenes* in a meat model system. *J. Food Prot*. 55:574–578.

37. Chi-Zhang, Y., K. L.Yam, and M. L. Chikindas. 2004. Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. *Int. J. Food Microbiol.* 90:15–22.

38. Conedera, G., E. Mattiazzi, F. Russo, E. Chiesa, I. Scorzato, S. Grandesso, A. Bessegato, A. Fioravanti, and A. Caprioli. 2007. A family outbreak of *Escherichia coli* O157 haemorrhagic colitis caused by pork meat salami. *Epidemiol. Infect.* 135:311–314.

39. Cursino, L., D. Smajs, J. Smarda, R. M. Nardi, J. R. Nicoli, E. Chartone-Souza, and A. M. Nascimento. 2006. Exoproducts of the *Escherichia coli* strain H22 inhibiting some enteric pathogens both in vitro and in vivo. *J Appl Microbiol.* 100:821–829.

40. Cutler, S. A., S. M. Lonergan, and C. H. Stahl. 2007. Dietary inclusion of Colicin E1 prevents post-weaning diarrhea in pigs. Iowa State University A. S. Leaflet R2227. Available online at http://www.ans.iastate.edu/report/air/2007pdf/R2227.pdf

41. Davies, J. K., and P. Reeves. 1975. Genetics of resistance to colicins in *Escherichia coli* K–12. Cross-resistance among colicins of group A. *J. Bacteriol*. 123:102–117.

42. Daw, M. A., and F. R. Falkiner. 1996. Bacteriocins: nature, function and structure. *Micron.* 27:467–479.

43. Deich, R. A., B. J. Metcalf, C. W. Finn, J. E. Farley, and B. A. Green. 1988. Cloning of genes encoding a 15,000-dalton peptidoglycan-associated outer membrane lipoprotein and an antigenically related 15,000-dalton protein from Haemophilus influenzae. *J. Bacteriol.* 170:489–498.

المنارة للاستشارات
www.manaraa.com

44. DiMasi, D. R., J. C. White, C. A. Schnaitman, and C. Bradbeer.1973. Transport of vitamin B12 in *Escherichia coli*: common receptor sites for vitamin B12 and the E colicins on the outer membrane of the cell envelope. *J. Bacteriol*. 115:506–513.

45. Doane, C. A., P. Pangloli, H. A. Richards, J. R. Mount, D. A. Golden, and F. A. Draughon. 2007. Occurrence of *Escherichia coli* O157:H7 in diverse farm environments. *J. Food Prot.* 70:6–10.

46. Dunny, G. M., and B. A. Leonard. 1997. Cell-cell communication in gram-positive bacteria. *Annu Rev. Microbiol*. 51:527–564.

47. Dykes, G. A. 1995. Bacteriocins: ecological and evolutionary significance. *Trends Ecol. Evol*. 10:186–189.

48. Ebina, Y., F. Fishi, T. Miki, H. Kagamiyama, T. Nakazawa, and A. Nakazawa. 1981. The nucleotide sequence surrounding the promoter region of colicin E1 gene. *Gene.* 15:119–126.

49. Edmiston, C. E., G. R. Seabrook, M. P. Goheen, C. J. Krepel, C. P. Johnson, B. D. Lewis, K. R. Brown, and J. B. Towne. 2006. Bacterial adherence to surgical sutures: can antibacterial-coated sutures reduce the risk of microbial contamination? *J. Am. Coll. Surg*. 203:481–489.

50. El-Shenawy, M. A., and E. H. Marth. 1989. Inhibition or inactivation of *Listeria monocytogenes* by sodium benzoate together with some organic acids. *J. Food Prot*. 52:771–776.

المنارة للاستشارات

www.manaraa.com

51. Elam, N. A., J. F. Gleghorn, J. D. Rivera, M. L. Galyeah, P. J. Defoor, M. M. Brashears, and S. M. Younts-Dahl. 2003. Effects of live cultures of *Lactobacillus acidophilus* (strains NP45 and NP51) and *Propionibacterium freudenreichii* on performance, carcass, and intestinal characteristics, and *Escherichia coli* strain O157 shedding of finishing beef steers. *J. Anim. Sci*. 81:2686–2698.

52. Elkins, P., A. Bunker, W. A. Cramer, and C.V. Stauffacher. 1997. A mechanism for toxin insertion into membranes is suggested by the crystal structure of the channel-forming domain of colicin E1. *Structure.* 5:443–458.

53. Ennahar, S., K. Sonomoto, and A. Ishizaki. 2000. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. *J. Biosci. Bioeng*. 87:705–716.

54. Ercolini, D., A. Storia, F. Villani, and G. Mauriello. 2006. Effect of a bacteriocin-activated polythene film on *Listeria monocytogenes* as evaluated by viable staining and epifluorescence microscopy. *J. Appl. Microbiol*. 100:765–772.

55. Fairbrother, J. M., and E. Nadeau. 2006. *Escherichia coli*: on-farm contamination of animals. *Rev Sci Tech*. 25:555–569.

56. Farber, J. M., and P. I. Peterkin. 1991. *Listeria monocytogenes*, a food-borne pathogen. *Microbiol. Rev*. 55:476–511.

57. Fernandez-Prada, C., B. D. Tall, S. E. Elliott, D. L. Hoover, J. P. Nataro, and M. M. Venkatesan. 1998. Hemolysin-positive enteroaggregative and cell-detaching *Escherichia coli* strains cause oncosis of human monocytederived macrophages and apoptosis of murine J774 cells. *Infect. Immun*. 66:3918–3924.

المنارة للاستشارات

www.manaraa.com

58. Foxman, B., L. Zhang, P. Tallman, K. Palin, C. Rode, C. Bloch, B. Gillespie, and C. F. Marrs. 1995. Virulence characteristics of *Escherichia coli* causing first urinary tract infection predict risk of second infection. *J. Infect. Dis*. 172:1536–1541.

59. Frana, T. S., S. A. Carlson, D. C. Rauser, B. D. Jones, B. J. Fergen, and R. W. Griffith. 2004. Effects of microcin 24-producing *Escherichia coli* on shedding and multiple-antimicrobial resistance of *Salmonella* enterica serotype *Typhimurium* in pigs. *Am. J. Vet. Res*. 65:1616–1620.

60. Fuller, R. 1989. Probiotics in man and animals. *J. Appl. Bacteriol*. 66:365–378.

61. Gaastra, W., and A. M. Svennerholm. 1996. Colonization factors of human enterotoxigenic *Escherichia coli* (ETEC). *Trends Microbiol*. 4:444–452.

62. Gandhi, M., and M. L. Chikindas. 2007. *Listeria*: a foodborne pathogen that knows how to survive. *Int. J. Food Microbiol*. 113:1–15.

63. Gellin, B. G. and C. V. Broome. 1989. Listeriosis. *JAMA*. 261:1313–1320.

64. Geornaras, I., K. E. Belk, J. A. Scanga, P. A. Kendell, G. C. Smith, and J. N. Sofos. 2005. Post-processing antimicrobial treatments to control *Listeria monocytogenes* in commercial vacuum-packaged bologna and ham stored at 10°C. *J. Food Prot*. 68:991–998.

65. Golnazarian, C. A., C. W. Donnelly, S. J. Pintauro, and D. B. Howard. 1989. Comparison of infectious dose of *Listeria monocytogenes* F5817 as determined for normal versus compromised C57B1/6J mice. *J. Food. Prot*. 52:696–701.

66. González-Garcia, E. A. 2002. Animal health and foodborne pathogens: enterohaemorrhagic O157:H7 strains and other pathogenic *Escherichia coli* virotypes (EPEC, ETEC, EIEC, EHEC). *Pol. J. Vet Sci*. 5:103–115.

المنارة للاستشارات

www.manaraa.com

67. Gottlieb, S. L., E. C. Newbern, P. M. Griffin, L. M. Graves, R. M. Hoekstra, N. L. Baker, S. B. Hunter, K. G. Holt, F. Ramsey, M. Head, P. Levine, G. Johnson, D. Schoonmaker-Bopp, V. Reddy, L. Kornstein, M. Gerwel, J. Nsubuga, L. Edwards, S. Stonecipher, S. Hurd, D. Austin, M. A. Jefferson, S. D. Young, K. Hise, E. D. Chernak, and J. Sobel. 2006. Multistate outbreak of Listeriosis linked to turkey deli meat and subsequent changes in US regulatory policy. *Clin. Infect. Dis*. 42:29–36.

68. Harbottle, H., S. Thakur, S. Zhao, and D. G. White. 2006. Genetics of antimicrobial resistance. *Anim. Biotech*. 17:111–124.

69. Hardy, K. G., G. G. Meynell, J. E. Dowman, and B. G. Spratt. 1973. Two major groups of colicinogenic factors: their evolutionary significance. *Mol. Gen. Genet*. 125:217–230.

70. Harris, K., M. F. Miller, G. H. Loneragan, and M. M. Brashears. 2006. Validation of the use of organic acids and acidified sodium chlorite to reduce *Escherichia coli* O157 and *Salmonella typhimurium* in beef trim and ground beef in a simulated processing environment. *J. Food Prot* 69:1802–1807.

71. Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, and E. Breukink. 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. *Science*. 313:1636–1637.

72. Héchard, Y., and H. G. Sahl. 2002. Mode of action of modified and unmodified bacteriocins from gram-positive bacteria. *Biochimie*. 84:545–557.

73. Hicks, S., D. C. Candy, and A. D. Phillips. 1996. Adhesion of enteroaggregative *Escherichia coli* to pediatric intestinal mucosa in vitro. *Infect. Immun*. 64:4751–4760.

المنارة للاستشارات
www.manaraa.com

74. Hilborn, E. D., J. H. Mermin, P. A. Mshar, J. L. Hadler, A. Voetsch, C. Wojtkunski, M. Swartz, R. Mshar, M. A. Lambert-Fair, J. A. Farrar, M. K. Glynn, and L. Slutsker. 1999. A multistate outbreak of *Escherichia coli* O157:H7 infections associated with consumption of mesclun lettuce. *Arch. Intern. Med*. 159:1758–1764.

75. Hilborn, E. D., P. A. Mshar, T. R. Fiorentino, Z. F. Dembek, T. J. Barrett, R. T. Howard, and M. L. Cartter. 2000. An outbreak of *Escherichia coli* O157:H7 infections and haemolytic uraemic syndrome associated with consumption of unpasteurized apple cider. *Epidemiol*. *Infect*. 124:31–36.

76. Hirsch, J. G. 1980. The greatest success story in the history of medicine. *Med Times*. 108:36–43.

77. Hofinger, C., H. Karch, and H. Schmidt. 1998. Structure and function of plasmid pColD157 of enterohemorrhagic *Escherichia coli* O157 and its distribution among strains from patients with diarrhea and hemolytic-uremic syndrome. *J. Clin. Microbiol*. 36:24–29.

78. Housden, N. G., S. R. Loftus, G. R. Moore, R. James, and C. Kleanthous. 2005. Cell entry mechanism of enzymatic bacterial colicins: porin recruitment and the thermodynamics of receptor binding. *Proc. Natl. Acad. Sci. USA*. 102:13849–13854.

79. Hudson, J. A., C. Billington, G. Carey-Smith, and G. Greening. 2005. Bacteriophages as biocontrol agents in food. *J. Food Prot*. 68:426–437.

80. Ireton, K. 2007. Entry of the bacterial pathogen *Listeria monocytogenes* into mammalian cells. *Cell Microbiol*. 9:1365–1375.

81. Jack, R. W., J. R.Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev*. 59:171–200.

المنارة للاستشارات

www.manaraa.com

82. Jacob, F., A. Lwoff, L. Siminovitch, and E. Wollman. 1953. De´finition de quelques termes relatifs a` la lysoge´nie. *Ann. Inst. Pasteur* (Paris) 84:222–224.

83. James, R., C. N. Penfold, G. R. Moore, and C. Kleanthous. 2002. Killing of *E. coli* cells by E group nuclease colicins. *Biochimie.* 84:381–389.

84. Juneja, V. K., and H. Thippareddi. 2004. Inhibitory effects of organic acid salts on growth of *Clostridium perfringens* from spore inocula during chilling of marinated ground turkey breast. *Int. J. Food Microbiol*. 93:155–163.

85. Kanoh, S., H. Masaki, S. Yajima, T. Ohta, and T. Uozumi. 1991. Signal peptide of the colicin-E2 lysis protein causes host-cell death. *Agric. Biol. Chem*. 55:1607–1614.

86. Karch, H. 2001. The role of virulence factors in enterohemorrhagic *Escherichia coli* (EHEC)--associated hemolytic-uremic syndrome. *Semin. Thromb. Hemost.* 27:207–213.

87. Keene, W. E., K. Hedberg, D. E. Herriott, D. D. Hancock, R. W. McKay, T. J. Barrett, and D. W. Fleming. 1997. A prolonged outbreak of *Escherichia coli* O157:H7 infections caused by commercially distributed raw milk. *J. Infect. Dis*. 176:815–818.

88. Klaenhammer, T. R. 1988. Bacteriocins of lactic-acid bacteria. *Biochimie*. *Paris*. 70:337–349.

89. Kotloff, K. L., J. P. Winickoff, B. Ivanoff, J. D. Clemens, D. L. Swerdlow, P. J. Sansonetti, G. K. Adak, and M. M. Levine. 1999. Global burden of Shigella infections: implications for vaccine development and implementation of control strategies. *Bull. World Health Organization.* 77:651–666.

90. Kruse, H., and H Sørum. 1994. Transfer of multiple drug resistance plasmids between bacteria of diverse origins in natural microenvironments. *Appl. Environ. Microbiol*. 60:4015–4021.

المنارة للاستشارات
www.manaraa.com

91. Kudva, I. T., S. Jelacic, P. I. Tarr, P. Youderian, and C. J. Hovde. 1999. Biocontrol of *Escherichia coli* O157 with O157-specific bacteriophages. *Appl. Environ. Microbiol*. 65:3767–3773.

92. Laine, E. S., J. M. Scheftel, D. J. Boxrud, K. J. Vought, R. N. Danila, K. M. Elfering, and K. E. Smith. 2005. Outbreak of *Escherichia coli* O157:H7 infections associated with nonintact blade-tenderized frozen steaks sold by door-to-door vendors. *J Food Prot*. 68:1198–1202.

93. Lathers, C. M. 2002. Clinical pharmacology of antimicrobial use in humans and animals. *J. Clin. Pharmacol*. 42:587.

94. Lorber, B. 2007. Community-acquired *Listeria monocytogenes* meningitis in adults. *Clin. Infect. Dis*. 44:765–766.

95. Lu, Z., J. G. Sebranek, J. S. Dickson, A. F. Mendonca, and T. B. Bailey. 2005. Inhibitory effects of organic acid salts for control of *Listeria monocytogenes* on frankfurters. *J. Food Prot*. 68:499–506.

96. Luirink, J., B. Duim, J. W. L. de Gier, and B. Oudega. 1991. Functioning of the stable signal peptide of the pCIoDF13-encoded bacteriocin release protein. *Mol. Microbiol*. 5:393–399.

97. Mead, P. S., L. Slutsker, V. Dietz, L. F. McCaig, J. S. Bresee, C. Shapiro, P. M. Griffin, and R. V. Tauxe. 1999. Food-related illness and death in the United States. *Emerg. Infect. Dis.* 5:607–625.

98. Miller, M., and B. Bassler. 2001. Quorum sensing in bacteria, *Annu. Rev. Microbiol.* 55:165–199.

المنارة للاستشارات
www.manaraa.com

99. Mims, C. A., A. Nash, and J. Stephen. 2001. Mims' pathogenesis of infectious disease. Academic Press, London.

100. Ming, X. T., and M. A. Daeschel. 1993. Nisin resistance of foodborne bacteria and the specific resistance responses of *Listeria monocytogenes* Scott-A. *J. Food Prot*. 56:944–948.

101. Mohanty, A. K., C. M. Bishop, T. C. Bishop, W. C. Wimley, and M. C. Wiener. 2003. Enzymatic E-colicins bind to their target receptor BtuB by presentation of a small binding epitope on a coiled-coil scaffold. *J. Biol Chem*. 278:40953–40958.

102. Moon, G. S., W. J. Kim, and M. Kim. 2002. Synergistic effects of bacteriocin-producing *Pediococcus acidilactici* K10 and organic acids on inhibiting *Escherichia coli* O157:H7 and applications in ground beef. *J. Micro. Biotech*. 12:936–942.

103. Mosbahi, K., C. Lemaitre, A. H. Keeble, H. Mobasheri, B. Morel, R. James, G. R. Moore, E. J. Lea, and C. Kleanthous. 2002. The cytotoxic domain of colicin E9 is a channel-forming endonuclease. *Nat. Struct. Biol*. 9:476–484.

104. Mota-Meira, M., G. Lapointe, C. Lacroix, and M. C. Lavoie. 2000. MICs of mutacin B-Ny266, nisin A, vancomycin, and oxacillin against bacterial pathogens. *Antimicrob. Agents Chemother*. 44:24–29.

105. Murinda, S. E., R. F. Roberts, and R. A. Wilson. 1996. Evaluation of colicins for inhibitory activity against diarrheagenic *Escherichia coli* strains, including serotype O157:H7. *Appl. Environ. Microbiol*. 62:3196–3202.

106. Murinda, S. E., K. A. Rashid, and R. F. Roberts. 2003. In vitro assessment of the cytotoxicity of nisin, pediocin, and selected colicins on simian virus 40-transfected human colon and Vero monkey kidney cells with trypan blue staining viability assays. *J. Food Prot*. 66:847–853.

المنارة للاستشارات

www.manaraa.com

107. Nagy, B., and P. Z. Fekete. 2005. Enterotoxigenic *Escherichia coli* in veterinary medicine. *Int. J. Med. Microbiol*. 295:443–454.

108. Nataro, J. P., J. B. Kaper, R. Robins-Browne, V. Prado, P. Vial, and M. M. Levine. 1987. Patterns of adherence of diarrheagenic *Escherichia coli* to HEp-2 cells. *Pediatr. Infect. Dis. J*. 6:829–831.

109. Nataro, J. P., Y. Deng, D. R. Maneval, A. L. German, W. C. Martin, and M. M. Levine. 1992. Aggregative adherence fimbriae I of enteroaggregative *Escherichia coli* mediate adherence to HEp-2 cells and hemagglutination of human erythrocytes. *Infect. Immun.* 60:2297–2304.

110. Nataro, J. P., and J. B. Kaper. 1998. Diarrheagenic *Escherichia coli*. *Clinical Micro. Reviews.* 11:142–201.

111. Nigútová, K., P. Pristas, and P. Javorský. 2005. Bacteriocin-like activity production and resistance in selected enterococci and streptococci of animal origin. *Arch. Anim. Nutr*. 59:205–211.

112. Ogawa, T., K. Tomita, T. Ueda, K. Watanabe, T. Uozumi, and H. Masaki. 1999. Acytotoxic ribonuclease targeting specific transfer RNA anticodons. *Science*. 283:2097–2100.

113. Padilla, C., O. Lobos, P. Brevis, P. Abaca, and E. Hubert. 2004. In vitro antibacterial activity of the peptide PsVP-10 against antimicrobial-resistant *Enterococcus faecalis* isolated from clinical samples. *J. Antimicrob. Chemother*. 53:390–392.

114. Parker, M. W., F. Pattus, A. D. Tucker, and D. Tsernoglou. 1989. Structure of the membrane pore-forming fragments of colicin A. *Nature*. 337:93–96.

المنارة للاستشارات
www.manaraa.com

115. Patton, B. S., J. S. Dickson, S. M. Lonergan, S. A. Cutler, and C. H. Stahl. 2007. Inhibitory activity of colicin E1 against *Listeria monocytogenes*. *J Food Prot*. 70:1256–1262.

116. Pattus, F., D. Massotte, H. U. Wilmsen, J. Lakey, D. Tsernoglou, A. Tucker, and M. W. Parker. 1990. Colicins: prokaryotic killer-pores. *Experientia*. 46:180–192.

117. Peterson, R. E., T. J. Klopfenstein, G. E. Erickson, J. Folmer, S. Hinkley, R. A. Moxley, and D. R. Smith. 2007. Effect of Lactobacillus acidophilus strain NP51 on *Escherichia coli* O157:H7 fecal shedding and finishing performance in beef feedlot cattle. *J. Food Prot.* 70:287–291.

118. Qadri, F., A. M. Svennerholm, A. S. Faruque, and R. B. Sack. 2005. Enterotoxigenic *Escherichia coli* in developing countries: epidemiology, microbiology, clinical features, treatment, and prevention. *Clin. Microbiol. Rev.* 18:465–483.

119. Rafelski, S. M., and J. A. Theriot. 2006. Mechanism of polarization of *Listeria monocytogenes* surface protein ActA. *Mol. Microbiol*. 59:1262–1279.

120. Ramaswamy, V., V. M. Cresence, J. S. Rejitha, M. U. Lekshmi, K. S. Dharsana, S. P. Prasad, and H. M. Vijila. 2007. *Listeria*—review of epidemiology and pathogenesis. *J. Microbiol. Immunol. Infect*. 40:4–13.

121. Riley, M. A., and D. M. Gordon. 1996. The ecology and evolution of bacteriocins. *J. Ind. Microbiol*. 17:151–158.

122. Riley, M. A. 1998. Molecular mechanisms of bacteriocin evolution. *Annu. Rev. Genet*. 32:255–278.

123. Riley, M. A., and J. E. Wertz. 2002. Bacteriocin diversity: ecological and evolutionary perspectives. *Biochimie.* 84:357–364.

المنارة للاستشارات
www.manaraa.com

124. Robinson, S. E., E. J. Wright, C. A. Hart, M. Bennett, and N. P. French. 2004. Intermittent and persistent shedding of *Escherichia coli* O157 in cohorts of naturally infected calves. *J. Appl. Microbiol*. 97:1045–1053.

125. Roos, U., R. E. Harkness, and V. Braun. 1989. Assembly of colicin genes from a few DNA fragments. Nucleotide sequence of colicin D. *Mol. Microbiol*. 3:891–902.

126. Saeed, A. M., N. S. Magnuson, C. C. Gay, and R. N. Greenberg. 1986. Characterization of heat-stable enterotoxin from a hypertoxigenic *Escherichia coli* strain that is pathogenic for cattle. *Infect. Immun*. 53:445–447.

127. Samelis, J., P. Kendall, G. C. Smith, and J. N. Sofos. 2004. Acid tolerance of acid-adapted and nonadapted *Escherichia coli* O157:H7 following habituation (10 degrees C) in fresh beef decontamination runoff fluids of different pH values. *J. Food Prot*. 67:638–645.

128. Savarino, S. J., A. Fasano, D. C. Robertson, and M. M. Levine. 1991. Enteroaggregative *Escherichia coli* elaborate a heat-stable enterotoxin demonstrable in an in vitro rabbit intestinal model. *J. Clin. Invest*. 87:1450–1455.

129. Schaller, K., and M. Nomura. 1976. Colicin E2 is DNA endonuclease. *Proc. Natl. Acad. Sci.* 73:3989–3993.

130. Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez. 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. *Appl Environ. Microbiol*. 70:6053–6060.

131. Schamberger, G. P., and F. Diez-Gonzalez. 2004. Characterization of colicinogenic *Escherichia coli* strains inhibitory to enterohemorrhagic *Escherichia coli*. *J. Food. Prot.* 67:486–492.

المنارة للاستشارات
www.manaraa.com

132. Schamberger, G. P., and F. Diez-Gonzalez. 2005. Assessment of resistance to colicinogenic *Escherichia coli* by *E. coli* O157:H7 strains. *J. Appl. Microbiol*. 98:245–252.

133. Schein, S. J., B. L. Kagan, and A. Finkelstein. 1978. Colicin K acts by forming voltage-dependent channels in phospholipid bilayer membranes. *Nature.* 276:159–163.

134. Servin, A. L. 2005. Pathogenesis of Afa/Dr diffusely adhering *Escherichia coli*. *Clin. Microbiol. Rev*. 18:264–292.

135. Sheldon, A. T. Jr. 2005. Antibiotic resistance: a survival strategy. *Clin. Lab. Sci*. 18:170–180.

136. Sheng, H. Q., H. J. Knecht, I. T. Kudva, and C. J. Hovde. 2006. Application of bacteriophages to control intestinal *Escherichia coli* O157:H7 levels in ruminants. *Appl. Environ. Microbiol*. 72:5359–5366.

137. Sobko, A. A., E. A. Kotova, Y. N. Antonenko, S. D. Zakharov, and W. A. Cramer. 2004. Effect of lipids with different spontaneous curvature on the channel activity of colicin E1: evidence in favor of a toroidal pore. *FEBS Lett*. 576:205–210.

138. Srinivasan, V., L. T. Nguyen, S. I. Headrick, S. E. Murinda, and S. P. Oliver. 2007. Antimicrobial resistance patterns of Shiga toxin-producing *Escherichia coli* O157:H7 and O157:H7- from different origins. *Microb. Drug Resist*. 13:44–51.

139. Stahl, C. H., T. R. Callway, L. M. Lincoln, S. M. Lonergan, and K. J. Genovese. 2004. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhea and edema disease in swine. *Antimicrob. Agents Chemother.* 48:3119–3121.

140. Tagg, J. R., A. S. Dajani, and L. W. Wannamaker. 1976. Bacteriocins of gram-positive bacteria. *Bacteriol. Rev*. 40:722–756.

المنارة للاستشارات

www.manaraa.com

141. Tannock, G. W. 1995. Normal microflora: an introduction to microbes inhabiting the human body, p. 1-36. In G. W. Tannock (ed). Chapman and Hall, London.

142. Taylor, C. J., A. Hart, R. M. Batt, C. McDougall, and L. McLean. 1986. Ultrastructural and biochemical changes in human jejunal mucosa associated with enteropathogenic *Escherichia coli* (O111) infection. *J. Pediatr. Gastroenterol. Nutr.* 5:70–73.

143. Theron, M. M., and J. F. R. Lues. 2007. Organic acids and meat preservation: a review. *Food Rev. Intl*. 23:141–158.

144. Threlfall, E. J., L. R. Ward, J. A. Frost, and G. A. Willshaw. 2000. The emergence and spread of antibiotic resistance in food-borne bacteria. *Int. J. Food Microbiol*. 62:1–5.

145. Tkalcic, S., T. Zhao, B. G. Harmon, M. P. Doyle, C. A. Brown, and P. Zhao. 2003. Fecal shedding of enterohemorrhagic *Escherichia coli* in weaned calves following treatment with probiotic *Escherichia coli*. *J. Food Prot.* 66:1184–1189.

146. Tompkin, R. B. 2002. Control of *Listeria monocytogenes* in the food-processing environment. *J. Food Prot*. 65:709–725.

147. Turner, S. M., A. Scott-Tucker, L. M. Cooper, and I. R. Henderson. 2006. Weapons of mass destruction: virulence factors of the global killer enterotoxigenic *Escherichia coli*. *FEMS Microbiol Lett*. 263:10–20.

148. Tuttle, J., T. Gomez, M. P. Doyle, J. G. Wells, T. Zhao, R. Tauxe, and P. M. Griffin. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol*. *Infect*. 122:185–192.

المنارة للاستشارات

www.manaraa.com

149. U.S. Department of Agriculture, Food Safety and Inspection Service. 1993. Immediate actions: cattle clean meat program. FSIS correlation packet, interim guidelines for inspectors. U.S. Department of Agriculture, Food Safety and Inspection Service, Washington, D.C.

150. van der Wal, F. J., J. Luirink, and B. Oudega. 1995. Bacteriocin release proteins: mode of action, structure, and biotechnological application. *FEMS Microbiol. Rev*. 17:381–399.

151. Vermeiren, L., F. Devlieghere, and J. Debevere. 2004. Evaluation of meat born lactic acid bacteria as protective cultures for the biopreservation of cooked meat products. *Int. J. Food Microbiol.* 96:149–164.

152. Vogt, R. L., and L. Dippold. 2005. *Escherichia coli* O157:H7 outbreak associated with consumption of ground beef, June-July 2002. *Public Health Rep*. 120:174–178.

153. Woodford, N., and M. J. Ellington. The emergence of antibiotic resistance by mutation. *Clin. Micr. Infec*. 13:5–18.

154. Wu, S. X., and R. Q. Peng. 1992. Studies on an outbreak of neonatal diarrhea caused by EPEC 0127:H6 with plasmid analysis restriction analysis and outer membrane protein determination. *Acta. Paediatr*. 81:217–221.

155. Wu, X. Y., T. Chapman, D. J. Trott, K. Bettelheim, T. N. Do, S. Driesen, M. J. Walker, and J. Chin. 2007. Comparative analysis of virulence genes, genetic diversity, and phylogeny of commensal and enterotoxigenic *Escherichia coli* isolates from weaned pigs. *Appl. Environ. Microbiol*. 73:83–91.

156. Younts-Dahl, S. M., G. D. Osborn, M. L. Galyean, J. D. Rivera, G. H. Loneragan, and M. M. Brashears. 2005. Reduction of *Escherichia coli* O157 in finishing beef cattle by various doses of *Lactobacillus acidophilus* in direct-fed microbials. *J. Food. Prot*. 68:6–10.

المنارة للاستشارات
www.manaraa.com

157. Zakharov, S. D., M. Lindeberg, Y. Griko, Z. Salamon, G. Tollin, F. G. Prendergast, and W. A. Cramer. 1998. Membrane-bound state of the colicin E1 channel domain as an extended two-dimensional helical array. *Proc. Natl. Acad. Sci*. 95:4282–4287.

158. Zakharov, S. D., M. Lindeberg, and W.A. Cramer. 1999. Kinetic description of structural changes linked to membrane import of the colicin E1 channel protein. *Biochemistry.* 36:11325–11332.

159. Zakharov, S. D., and W. A. Cramer. 2002. Colicin crystal structures: pathways and mechanisms for colicin insertion into membranes. *Biochim. Biophys. Acta*. 1565:333–346.

160. Zakharov, S. D., V. Y. Eroukova, T. I. Rokitskaya, M. V. Zhalnina, O. Sharma, P. J. Loll, H. I. Zgurskaya, Y. N. Antonenko, and W. A. Cramer. 2004. Colicin occlusion of OmpF and TolC channels: outer membrane translocons for colicin import. *Biophys Journ.* 87:3901–3911.

161. Zarivach, R., E. Ben-Zeev, N. Wu, T. Auerbach, A. Bashan, K. Jakes, K. Dickman, A. Kosmidis, F. Schluenzen, A. Yonath, M. Eisenstein, and M. Shoham. 2002. On the interaction of colicin E3 with the ribosome. *Biochimie*. 84:447–454.

162. Zhang, S., and A. Mustapha. 1999. Reduction of *Listeria monocytogenes* and *Escherichia coli* O157:H7 numbers on vacuum-packaged fresh beef treated with nisin or nisin combined with EDTA. *J. Food Prot*. 62:1123–1127.

163. Zhao, T., M. P. Doyle, B. G. Harmon, C. A. Brown, P. O. E. Mueller, and A. H. Parks. 1998. Reduction of carriage of enterohaemorrhagic *Escherichia coli* O157:H7 in cattle by inoculation with probiotic bacteria. *J. Clin. Microbiol*. 36:641–647.

المنارة للاستشارات
www.manaraa.com

164. Zhao, T., S. Tkalcic, M. P. Doyle, B. G. Harmon, C. A. Brown, and P. Zhao. 2003. Pathogenicity of enterohemorrhagic *Escherichia coli* in neonatal calves and evaluation of fecal shedding by treatment with probiotic *Escherichia coli*. *J. Food Prot*. 66:924–930.

165. Zhao, T., P. Zhao, J. W. West, J. K. Bernard, H. G. Cross, and M. P. Doyle. 2006. Inactivation of enterohemorrhagic *Escherichia coli* in rumen content- or feces-contaminated drinking water for cattle. *Appl Environ Microbiol.* 72:3268–3273.

Figure Legends

Figure 1. BtuB, Prokaryotic Vitamin B12 receptor. a) unbound BtuB; b) BtuB bound with Vitamin B12 (red). From Mohanty et al., 2003, with permission.

Figure 2. Colicin E1 translocation events. Pathway of the distinguishable structure transitions of P178 in the course of binding to the membrane surface and insertion into the bilayer. Rate constants for defined steps are for pH 4.0, I = 0.1 M and 25°C. Copied with permission from Zakharov et al., 1999.

المنارة للاستشارات

www.manaraa.com

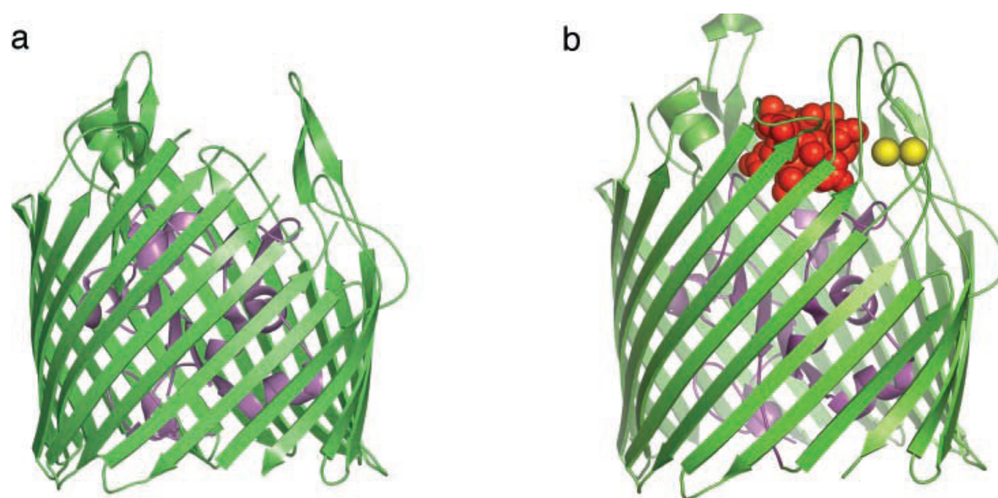


Figure 1. BtuB, Prokaryotic Vitamin B12 receptor.

المنارة للاستشارات
www.manaraa.com

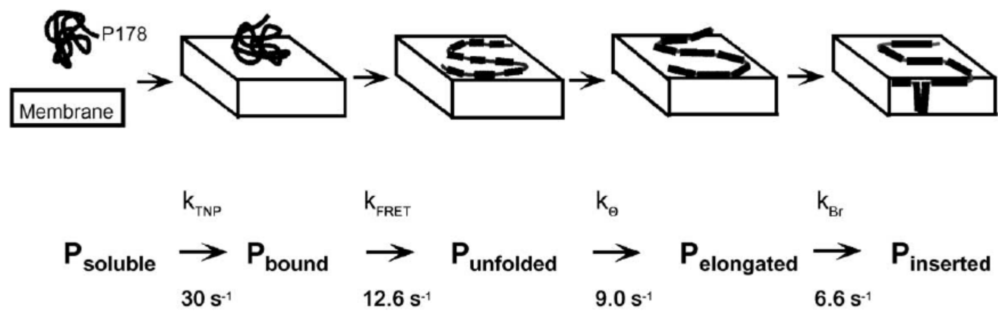


Figure 2. Colicin E1 translocation events.

المنارة للاستشارات
www.manaraa.com

CHAPTER 2. APPLICATION OF COLICIN E1 AS A CARCASS WASH INTERVENTION STRATEGY

A paper to be submitted to the *Journal of Food Protection*

Brenda S. Patton[1,2], Steven M. Lonergan[3], Sara A. Cutler[3], Chad H. Stahl[3], and James S. Dickson[2,*]

Abstract

Colicin E1 (ColE1) is a bacteriocin produced by and effective against *Escherichia coli* and related species. The current study examined ColE1 as a potential carcass intervention strategy in controlling the contamination of *E. coli* O157:H7 on beef carcasses. Untrimmed beef round roasts were cut into sample sizes of 12.5 x 7.5 x 2.5 cm with an adipose layer covering an entire surface of lean. Samples were placed on sterile metal hooks hung in the same orientation as the tissue would hang on a carcass. Samples were inoculated with 1ml TSB (control) or 1mL TSB, containing 5 $\log_{10}$ CFU *E. coli* O157:H7 strain WS 3062 or WS 3331. After inoculum attachment, 1mL ColE1 (in doses of 0, 100µg, 500µg) and 1mg/mL 10mM Tris, pH 7.6 were sprayed on the samples and allowed to associate for a period of 10 minutes. Samples were evaluated at 0 and 30minutes, and at 1, 2, 3, 4, and 5 days post spray at 10°C for *E. coli* O157:H7 inhibition.

Treatment of samples with 500µg and 1mg ColE1 effectively inhibited *E. coli* O157:H7 growth. When these doses were applied to the samples inoculated with *E. coli*

---

[1]Graduate research assistant.
[2]Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, 50011.
[3]Department of Animal Sciences, Iowa State University, Ames, IA, 50011.
[*]Author for correspondence, principal investigator.

www.manaraa.com

WS 3331, *E. coli* contamination was reduced by 4 and 7 log_{10} CFU/cm^2, respectively, compared to the untreated control samples. In strain WS 3062, treatment with 1mg ColE1 significantly inhibited growth of *E. coli* O157:H7 compared to the untreated control for the entirety of the study. Colicin E1 provided powerful reduction of E. *coli* O157:H7 as a beef carcass spray intervention.

Introduction

In the United States during 1992 and 1993, outbreaks of *Escherichia coli* O157:H7 infection associated with human consumption of ground beef caused hundreds of illnesses and four deaths (30). As a result of these and similar outbreaks, the U.S. Department of Agriculture Food Safety and Inspection Service (USDA-FSIS) mandated the Cattle Clean Meat Program and a zero-tolerance standard regarding the complete removal of fecal material, ingesta, and udder fluids from beef carcasses, as well as detectable growth of *E. coli* O157:H7 (31).

Cattle are a major reservoir for *E. coli* O157:H7, and contamination of the beef carcass at slaughter occurs during such processes as hide removal and evisceration (2, 3). In an effort to reduce *E. coli* O157:H7 as well as other harmful pathogenic bacteria, processors are incorporating antimicrobial intervention strategies into their slaughter processes. Commonly used carcass interventions include trimming, steam vacuuming, steam pasteurization, water washes, and organic acid washes (5, 8, 9). Recent studies have demonstrated that combinations of antimicrobial interventions are more effective at reducing surface contamination on beef tissue than individual interventions (9, 14, 19). Of these antimicrobial interventions, organic acid washes, such as lactic and acetic acids, are the most common. With the use of multiple or "hurdle" antimicrobials, carcasses have an average

المنارة للاستشارات
www.manaraa.com

overall reduction in surface contamination by 30% and up to 2 log cycles (4, 5, 27). Current drawbacks to the use of organic acids are the increase of acid-resistant *E. coli* O157:H7, the efficacy of these organic acids over the fabrication and retail packaging times, and the adverse sensory properties detectable on some fresh meat cuts after organic acid treatment (11, 24, 27).

Colicins are antimicrobial proteins produced by, and effective against, *E. coli* and other members of the *Enterobacteriaceae* family (6, 16). Pore-forming colicins, such as colicin E1 (ColE1), function as potent bacteriocidal agents via the formation of depolarizing ion channels in the cytoplasmic membrane (17). Colicins have long been viewed as potent killers to *E. coli*, and more recently to enterohemorragic and shiga-producing strains (7, 23, 28). Given the efficacy of these proteins against various *E. coli* strains in vitro, the objective of this study was to examine the efficacy of ColE1 against *E. coli* O157:H7 on beef tissue. The current experiments evaluated the use of ColE1 as a spray to reduce *E. coli* O157:H7 populations.

Materials and Methods

Colicin production and purification. Colicin E1 was produced and purified by the method of Stahl et al. (28). Briefly, ColE1 was produced from an *E. coli* K-12 strain containing plasmid pColE1-K53, obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, UK). Colicin expression was induced with mitomycin C, and the ColE1 was purified from the cell-free supernatant by ion exchange chromatography using Q Sepharose (Amersham Biosciences, Piscataway, N.J.) and then concentrated and desalted by ultra-filtration across a regenerated cellulose membrane in a stir

المنارة للاستشارات
www.manaraa.com

cell apparatus (Amicon, Millipore, Bedford, M.A.). The ColE1 preparation used in this study was purified to near homogeneity (98% pure).

Inhibition study. For each of the two *E. coli* O157:H7 cultures, WS 3331 and WS 3062, (obtained from FSIS, Beltsville, MD, isolated from ground beef outbreak), 100 µL of frozen stock culture was individually added to 9.9mL of sterile tryptic soy broth (TSB) and incubated at 35º C for 24 hours. These cultures were then diluted 1:100 into fresh TSB to 4 $\log_{10}$ CFU/mL. From these cultures 7.9 mL aliquots of each E. coli culture were placed into 10 mL culture tubes containing either 0, 0.8, 8, or 80µg colicin E1 in 100µl of TSB; therefore, the final concentrations of the colicin E1 were 0, 0.1, 1, and 10µg ColE1/mL culture. These cultures were then incubated at 35º C, and CFU/mL of *E. coli* O157:H7 was determined initially, after 30 minutes, and hourly for five hours of incubation. Colony forming units/mL were determined by serial dilution and plating in triplicate onto sorbitol-Mackonkey agar (sMAC). Plates were incubated for 24 hours prior to counting for CFU determination.

Beef sample preparation. Untrimmed beef outside round roasts (IMPS/NAMP 171B) were obtained from the Iowa State University Meat Laboratory and processed by irradiation to an absorbed dose of 9 kGy to eliminate naturally occurring bacteria. Roasts were aseptically cut into sample sizes of 12.5 x 7.5 x 2.5 cm with an adipose layer covering an entire surface of lean in order to mimic carcass conditions. Samples were tempered at 35°C for two hours to reach temperatures similar to that of beef carcasses directly post slaughter (18). Upon reaching abattoir muscle temperatures, the samples were placed on sterile metal hooks and hung in the same orientation as the tissue would hang on a carcass. Each sample was separated by a square piece of high-density polyethylene plastic (15 x 15

المنارة للاستشارات
www.manaraa.com

cm) to prevent aerosol contamination from the other organisms during spray treatment. After placement on sterile hooks, samples were directly moved to 4°C to simulate movement through an abbatoir. Tissue samples were hung in accordance with sampling time, so that duplicate samples would be evaluated at 0, 30 minutes, and at 1, 2, 3, 4, and 5 days.

***E. coli* inoculum preparation and addition.** For each of the two *E. coli* O157:H7 cultures, (WS 3331 and WS 3062), 100µL *E. coli* was added to 9.9mL of sterile tryptic soy broth (TSB) and incubated at 35º C for 24 hours. Each culture was then diluted 1:100 in fresh TSB to 5 $\log_{10}$ CFU/mL. The cultures were then transferred into sterile plastic spray bottles for sample application. To ensure that the anticipated inoculation levels were obtained, a sample from the spray bottle was serially diluted and plated onto tryptic soy agar (TSA) as well as sMAC and incubated for 24 hours at 35°C. For each sample, 1mL of the *E. coli* inoculum was sprayed on surfaces of the sample. One mL of culture was sufficient in covering all surfaces of the carcass samples. Inoculated samples were allowed to attach for a period of 10 minutes prior to ColE1 addition.

Colicin preparation and addition. Sterile plastic spray bottles were filled with 2mL of 10mM Tris (pH 7.6) containing either 0, 100µg, 500µg, and 1mg/mL of ColE1. A total of 1mL of these washes, which was sufficient to cover all of the surfaces of the sample, was applied to each sample. After spraying, samples were allowed to stand at room temperature for 10 minutes prior to being placed in 10°C refrigeration to simulate movement through an abattoir.

Bacterial enumeration. Entire samples (12.5 cmx 7.5 cmx 2.5 cm) weighing approximately 1.35 grams were placed in 25mL 0.1% peptone water and stomached (Seward Stomacher 3500, Worthing, West Sussex, UK) for 60 seconds. Samples were then serially

www.manaraa.com

diluted in 0.1% peptone water, plated on both TSA and sMAC, and incubated at 35°C for 24 hours. Samples were plated in triplicate for each ColE1 treatment.

Statistical analysis. Broth and carcass experiments were conducted two times. Data were analyzed using the general linear model (GLM) procedures of SAS (SAS Institute, Cary, NC). Colicin E1 dose was considered the fixed effect. Tukey's standardized range test was used to determine statistical differences (alpha level = 0.01). Colony forming unit data were transformed into $\log_{10}$ CFU/cm^2.

Results

Inhibition study. Figures 1a and 1b indicate inhibition of *E. coli* O157:H7 WS 3331 (a) and WS 3062 (b) by various concentrations of colicin E1. Although colicin E1 signficantly inhibited growth of *E. coli* O157 WS 3331 compared to the untreated control, no colicin E1 dose provided complete inhibition at any timepoint. Log_{10} reductions of 1.25, 2.25, and 3.75 were observed with treatments of 0.1, 1.0, and 10µg colicin E1/mL, respectively. These results differed from strain WS 3062 in which 10µg colicin E1/mL was sufficient to completely inhibit growth throughout the five hours of the experiment.

Beef sample study. Treatment of samples with ColE1 effectively inhibited *E. coli* O157:H7 growth at all applied dosage levels (Figures 2a and 2b). In both of the *E. coli* O157:H7 strains evaluated, control (0µg ColE1) samples had initial populations of ~3 $\log_{10}$ CFU/cm^2 and grew to a maximum of ~6 $\log_{10}$ CFU/cm^2. Strain WS 3331 appeared to be less susceptible to ColE1, as the 100µg dose was unable to significantly deter growth compared to the untreated control (Figure 2a). In this strain, at five days post colicin spray, the 500µg and 1mg doses were able to reduce contamination by 4 and 5 $\log_{10}$ CFU/cm^2 compared to the control samples. Growth was inhibited below detection limits (1 log CFU/cm^2) until five

المنارة للاستشارات
www.manaraa.com

days post-spray with a colicin E1 dose of 1mg. Among the samples inoculated with strain WS 3062, treatment with 1mg ColE1 significantly eliminated detectable growth of *E. coli* O157:H7 compared to the untreated control for the entirety of the study (Figure 2b). Samples sprayed with 100µg and 500µg ColE1/mL had detectable *E. coli* growth post-application, but had 3 and 5 $\log_{10}$ CFU/cm^2 lower *E. coli* levels, respectively, than the control samples at the five day sampling.

Discussion

The use of antimicrobials as an intervention on beef carcasses is well documented (9, 11, 14). Cattle are known reservoirs for *E. coli* O157:H7, and if infected carcasses and hides are not properly handled at the time of slaughter, this pathogen can continue to grow and be found on beef samples during the fabrication process and into retail products (12, 22). The most prevalent antimicrobial strategy in use in beef processing is the use of combinations of organic acids as a carcass intervention strategy. In modern beef processing plants, 2% lactic, acetic, and/or various organic acids are applied to pre-evisceration carcasses via an online spray cabinet that warms the lactic acid to approximately 42°C (10). Treating carcasses with 0.1–2.0% organic acids has been shown to reduce *E. coli* O157:H7 populations by 30% and up to 2 log cycles (5, 27). In 1998, Smulders and Greer (27) hypothesized that using organic acids in this manner may induce the emergence of acid-resistant pathogens including *E. coli* O157:H7. Current research has supported this hypothesis as acid-adapted and acid-tolerant *E. coli* O157:H7 are readily found in beef processing facilities using organic acid interventions, and the use of organic acids against these *E. coli* strains can induce further acid-adaptation and survival of the pathogen at refrigeration temperatures (24, 25, 29). In the hopes of overcoming the limitations of organic acids, bacteriocins are being examined as

المنارة للاستشارات
www.manaraa.com

potential interventions for *E. coli* O157:H7 on beef carcasses. Barboza de Martinez et al. (1) reported that the use of nisin, a gram-positive bacteriocin produced by lactic acid bacteria, was effective at reducing microbial populations on beef carcasses by 2 log CFU/gram when applied at 500µg/mL in combination with 2% lactic acid. Other bacteriocins such as sakacin P and curvacin A have also been shown to be effective against a wide range of microorganisms including *E. coli* O157:H7 if applied in combination with other antimicrobials (13).

Colicins are highly effective against *E. coli* O157:H7 (7, 15, 28). Much of the antimicrobial efficacy has been evaluated in vitro. Recent work on the use of colicins in pre-harvest intervention strategies, as well as vegetable applications, is being evaluated. Colicins have been fed to beef cattle to prevent *E. coli* O157:H7 shedding (26), as well as being used as a sanitizer in alfalfa sprout washes (21). These applications have widened the potential for colicins against enterhemorrhagic *E. coli*.

Evidence supporting colicin E1 use against E. coli O157:H7 is shown in Figures 1a and 1b. Inhibition of both strains of E. coli O157:H7 was apparent at all doses compared to the untreated controls; however, in both strains treating with at least 10µg ColE1/mL provided reductions of ~5-6 $\log_{10}$ CFU/cm^2.

In beef sample study, ColE1 was able to completely inhibit growth—below detectable levels—of *E. coli* O157:H7 on beef samples at a dose of 1mg ColE1 (Figure 2). In strain WS 3331, 1 mg ColE1 reduced *E. coli* O157:H7 to undetectable levels until five days post-ColE1 application. After five days, the 1mg ColE1 inhibited *E. coli* O157:H7 by 5 $\log_{10}$ CFU/cm^2. Growth of both control treatments reached a level of ~6 $\log_{10}$ CFU/cm^2 after five days of growth at 4°C. At first glance, the growth of these *E. coli* strains seems extremely rapid

المنارة للاستشارات
www.manaraa.com

under the experimental treatments. One reason for the resulting growth patterns was the doubling time of these strains, which we determined to be 16 and 20 minutes for strain WS 3331 and WS 3062, respectively (data not shown). Additionally, samples were tempered to 35°C prior to sampling. Although samples were moved to 4°C after inoculation, the latent heat would be sufficient to allow for continued growth for ~12 hours.

These data prove that ColE1 has potential as an effective antimicrobial carcass spray and is at least as effective as other bacteriocins previously evaluated for the same use (1). Also, the highest level of ColE1 used in this study (1mg) is more effective against *E. coli* O157:H7 than typically used levels of organic acids. Smulders and Greer (27) reported that organic acids typically reduce *E. coli* O157;H7 populations by 2 logs. In this study, at certain points colicin E1 was able to reduce *E. coli* O157 populations by almost 7 log_{10} CFU/cm^2.

While the results demonstrate the efficacy of ColE1 against *E. coli* O157:H7, the safety of this protein for human consumption is also of particular importance for its use on food. There is great support for the safety of ColE1 for use in or on food. Murinda et al. (20) compared the cytoxicity of ColE1, nisin, and pediocin in mammalian cell culture. In that study, ColE1 demonstrated significantly less cytotoxicity than both nisin and pediocin. Coupled with the long history of exposure that humans have to colicins produced by commensal organisms in their gastrointestinal tract (23), this suggests that there should be no concerns for the use of this protein as an antimicrobial carcass spray.

Given the limited efficacy of organic acids and the emergence of acid-resistant pathogens, there is a critical need for a safe, potent, and effective carcass intervention

المنارة للاستشارات
www.manaraa.com

strategy that will not only improve abilities to meet zero-tolerance requirements for *E. coli* O157:H7 but also guarantee a safer retail product.

Conclusions

Colicin E1 is an effective antimicrobial against *E. coli* O157:H7. Treatment with ColE1 provided effective reductions against *E. coli* O157:H7 as a carcass spray intervention. While the 500µg/mL dose provided a ~5 $\log_{10}$ CFU/cm^2 reduction compared to the untreated control samples, dosing with 1mg ColE1 inhibited growth below detection limits for the duration of the study. Further work is needed to identify optimum dose requirements against multiple strains of *E. coli* O157:H7 to understand if inoculum type and level will affect the antimicrobial activities of ColE1.

Acknowledgements

We acknowledge Steven Niebuhr and Food Safety Research Laboratory personnel for assistance in microbiological preparation and technical help, as well as Randy Petersohn and the employees of the Iowa State Meats Laboratory.

This work was funded in part by the Biotechnology Research and Development Corporation (BRDC), the Tri-State Food Safety Consortium, and the Institute of Food Safety, Iowa State University.

References

1. Barboza de Martinez, Y., K. Ferrer, and E. M. Salas. 2002. Combined effects of lactic acid and nisin solution in reducing levels of microbiological contamination in red meat carcasses. *J. Food Prot*. 65:1780–1783.

المنارة للاستشارات
www.manaraa.com

2. Barkocy-Gallagher, G. A., T. M. Arthur, M. Rivera-Betancourt, X., Nou, S. D. Shackelford, T. Wheeler, and M. Koohmaraie. 2003. Seasonal prevalence of Shiga toxin-producing *Escherichia coli*, including O157:H7 and non-O157 serotypes, and Salmonella in commercial beef processing plants. *J. Food Prot*. 66:1978–1986.

3. Bell, R. G. 1997. Distribution and sources of microbial contamination on beef carcasses. *J. Appl. Microbiol*. 82:292–300.

4. Bosilevac, J. M., T. M. Arthur, T. L. Wheeler, S. D. Shackelford, M. Rossman, J. O. Reagan, and M. Koohmaraie. 2004. Prevalence of *Escherichia coli* O157 and levels of aerobic bacteria and Enterobacteriaceae are reduced when hides are washed and treated with cetylpyridinium chloride at a commercial beef processing plant. *J. Food Prot*. 67:646–650.

5. Bosilevac, J. M., X. Nou, G. A. Barkocy-Gallagher, T. M. Arthur, and M. Koohmaraie. 2006. Treatments using hot water instead of lactic acid reduce levels of aerobic bacteria and Enterobacteriaceae and reduce the prevalence of Escherichia coil O157:H7 on preevisceration beef carcasses. *J Food Prot*. 69:1808–1813.

6. Breukink, E., and B. de Kruijff. 2006. Lipid II as a target for antibiotics. *Nature Reviews Drug Discovery*. 5:321–332.

7. Callaway, T. R., C. H. Stahl, T. S. Edrington, K. J. Genovese, L. M. Lincoln, R. C. Anderson, S. M. Lonergan, T. L. Poole, R. B. Harvey, and D. J. Nisbet. 2004. ColE1 concentrations inhibit growth of *Escherichia coli* O157:H7 in vitro. *J. Food Prot*. 67:2603–2607.

8. Castillo, A., L. M. Lucia, K. J. Goodson, J. W. Savell, and G. R. Acuff. 1998. Comparison of water wash, trimming, and combined hot water and lactic acid treatments for reducing bacteria of fecal origin on beef carcasses. *J Food Prot*. 61:823–828.

المنارة للاستشارات
www.manaraa.com

9. Castillo, A., L. M. Lucia, K. J. Goodson, J. W. Savell, and G. R. Acuff. 1999. Decontamination of beef carcass surface tissue by steam vacuuming alone and combined with hot water and lactic acid sprays. *J Food Prot*. 62:146–151.

10. Dickson, J. S., and M. E. Anderson. 1992. Microbiological decontamination of food animal carcasses by washing and sanitizing systems: a review. *J. Food Prot*. 55:133–140.

11. Dorsa, W. J., C. N. Cutter, and G. R. Siragusa. 1998. Long-term effect of alkaline, organic acid, or hot water washes on the microbial profile of refrigerated beef contaminated with bacterial pathogens after washing. *J. Food Prot*. 61:300–306.

12. Elder, R. O. 2000. Correlation of enterohemorrhagic *Escherichia coli* O157 prevalence in feces, hides, and carcasses of beef cattle during processing. *PNAS* 97:2999.

13. Ganzle, M. G., S. Weber, and W. P. Hammes. 1999. Effect of ecological factors on the inhibitory spectrum and activity of bacteriocins. *Int. J. Food. Microbiol*. 46:207–217.

14. Harris, K., M. F. Miller, G. H. Loneragan, and M. Brashears. 2006. Validation of the use of organic acids and acidified sodium chlorite to reduce *Escherichia coli* O157 and Salmonella typhimurium in beef trim and ground beef in a simulated processing environment. *J. Food Prot*. 69:1802–1807.

15. Jordi, B. J., K. Boutaga, C. M. van Heeswijk, F. van Knapen, and L. J. Lipman. 2001. Sensitivity of Shiga toxin-producing *Escherichia coli* (STEC) strains for colicins under different experimental conditions. *FEMS Microbiol Lett*. 204:329–334

16. Konisky, J. 1982. ColE1s and other bacteriocins with established modes of action. *Ann. Rev. Microbiol*. 36:125–144.

17. Lazdunski, C. J. 1988. Pore-forming ColE1s: synthesis, extracellular release, mode of action, immunity. *Biochimie*. 70:1291–1296.

المنارة للاستشارات

www.manaraa.com

18. Locker, R. H., and C. Hagyard. 1963. A cold shortening effect in beef muscles. *J. Sci. Food Agr*. 14:787–793.

19. Marshall, K. M., S. E. Niebuhr, G. R. Acuff, L. M. Lucia, and J. S. Dickson. 2005. Identification of *Escherichia coli* O157:H7 meat processing indicators for fresh meat through comparison of the effects of selected antimicrobial interventions. *J. Food Prot*. 68:2580–2586.

20. Murinda, S. E., K. A. Rashid, and R. F. Roberts. 2003. In vitro assessment of the cytotoxicity of nisin, pediocin, and selected colicins on simian virus 40-transfected human colon and Vero monkey kidney cells with trypan blue staining viability assays. *J. Food Prot*. 66:847–853.

21. Nandiwada, L. S., G. P. Schamberger, H. W. Schafer, and F. Diez-Gonzalez. 2004. Characterization of an E2-type colicin and its application to treat alfalfa seeds to reduce *Escherichia coli* O157:H7. *Int. J.Food Microbiol*. 93:267–279.

22. Phillips, C. A. 1999. The epidemiology, detection, and control of *Escherichia coli* O157. *J. Sci. Food Agr*. 79:1367–1381.

23. Riley, M. A. 1996. The ecology and evolution of bacteriocins. *J. Ind. Microbiol*. 17:151.

24. Samelis, J., P. Kendall, G. C. Smith, and J. N. Sofos. 2004. Acid tolerance of acid-adapted and nonadapted *Escherichia coli* O157:H7 following habituation (10 degrees C) in fresh beef decontamination runoff fluids of different pH values. *J. Food Prot. 67:638–645.*

المنارة للاستشارات

www.manaraa.com

25. Samelis, J., J. N. Sofos, P. A. Kendall, and G. C. Smith. 2005. Survival or growth of *Escherichia coli* O157:H7 in a model system of fresh meat decontamination runoff waste fluids and its resistance to subsequent lactic acid stress. *Appl Environ Microbiol.* 71:6228–6234

26. Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez. 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. *Appl. Environ. Microbiol*. 70:6053–6060.

27. Smulders, F. J., and G. G. Greer. 1998. Integrating microbial decontamination with organic acids in HACCP programmes for muscle foods: prospects and controversies. *Int. J. Food Microbiol*. 44:149–169.

28. Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. Genovese. 2004. Inhibitory activities of ColE1s against *Escherichia coli* strains responsible for post-weaning diarrhea and edema disease in swine. *Ant. Agts. and Chem*. 48:3119–3121.

29. Stopforth, J. D., J. Samelis, J. N. Sofos, P. A. Kendall, and G. C. Smith. 2003. Influence of organic acid concentration on survival of *Listeria monocytogenes* and *Escherichia coli* O157:H7 in beef carcass wash water and on model equipment surfaces. *Food Microbiol*. 20:651–660

30. Tuttle, J., T. Gomez, M. P. Doyle, J. G. Wells, T. Zhao, R. Tauxe, and P. M. Griffin. 1999. Lessons from a large outbreak of *Escherichia coli* O157:H7 infections: insights into the infectious dose and method of widespread contamination of hamburger patties. *Epidemiol. Infect*. 122:185–192.

المنارة للاستشارات
www.manaraa.com

31. U.S. Department of Agriculture. Food Safety and Inspection Service. 1993. Immediate actions: cattle clean meat program. FSIS correlation packet, interim guidelines for inspectors. U.S. Department of Agriculture, Food Safety and Inspection Service. Washington, D.C.

Figure Legends

Figure 1. Colicin E1 inhibits *E. coli* O157:H7 in broth culture. Broth culture evaluation of WS 3331 (a) and WS 3062 (b) treated with concentrations of colicin E1. Samples were taken immediately post-application, 30 minutes, and hourly for five hours at 35°C. ♦ = no colicin (control); □ = 0.1µg colicin E1/mL; Δ = 1.0 µg colicin E1/mL; X = 10µg colicin E1/mL.

Figure 2. The effect of ColE1 against *E. coli* O157:H7 on beef carcass samples. Beef samples were initially inoculated with 5 log10 CFU/mL of E. coli O157:H7, and treated with 0, 100, 500µL or 1mg of ColE1. Detectable levels of *E. coli* WS 3331 (a) and WS 3062 (b) are represented over a five day period at 10°C. ♦ = 0 (control); □ = 100µg ColE1; Δ = 500µg ColE1; X = 1mg ColE1.

المنارة للاستشارات

www.manaraa.com

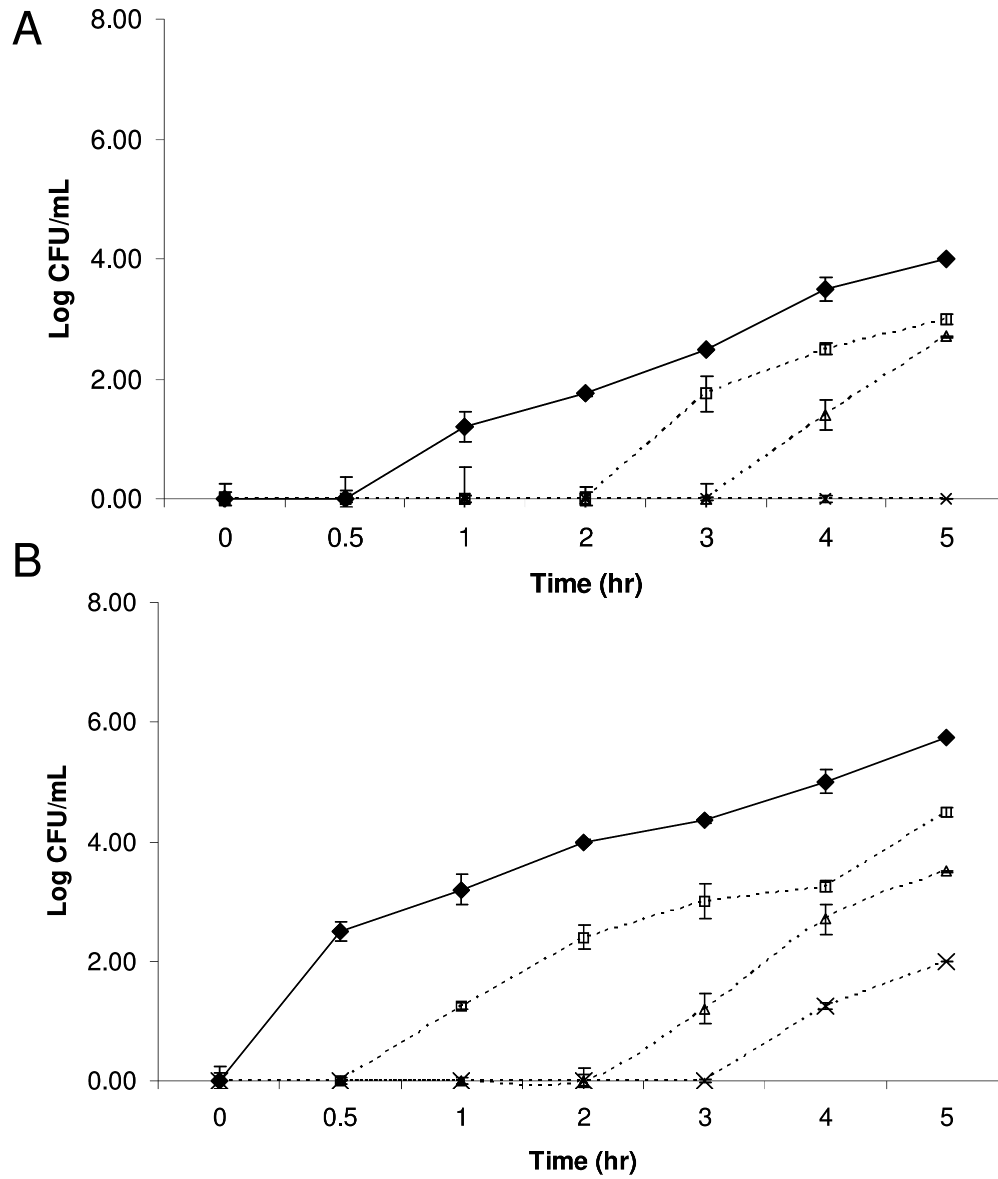


Figure 1. Colicin E1 inhibits *E. coli* O157:H7 in broth culture.

المنارة للاستشارات
www.manaraa.com

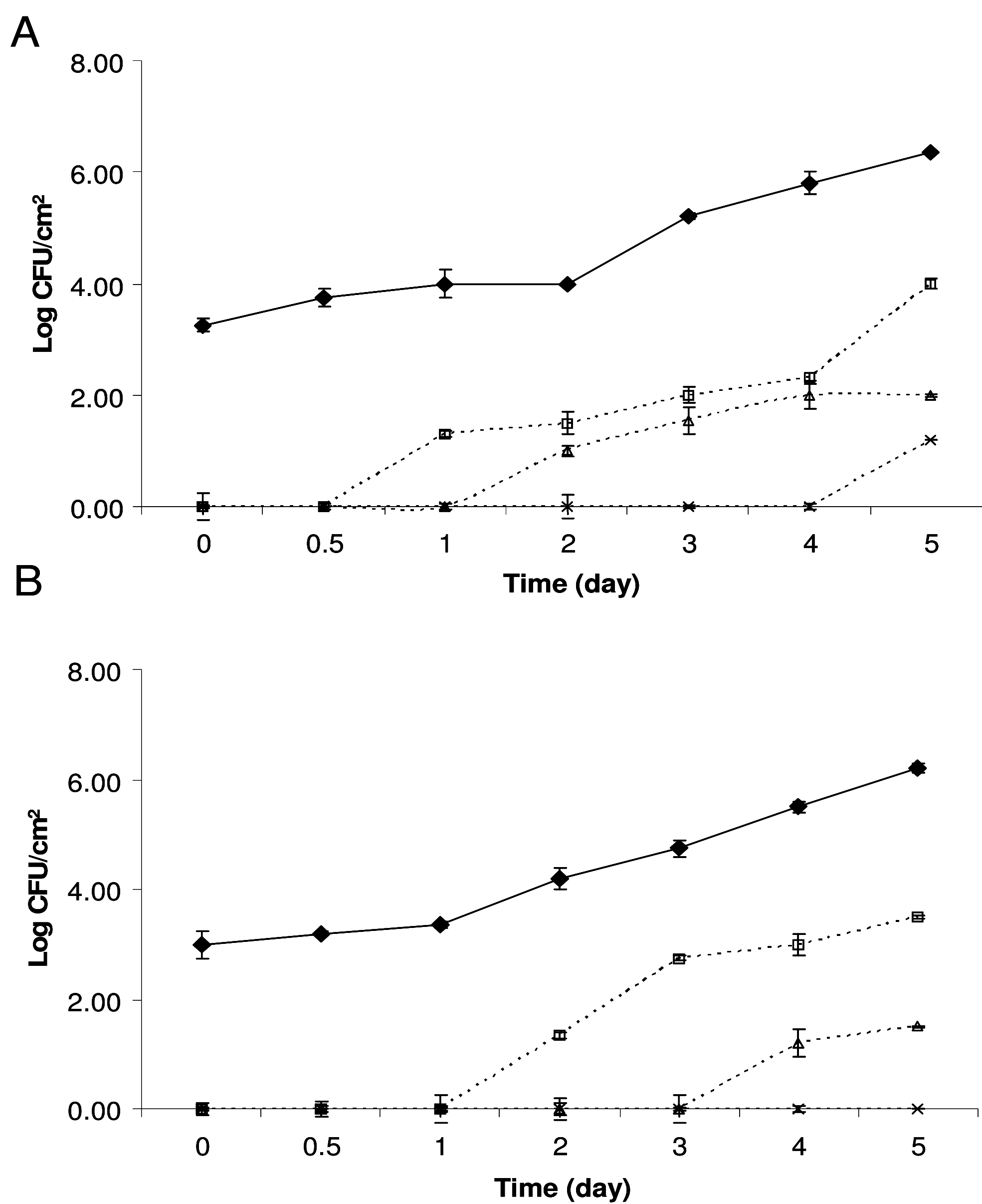


Figure 2. **The effect of ColE1 against *E. coli* O157:H7 on beef carcass samples**.

المنارة للاستشارات
www.manaraa.com

CHAPTER 3. INHIBITORY ACTIVITY OF COLICIN E1 AGAINST *LISTERIA MONOCYTOGENES*

A paper published in the Journal of Food Protection[4]

Brenda S. Patton[1,2], James S. Dickson[2], Steven M. Lonergan[3], Sara A. Cutler[3], and Chad H. Stahl[3,*]

Abstract

Colicins are gram-negative bacteriocins produced by, and effective against, *Escherichia coli* and related species. Colicin E1 (ColE1) is composed of three functional domains, which collectively cause a pore-forming effect on targeted bacteria. ColE1 binding and translocation domains are highly specific in contrast to the pore-forming domain, implying ColE1 could be broadly effective. This study evaluated the activity of ColE1 against *L. monocytogenes* in broth and on RTE product surfaces. Individual strains of *L. monocytogenes* were examined in broth containing 0, 0.1, 1, or 10µg ColE1/mL. While strain differences in sensitivity to ColE1 existed, growth was significantly reduced in all strains at doses as low as 0.1 µg/mL. Sterilized ham slices were submerged in a 5-strain *Listeria monocytogenes* cocktail (either 7 $\log_{10}$ CFU/ml or 4 $\log_{10}$ CFU/ml) and placed in vacuum packages containing 0, 1, 5, 10, 25, or 50µg ColE1. Ham slices were then stored at 4 or 10°C and sampled for presence of *L. monocytogenes* after 1, 3, 7, and 14 days.

---

[1]Graduate research assistant.
[2]Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, 50011.
[3]Department of Animal Sciences, Iowa State University, Ames, IA, 50011.
[4]Reprinted with permission from *J. Food Prot.*, 2007, 70(5), 1256–1262.
*Author for correspondence, principal investigator.

www.manaraa.com

Reduction of *L. monocytogenes* by ColE1 was dependant on initial inoculum level as well as storage temperature. For slices stored at 4°C, treatment with 25ug reduced *Listeria* growth below detection limits for the 4 $\log_{10}$ CFU/mL inoculum slices for the entire 14d, whereas for 7 $\log_{10}$ CFU/mL slices, growth was detected 7d post inoculation. For slices stored at 10°C, ColE1 significantly inhibited growth of *L. monocytogenes* at the 10µg/mL dose up to three days for both inoculums. These data indicate that ColE1 is highly effective against *Listeria*.

Introduction

Listeria monocytogenes is a foodborne pathogen responsible for human listeriosis, a severe gastrointestinal illness with a mortality rate of 30% (28). Numerous cases of foodborne illness have been linked to the consumption of RTE (RTE) products contaminated with *L. monocytogenes*. Among the many categories of RTE foods, deli meats have been identified as products of highest risk for causing listeriosis on both per serving and per annum basis (37). The resilience of this species has allowed for it to be ubiquitous in the food manufacturing and processing environments (18). *Listeria monocytogenes* resists the deleterious effects of high salt concentrations, pH extremes, freezing, and drying common to the manufacture of RTE meat products (22). Due to the widespread nature of *L. monocytogenes*, RTE products can become contaminated with this pathogen during peeling, slicing and repackaging, as well as at the retail and consumer levels during storage and preparation.

In response to this risk, the Food Safety and Inspection Service (FSIS) and the Food and Drug Administration (FDA) have set strict regulatory standards for RTE products including a "zero-tolerance" for *L. monocytogenes* on all RTE foods. Several antimicrobial

www.manaraa.com

methods are currently being used, and others are being evaluated for potential application to food products and packaging in order to meet these new FSIS standards. These include but are not limited to: steam pasteurization, hot water pasteurization, radiant heating and high pressure processing, UV and ionizing radiation, and the application of antimicrobial organic acids such as lactates, diacetates, and propionates. Costs associated with the implementation of the currently available methods, as well as their limited efficacy, clearly indicate a critical need for a cost-effective intervention capable of reducing high levels of *L. monocytogenes* on RTE products.

Colicins are antimicrobial proteins produced by, and effective against, *E. coli* and other members of the *Enterobacteriaceae* family (6, 24). Pore-forming colicins function as potent bacteriocidal agents via formation of depolarizing ion channels in the cytoplasmic membrane (25, 31). Given the efficacy of these proteins against various *E. coli* strains (32, 34), we were interested to determine if these bacteriocins could be efficacious against *L. monocytogenes*. The current study evaluated the activity of Colicin E1 (ColE1) against *L. monocytogenes* in both pure culture and on RTE product surfaces.

Materials and Methods

Colicin production and purification. Colicin E1 was produced and purified by the method of Stahl et al. (34). Briefly, ColE1 was produced from an *E. coli* K-12 strain containing plasmid pColE1-K53, obtained from the National Collection of Type Cultures (Public Health Laboratory Service, London, UK). Colicin expression was induced with mitomycin C, and the ColE1 was purified from the cell-free supernatant by ion exchange chromatography using Q Sepharose (Amersham Biosciences, Piscataway, N.J.) and then concentrated and desalted by ultrafiltration across a regenerated cellulose membrane in a stir

www.manaraa.com

cell apparatus (Amicon, Millipore, Bedford, M.A.). The purity of the ColE1 preparation used in this study can be seen in Figure 1.

Broth culture evaluation. Five isolated strains of *Listeria monocytogenes* were grown and evaluated for sensitivity to ColE1. The five strains included one human clinical isolate (2045 Scott A) and four meat product isolates (FSIS 1126 isolated from a beef carcass, H7769 isolated from a RTE poultry product, H7762 and H7764 both isolated from frankfurters) all carrying the serotypes (1/2a, 4b) for human clinical illness. All strains were obtained from National Animal Disease Center (NADC, Ames, IA) and the Food Safety Research Laboratory, Iowa State, Ames, IA). Growth curves for the individual strains were constructed to determine the time necessary to reach 4 $\log_{10}$ CFU/mL under the growth conditions utilized. For each of the five cultures examined, frozen stock cultures (9.5 $\log_{10}$ CFU/mL) were thawed at room temperature, and 100µL of the re-suspended stock culture was added to 9mL of sterile Trypticase soy broth (TSB, Becton-Dickinson, Sparks, MD) containing 0.6% yeast extract (YE, Becton-Dickinson, Sparks, MD) (TSBYE). Cultures were incubated at 37°C for 24 hours, and then 1mL of the culture was placed into 99mL of fresh TSBYE and changes in optical density and growth were determined hourly over a 24 hour incubation. Optical density was determined at 600nm (Spectronic 20D spectrophotometer, Thermo Spectronic Inc., Madison, WI) and growth (CFU/mL) was determined by serial dilution and plating onto Modified Oxford Listeria Selective Agar (MOX, Becton-Dickson, Sparks, MD) followed by incubation at 37ºC for 24 hours.

Colicin sensitivity testing. For each strain, 100 µL of frozen stock culture was individually added to 9.9mL of sterile TSBYE and incubated at 37º C for 24 hours. These cultures were then diluted 1:100 into fresh TSBYE and allowed to grow to 4 $\log_{10}$ CFU/mL.

المنارة للاستشارات
www.manaraa.com

From these cultures 7.9 mL aliquots were placed into 10 mL culture tubes containing either 0, 0.8, 8, or 80µg ColE1 in 100µl of 10 mM Tris, pH 7.6; therefore, the final concentrations of the ColE1 were 0, 0.1, 1, and 10µg ColE1/mL culture. These cultures were then incubated at 37° C and optical density and CFU/mL of *L. monocytogenes* determined initially and after 1, 3, and 6 hours of incubation. Optical density was determined at 600nm, and CFU/mL were determined by serial dilution and plating in triplicate onto MOX agar. Plates were incubated for 24 hours prior to counting for CFU determination.

Acquired resistance evaluation. In order to determine if surviving *L. monocytogenes* had altered metabolism or acquired resistance to ColE1, single isolates of *L. monocytogenes* that survived the 10µg ColE1/mL dose in the broth culture study were isolated from the MOX plates for each of the five strains tested. These isolates were then restreaked onto fresh MOX agar and allowed to grow at 37°C for 24 hours. Regrown isolates were then identified as *L. monocytogenes* using AccuProbe® *Listeria monocytogenes* culture identification tests (Gen-Probe Inc., San Diego, CA). Isolates were then analyzed for growth—in the same manner as the original strains—in TSBYE with either 0 (Control) or 1µg ColE1/mL. Optical density and CFU/mL of *L. monocytogenes* were determined initially and after 1, 3, and 6 hours of incubation at 37°C. Optical density was determined at 600 nm, and CFU/mL were determined by serial dilution and plating in triplicate onto MOX agar. Plates were incubated for 24 hours prior to counting for CFU determination. Resistance isolation and regrowth was repeated three times, with five isolates per strain in each replication. The original ColE1 sensitivity testing as well as the acquired resistance growth studies were each conducted on three separate occasions.

المنارة للاستشارات
www.manaraa.com

Ready-to-eat (RTE) product evaluation. For each of the five stock cultures, 100µL was added to 9.9mL of sterile TSBYE and incubated at 37° C for 24 hours. Each culture was then diluted 1:100 in fresh TSBYE and grown to either 4 $\log_{10}$ CFU/mL or 7 $\log_{10}$ CFU/mL. Within each inoculum level, 10mL of each culture was transferred into 90mL sterilized 0.1% peptone water, and the five individual strains were then combined (450mL total) for submerging ham slices. To ensure that the anticipated inoculation levels were obtained, a sample from each pooled inoculum culture was serially diluted and plated onto MOX agar. Ham slices were sterilized by irradiation to avoid potential pre-contamination with *Listeria* or other bacteria (15). Ham slices weighing 50g (surface area of approximately 232cm^2) were aseptically sliced in half, completely submerged in the five stain *L. monocytogenes* inoculum for 10 minutes to allow for bacterial attachment, then drained briefly, and transferred into sterile vacuum bags containing 1mL of various ColE1 doses in 10mM Tris, pH 7.6. The bags were then massaged (Seward Stomacher 3500, Worthing, West Sussex, UK) for 60 seconds, vacuum packaged, and placed into refrigeration. Slices stored at 4°C were treated with ColE1 concentrations of 0, 1, 5, 10, and 25µg, and sampled at 0, 1, 3, 7, and 14 days. Slices stored at 10°C were treated with ColE1 concentrations of 0, 1, 10, and 50µg ColE1, and sampled at 0, 1, and 3 days. For enumeration of *L. monoyctogenes*, ham slices were aseptically cored (surface area =12.5 cm^2, 2.5g), and the cores were serially diluted in 10mL peptone water, plated on MOX agar, and incubated at 37°C for 24 hours. All samples were prepared in triplicate for each refrigeration temperature-dose-day sampling point.

Statistical analysis. Data were analyzed as a split-plot design using the general linear model (GLM) procedures of SAS (SAS Institute, Cary, NC). Colicin E1 dose, temperature treatment (4° and 10°C), and storage time were considered fixed effects. Tukey's

المنارة للاستشارات
www.manaraa.com

standardized range test was used to determine statistical differences (alpha level = 0.01). Colony forming unit data from the RTE Product Evaluation were transformed into log_{10} CFU/cm^2.

Results

Broth culture evaluation. All strains were sensitive to ColE1; however, significant variation in strain susceptibility existed (Table 1 and Figure 2). Significant reductions in *L. monocytogenes* populations were seen in all of the strains tested with inclusion of 0.1µg ColE1/mL, with the exception of FSIS 1126, after one hour (Figure 2). Strain H7762 was the most susceptible strain, as 0.1 µg ColE1/mL culture resulted in approximately 3.5 log_{10} CFU/mL less *L. monocytogenes* than in the untreated control after seven hours of growth. Even among the more resistant *L. monocytogenes* strains, 10µg ColE1/mL culture resulted in an approximately 3–4.5 log_{10} CFU/mL reduction in *Listeria* counts after three hours of incubation. In all strains, the highest dose of ColE1 utilized (10µg/mL) was not able to eliminate all of the *Listeria* in any of the cultures.

Acquired resistance evaluation. All five strains were evaluated for susceptibility of regrown isolates to further ColE1 treatment. In all strains, growth of control isolates was not significantly altered from the original strain data, suggesting that no change in growth or metabolism occurred after treatment with ColE1 (Figure 3). Retreated isolates (n = 5) grew extremely similar to the original strains treated at 1µg ColE1/mL, and in certain cases appeared to have become more susceptible. In evaluation of strains Scott A and H7769, growth of retreated isolates was significantly lower ($P<0.01$) at six hours post-addition than the original strains.

المنارة للاستشارات
www.manaraa.com

RTE product evaluation. For the ham slices stored at stored at 4°C, growth of *L. monocytogenes* was reduced at every ColE1 dosage level (Figures 4a and 4b); however, the efficacy of the doses was dependant on the initial inoculum level. Despite this, the 5.0µg ColE1 dose was sufficient to reduce *L. monocytogenes* levels below our limits of detection for over 24 hours, regardless of the inoculation level of the ham slices. When 25µg ColE1 was applied on ham slices inoculated with 4 log_{10} CFU/mL (Figure 4b), no *Listeria* was detectable for the entirety of the 14 day study. Ham slices inoculated with 7 log_{10} CFU/mL had detectable *L. monocytogenes* growth at this same ColE1 treatment after three days. In the samples inoculated with 7 log_{10} CFU/mL, ColE1 application at 5, 10, and 25µg still caused a 4 log_{10} CFU/cm^2 reduction in *L. monocytogenes* compared to the control slices at the completion of the 14 day study.

Ham slices stored at 10°C were used to evaluate the efficacy of ColE1 against *L. monocytogenes* on RTE products stored under insufficient refrigeration (46). The 10 and 50µg ColE1 doses eliminated detectable *L. monocytogenes* for over 24 hours regardless of initial inoculum level. When the ham slices were stored at 10°C (Figure 3c and 3d), ColE1 significantly ($P<0.01$) inhibited growth of *L. monocytogenes* at doses as low as 1µg. After three days storage at this elevated temperature, ham slices treated with 1µg ColE1 had ~3 (c) and ~2.2 (d) log_{10} CFU/cm^2 lower CFU counts compared to the control slices inoculated at 7 and 4 log_{10} CFU/mL, respectively. After three days post-treatment, doses of 10 and 50µg ColE1 still significantly reduced the growth of *L. monocytogenes* by 3 log_{10} CFU/cm^2 and ~4 log_{10} CFU/cm^2, based on initial inoculum level (7 and 4 log_{10} CFU/mL, respectively). There were no significant differences between these two doses at any time point over the duration of this study.

www.manaraa.com

Discussion

Listeria monocytogenes is a prevailing environmental pathogen which poses tremendous challenges for the RTE food industry. This pathogen will grow at temperatures ranging from 1–45°C within a pH range of 4.1–9.6 and has been documented to survive in cured products up to 120 days (22, 27). Current intervention strategies for controlling *L. monocytogenes* in RTE meat products are the addition of (a) antimicrobials and chemical preservatives, which are generally recognized as safe substances (GRAS), or (b) a combination of two or more of these or like compounds (28, 30). The most common of these compounds used to combat *L. monocytogenes* in RTE products are organic acids and their salts (40).

Sodium, potassium, or other salts of lactic, acetic, and other organic acids have demonstrated significant antimicrobial activity in broth and meat product applications against *L. monocytogenes* (3, 7, 13, 35, 40). Although organic acids have proved to be effective anti-listerial agents, the levels required for this activity are extremely high. Additionally, several studies have shown that *L. monocytogenes* on RTE meat products survived in refrigerated storage despite the presence of organic acids that have been shown to have bacteriostatic activity against *L. monocytogenes* (3, 26, 39). Further limitations to the use of organic acids are their varying efficacy and sensory attributes. The antimicrobial ability of these acids is dramatically influenced by the presence of other antimicrobials or other ingredients commonly used in RTE meat manufacture (2, 3, 9, 16, 36). Current studies have indicated that RTE meat products formulated or applied with organic acids led to lower overall consumer acceptability compared to untreated products (17).

المنارة للاستشارات
www.manaraa.com

In hopes of overcoming the limitations of organic acids, bacteriocins are being examined as potential interventions for *L. monocytogenes* on RTE food products (10, 21). One such bacteriocin, nisin, was found to inhibit growth of *L. monocytogenes* on the surface of bologna by 2.4–3.8 $\log_{10}$ CFU/cm^2 at a concentration of 125µg/mL (17). Enterocin, another gram-positive bacteriocin of the pediocin family, has been shown to reduce *L. monocytogenes* growth in broth cultures by 3 $\log_{10}$ CFU/mL when included at 4µg/mL (14). Other pediocins have shown varied anti-Listerial activity, ranging from 0.5–4 $\log_{10}$ CFU/mL reductions when included at concentrations greater than 500µg/mL (1, 14, 20, 36).

Based on the efficacy of ColE1 against *L. monocytogenes* demonstrated in this study, it appears that ColE1 is more efficacious against *Listeria* than any of the previously reported bacteriocins. In our study, ColE1 was effective at reducing *L. monocytogenes* populations in pure culture, significantly reducing growth at doses of 0.1µg/mL (Figure 2). Among the five strains evaluated, reduction levels up to 5.5 $\log_{10}$ CFU/mL were observed with concentrations as low as 1µg ColE1/mL.

No other research has examined ColE1 for anti-Listerial activity. Since bacteriocins are frequently thought of as a means for similar bacteria to compete with each other for resources, it was surprising to see this high level of efficacy of an *E. coli* derived bacteriocin against *Listeria*. Colicin E1 exerts its cytotoxicity toward *E. coli* and other closely related bacteria through the formation of ion channels that depolarize the cytoplasmic membrane (8). Colicin E1 is composed of three functional domains that collectively cause a pore-forming effect on targeted bacteria (11). In gram-negative bacteria, pore-forming colicins must accomplish three tasks: binding to an outer membrane receptor, translocating across the periplasmic space, and inserting into the cytoplasmic membrane to form a highly conductive

المنارة للاستشارات
www.manaraa.com

ion channel (12, 31, 38). For binding and translocation purposes in gram-negative bacteria, ColE1 uses the vitamin B_{12} receptor, BtuB, and members of the Tol and OMP protein complexes (4, 11, 19, 33, 38). Future studies examining binding and attachment sites of ColE1 to *L. monocytogenes* are imperative to elucidating the mechanism of action of this bacteriocin against gram-positive organisms.

In our RTE product evaluation, significantly lower levels of ColE1 were required to reduce growth of *Listeria monocytogenes* compared to the previously mentioned antimicrobial studies (14, 16). With properly refrigerated ham slices that initially had approximately 2.5 $\log_{10}$ CFU/cm^2, treatment with 10µg of ColE1 reduced *L. monocytogenes* levels below the detection limits for three days (Figure 4b), and 25µg ColE1 reduced *L. monocytogenes* populations to undetectable levels for the entire 14 day study. Increasing refrigeration temperature from 4°C to 10°C had dramatic effects on product stability as well as ColE1 efficacy. At 10°C, 50µg of ColE1 reduced *L. monocytogenes* populations by 4 (Figure 4c) and 3 $\log_{10}$ CFU/cm^2 (Figure 4d) compared to the untreated control at three day post application (7 and 4 $\log_{10}$ CFU/mL inoculum, respectively). At this time, levels of *L. monocytogenes* reached approximately 9 $\log_{10}$ CFU/cm^2 on control slices. Common *L. monocytogenes* contamination levels in RTE products have been found anywhere from 1 to 5 $\log_{10}$ CFU/gram of product (37). In this study, our lower inoculum level (4 $\log_{10}$ CFU/mL) provided initial contamination levels (approximately 4.4 $\log_{10}$ CFU/gram) that would be at the higher end of this range. The ability of minute quantities of ColE1 to eliminate detectable *L. monocytogenes* from a RTE product contaminated at levels above the average seen among contaminated RTE products in the industry strongly supports its potential as an anti-*Listeria* agent for use in food.

المنارة للاستشارات
www.manaraa.com

Further supporting evidence on the value of ColE1 as a potent anti-listerial agent is the lack of acquired resistance demonstrated in this study (Figure 3a-e). Previous studies have shown that *L. monocytogenes* can spontaneously become highly resistant to bacteriocins such as nisin and pediocin (5, 23).

While we have demonstrated the efficacy of ColE1 against *L. monocytogenes*, the safety of this protein for human consumption is of particular importance for its use on food. There is great support for the safety of ColE1 for use in food. Murinda et al. (29) compared the cytoxicity of ColE1, nisin, and pediocin in mammalian cell culture. In this study, ColE1 demonstrated significantly less cytotoxicity than both nisin and pediocin. Coupled with the long history of exposure that humans have to colicins produced by commensal organisms in their gastrointestinal tracts (32), this finding suggests that there should be no concerns for the use of this protein as a biopreservative.

Conclusions

Colicin E1 effectively reduced populations of *L. monocytogenes* in broth culture as well as on RTE meat product surfaces. While strain differences in sensitivity to ColE1 existed, growth was significantly ($P < 0.01$) reduced in pure cultures of all strains tested with a ColE1 dose of 0.1µg/mL. The mechanism of action of ColE1 against gram-positive organisms such as *L. monocytogenes* has not yet been elucidated. Understanding this mechanism could lead to broader applications of ColE1 against many other bacterial pathogens. Colicin E1 is a safe and highly effective anti-L*isterial* agent, and its application to RTE meat products could provide greater safety against *L. monocytogenes* throughout the food processing and retail process.

المنارة للاستشارات

www.manaraa.com

Acknowledgements

We acknowledge Steven Niebuhr and Food Safety Research Laboratory personnel for assistance in microbiological preparation and technical help. This work was funded in part by the Biotechnology Research and Development Corporation (BRDC).

References

1. Bari, M. L., D. O. Ukuku, T. Kawasaki, Y. Inatsu, K. Isshiki, and S. Kawamoto. 2005. Combined efficacy of nisin and pediocin with sodium lactate, citric acid, phytic acid, and potassium sorbate and EDTA in reducing the *Listeria monocytogenes* population of inoculated fresh-cut produce. *J. Food Prot*. 68:1381–1387.

2. Barmpalia, I. M., I. Geornaras, K. E. Belk, J. A. Scanga, P. A. Kendall, G. C. Smith, and J. N. Sofos. 2004. Control of *Listeria monocytogenes* on frankfurters with antimicrobials in the formulation and by dipping in organic acid solutions. *J. Food Prot*. 67:2456–2464.

3. Bedie, G. K. 2001. Antimicrobials in the formulation to control *Listeria Monocytogenes* post-processing contamination on frankfurters stored at 4°C in vacuum packages. *J. Food Prot.* 64:1949–1955.

4. Benedetti, H., C. Lazdunski, and R. Lloubes. 1991. Protein import into *Escherichia-coli*: ColE1-a and ColE1-E1 interact with a component of their translocation system. *EMBO J.* 10:1989–1995.

5. Bonnet, M., M. M. Rafi, M. L. Chikindas, and T. J. Montville. 2006. Bioenergetic mechanism for nisin resistance, induced by the acid tolerance response of *Listeria monocytogenes. Appl. Environ. Microbiol.* 72:2556–2563.

6. Breukink, E., and B. de Kruijff. 2006. Lipid II as a target for antibiotics. *Nat. Rev. Drug Discov.* 5:321–332.

المنارة للاستشارات

www.manaraa.com

7. Buncic, S., C. M. Fitzgerald, R. G. Bell, and J. A. Hudson. 1995. Individual and combined listericidal effects of sodium lactate, potassium sorbate, nisin and curing salts at refrigeration temperatures. *J. Food Safety*. 15:247–264.

8. Cao, Z., and P. E. Klebba. 2002. Mechanisms of ColE1 binding and transport through outer membrane porins. *Biochimie*. 84:399–412.

9. Chen, N., and L. A. Shelef. 1992. Relationship between water activity, salts of lactic acid and growth of *Listeria monocytogenes* in a meat model system. *J. Food Prot*. 55:574–578.

10. Chi-Zhang, Y., K. L.Yam, and M. L. Chikindas. 2004. Effective control of *Listeria monocytogenes* by combination of nisin formulated and slowly released into a broth system. *Int. J. Food Microbiol*. 90:15–22.

11. Chimento, D. P., R. J. Kadner, and M. C. Wiener. 2003. The *Escherichia coli* outer membrane cobalamin transporter BtuB: structural analysis of calcium and substrate binding, and identification of orthologous transporters by sequence/structure conservation. *J. Mol. Biol.* 332:999–1014.

12. Cramer, W., J. Heymann, S. Schendel, B. Deriy, F. Cohen, P. Elkins, and C. Stauffacher. 1995. Structure-function of the channel-forming ColE1s. *Annu. Rev. Biophys. Biomol. Struct.* 24:611–664.

13. El-Shenawy, M. A., and E. H. Marth. 1989. Inhibition or inactivation of *Listeria monocytogenes* by sodium benzoate together with some organic acids. *J. Food. Prot*. 52:771–776.

14. Ennahar, S., K. Sonomoto, and A. Ishizaki. 2000. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. *J. Biosci. Bioeng*. 87:705–716.

المنارة للاستشارات

www.manaraa.com

15. Foong, S. C., G. L. Gonzalez, and J. S. Dickson. 2004. Reduction and survival of *Listeria monocytogenes* in ready-to-eat meats after irradiation. *J. Food Prot*. 67:77–82

16. Geornaras, I., P. N. Skandamis, K. E. Belk, J. A. Scanga, P. A. Kendall, G. C. Smith, and J. N. Sofos. 2006. Postprocess control of *Listeria monocytogenes* on commercial frankfurters formulated with and without antimicrobials and stored at 10°C. *J. Food Prot*. 69:53–61.

17. Geornaras, I., K. E. Belk, J. A. Scanga, P. A. Kendall, G. C. Smith, and J. N. Sofos. 2005. Post-processing antimicrobial treatments to control *Listeria monocytogenes* in commercial vacuum-packaged bologna and ham stored at 10°C. *J. Food Prot*. 68:991–998.

18. Gray, M. J., N. E. Freitag, and K. J. Boor. 2006. How the bacterial pathogen *Listeria monocytogenes* mediates the switch from environmental Dr. Jekyll to pathogenic Mr. Hyde. *Infect. Immun*. 74:2505–2512.

19. Griko, Y. V., S. D. Zakharov, and W. A. Cramer. 2000. Structural stability and domain organization of ColE1 E1. *J. Mol. Biol.* 302:941–953.

20. Gurira, O. Z., and E. M. Buys. 2005. Characterization and antimicrobial activity of *Pediococcus* species isolated from South African farm-style cheese. *Food Microbiol*. 22:159–168.

21. Jack, R. W., J. R. Tagg, and B. Ray. 1995. Bacteriocins of gram-positive bacteria. *Microbiol. Rev*. 59:171–200.

22. Jay, J. M. 2000. Foodborne Listeriosis, pp. 485–510. In J. M. Jay (ed.) Modern food microbiology (6th ed.). Aspen Publishers, Inc., Gaithersburg, MD.

المنارة للاستشارات
www.manaraa.com

23. Katla, T., K. Naterstad, M. Vancanneyt, J. Swings, and L. Axelsson. 2003. Differences in susceptibility of *Listeria monocytogenes* strains to sakacin P, sakacin A, pediocin PA-1, and nisin. *Appl. Environ. Microbiol.* 69:4431–4437.

24. Konisky, J. 1982. ColE1s and other bacteriocins with established modes of action. *Annu. Rev. Microbiol.* 36:125–144.

25. Lazdunski, C. J. 1988. Pore-forming ColE1s: synthesis, extracellular release, mode of action, immunity. *Biochimie.* 70:1291–1296.

26. Lu, Z., J. G. Sebranek, J. S. Dickson, A. F. Mendonca, and T. B. Bailey. 2005. Inhibitory effects of organic acid salts for control of *Listeria monocytogenes* on frankfurters. *J. Food Prot*. 68:499–506.

27. Mazzotta, A. S., and D. E. Gombas. 2001. Heat resistance of an outbreak strain of *Listeria monocytogenes* in hot dog batter. *J. Food Prot*. 64:321–324.

28. Mead, P. S., L. Slutsker, V. Dietz, F. McCaig, J. S. Bresee, and C. Shapiro. 1999. Food-related illness and death in the United States. *Emerging Infect. Dis*. 5:607–625.

29. Murinda, S. E., K. A. Rashid, and R. F. Roberts. 2003. In vitro assessment of the cytotoxicity of nisin, pediocin, and selected colicins on simian virus 40-transfected human colon and Vero monkey kidney cells with trypan blue staining viability assays. *J. Food Prot*. 66:847–853.

30. Nuñez de Gonzalez, M. T., J. T. Keeton, G. R. Acuff, L. J. Ringer, and L. M. Lucia. 2004. Effectiveness of acidic calcium sulfate with propionic and lactic acid and lactates as post-processing dipping solutions to control *Listeria monocytogenes* on frankfurters with or without potassium lactate and stored vacuum packaged at 4.5°C. *J. Food Prot*. 67:915–921.

المنارة للاستشارات
www.manaraa.com

31. Pugsley, A. P. (1984). The ins and outs of ColE1s. Part II. Lethal action, immunity and ecological implications. *Microbiol. Sci.* 1:203–205.

32. Riley, M. A., and J. E. Wertz. 2002. Bacteriocins: evolution, ecology, and application. *Annu. Rev. Microbiol*. 56:117–137.

33. Rodionov, D. A., A. G. Vitreschak, A. A. Mironov, and M. S. Gelfand. 2003. Comparative genomics of the Vitamin B12 metabolism and regulation in prokaryotes. *J. Biol. Chem.* 278:41148–41159.

34. Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. Genovese. 2004. Inhibitory activities of ColE1s against *Escherichia coli* strains responsible for post-weaning diarrhea and edema disease in swine. *Antimicrob. Agents Chemother*. 48:3119–3121.

35. Stekelenburg, F. K. 2003. Enhanced inhibition of *Listeria monocytogenes* in frankfurter sausage by the addition of potassium lactate and sodium diacetate mixtures. *Food Microbiol*. 20:133–137.

36. Uhart, M., S. Ravishankar, and N. D. Marks. 2004. Control of *Listeria monocytogenes* with combined antimicrobials on beef franks stored at 4 degrees C. *J. Food Prot*. 67:2296–2301.

37. U.S. FDA/CFSAN. September 2003, posting date. Quantitative assessment of the relative risk to public health from foodborne *Listeria monocytogenes* among selected categories of ready-to-eat foods. [Online.] http://www.foodsafety.gov/;dms/lmr2 toc.html. Accessed 11 April 2006.

38. Zakharov, S. D., E. A. Kotova, Y. N. Antonenko, and W. A. Cramer. 2004. On the role of lipid in ColE1 pore formation. *Biochim. Biophys. Acta.* 1666:239–249.

المنارة للاستشارات

www.manaraa.com

39. Zaika, L. L., S. A. Palumbo, J. L. Smith, F. Del Corral, S. Bhaduri, C. O. Jones, and A. H. Kim. 1990. Destruction of *L. monocytogenes* during frankfurter processing. *J. Food Prot.* 53:18–21.

40. Zhu, M. J., M. Du, J. Cordray, and D.U. Ahn. 2005. Control of *Listeria monocytogenes* contamination in ready-to-eat meat products. *Comp. Rev. Food Sci. Food Saf.* 4:34–42.

Figure Legends

Figure 1. **Purity of colicin E1 preparation**. Purity of ColE1 was assessed by SDS-Polyacrylamide gel electrophoresis utilizing a 12% polyacrylamide gel loaded with A: 5µg BenchMark Pre-Stained Protein Ladder (Invitrogen) and B: 30µg Colicin E1 in 10mM Tris, pH 7.6.

Figure 2. **The effects of ColE1 on *Listera monocytogenes* in broth culture**. Changes in growth (Log_{10} CFU/mL) were measured at 1, 3 and 6 hrs after treatment with different concentrations of ColE1 added to TSBYE. Error bars represent SE (n = 3). Graph A = *L. monocytogenes* FSIS 1126; Graph B = *L. monocytogenes* Scott A; Graph C = *L. monocytogenes* NADC H7769; Graph D = *L. monocytogenes* NADC H7762; and Graph E = *L. monocytogenes* NADC H7764.

Figure 3. **Acquired resistance evaluation of *Listeria monocytogenes* to ColE1**. Changes in growth (Log_{10} CFU/mL) of previously ColE1 treated *L. monocytogenes* isolates were measured at 1, 3, and 6 hrs after re-treatment with either 0 or 1µg ColE1/mL. Error bars represent SE (n = 3). Graph A = *L. monocytogenes* FSIS 1126; Graph B = *L. monocytogenes* Scott A; Graph C = *L. monocytogenes* NADC H7769; Graph D = *L.*

المنارة للاستشارات
www.manaraa.com

monocytogenes NADC H7762; and Graph E = *L. monocytogenes* NADC H7764.

Figure 4. The efficacy of ColE1 against *L. monocytogenes* in RTE products.

Reductions of *L. monocytogenes* (Log_{10} CFU/cm^2) on ham slices after treatment with different concentrations of ColE1. Graphs A and C represent the ham slices originally inoculated with 7 log_{10} CFU/mL and Graphs B and D represent the ham slices inoculated in 4 log_{10} CFU/mL. Graphs A and B depict ham slices refrigerated at 4°C for 14 days, and Graphs C and D depict ham slices refrigerated at 10°C for three days. Error bars represent SE (n = 3).

المنارة للاستشارات
www.manaraa.com

Table 1. Optical Density (600nm) of *Listeria monocytogenes* strains treated with ColE1 at 0, 1, 3, and 6 hours.

	Sampling time (hr)			
	0	**1**	**3**	**6**
FSIS 1126				
Dose (µg/ml)				
0.00	0	0.008	0.218[a]	0.712[a]
0.10	0	0	0.064[b]	0.132[b]
1.0	0	0	0.027[c]	0.176[c]
10.0	0	0	0.010[b]	0.117[b]
Scott A				
0.00	0	0.050[a]	.325[a]	1.350[a]
0.10	0	0[b]	.223[b]	0.776[b]
1.0	0	0[b]	.303[a]	0.709[c]
10.0	0	0[b]	.218[b]	0.590[d]
H7769				
0.00	0	.0100	0.154[a]	1.25[a]
0.10	0	0	0.021[b]	0.061[b]
1.0	0	0	0.020[b]	0.600[c]
10.0	0	0	0.130[a]	0.500[d]
H7762				
0.00	0	0.011	0.057[a]	1.28[a]
0.10	0	0	0.020[b]	0.100[b]
1.0	0	0	0[b]	0.005[c]
10.0	0	0	0[b]	0.004[c]
H7764				
0.00	0	0	0	0
0.10	0	0	0.002	0.018
1.0	0	0	0.002	0.018
10.0	0	0	0	0.005

[a,b,c,d] Within a given strain and time point, means not sharing a common superscript are significantly different (P<0.01).

www.manaraa.com

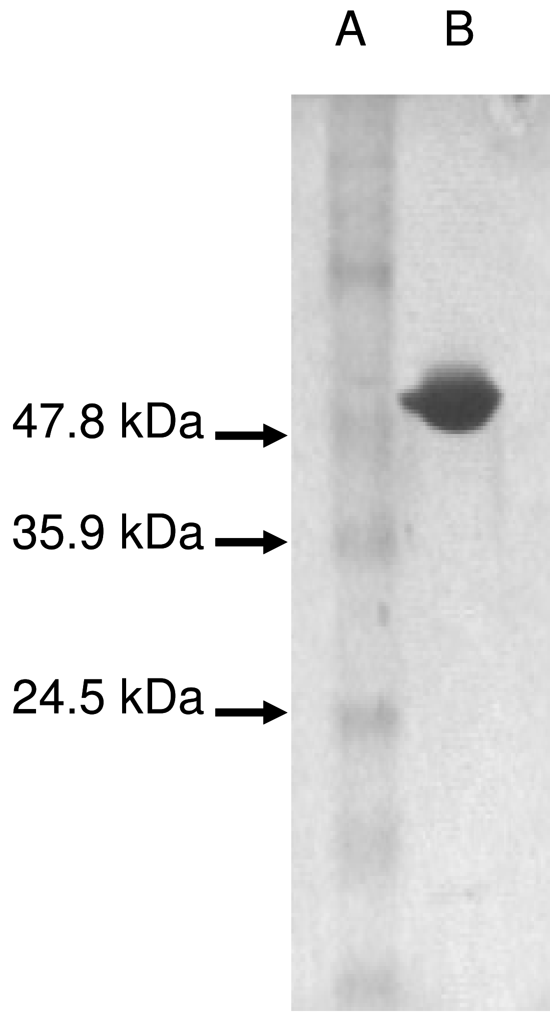


Figure 1. Purity of Colicin E1 preparation.

www.manaraa.com

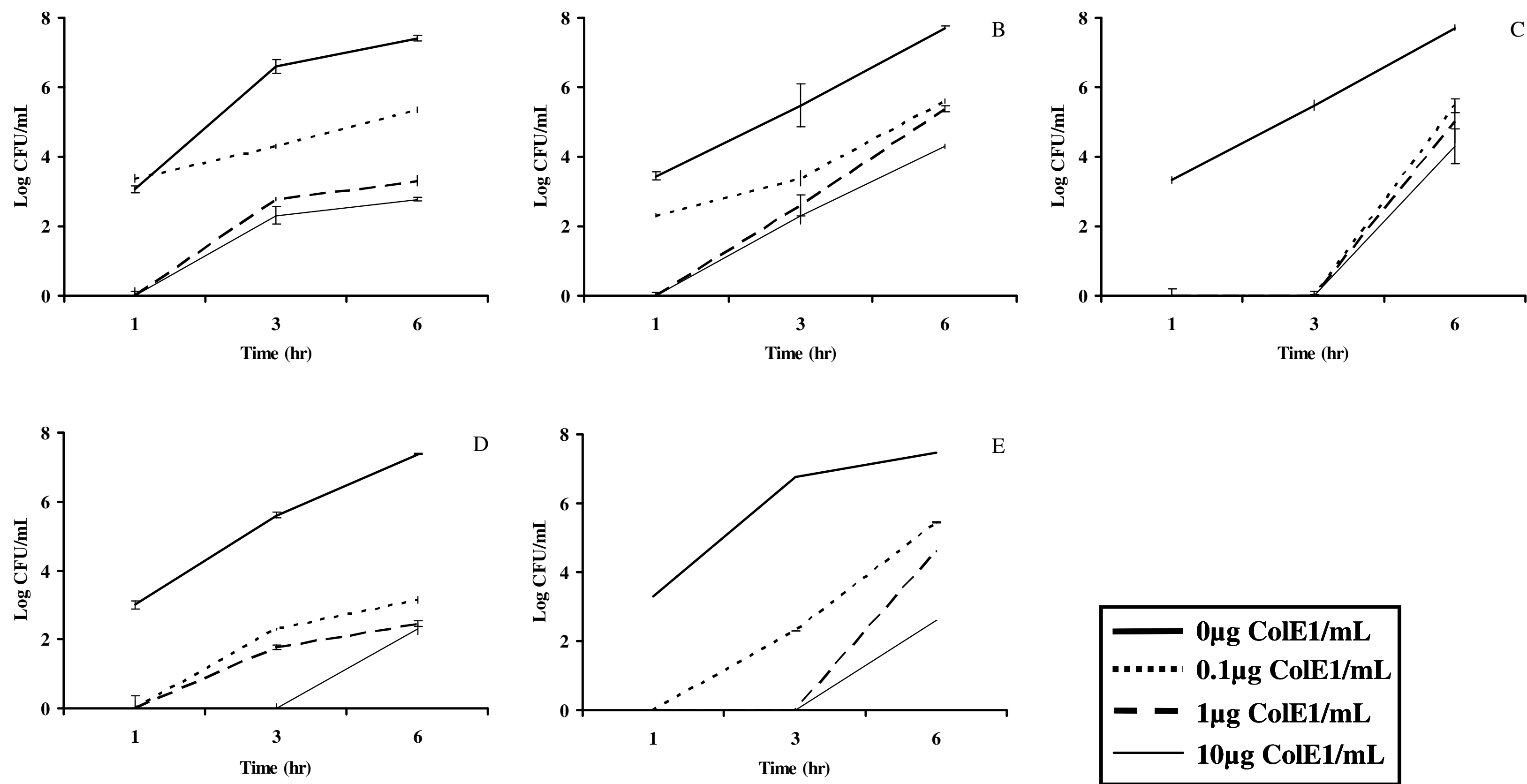


Figure 2. **The effects of ColE1 on *Listera monocytogenes* in broth culture**.

المنارة للاستشارات
www.manaraa.com

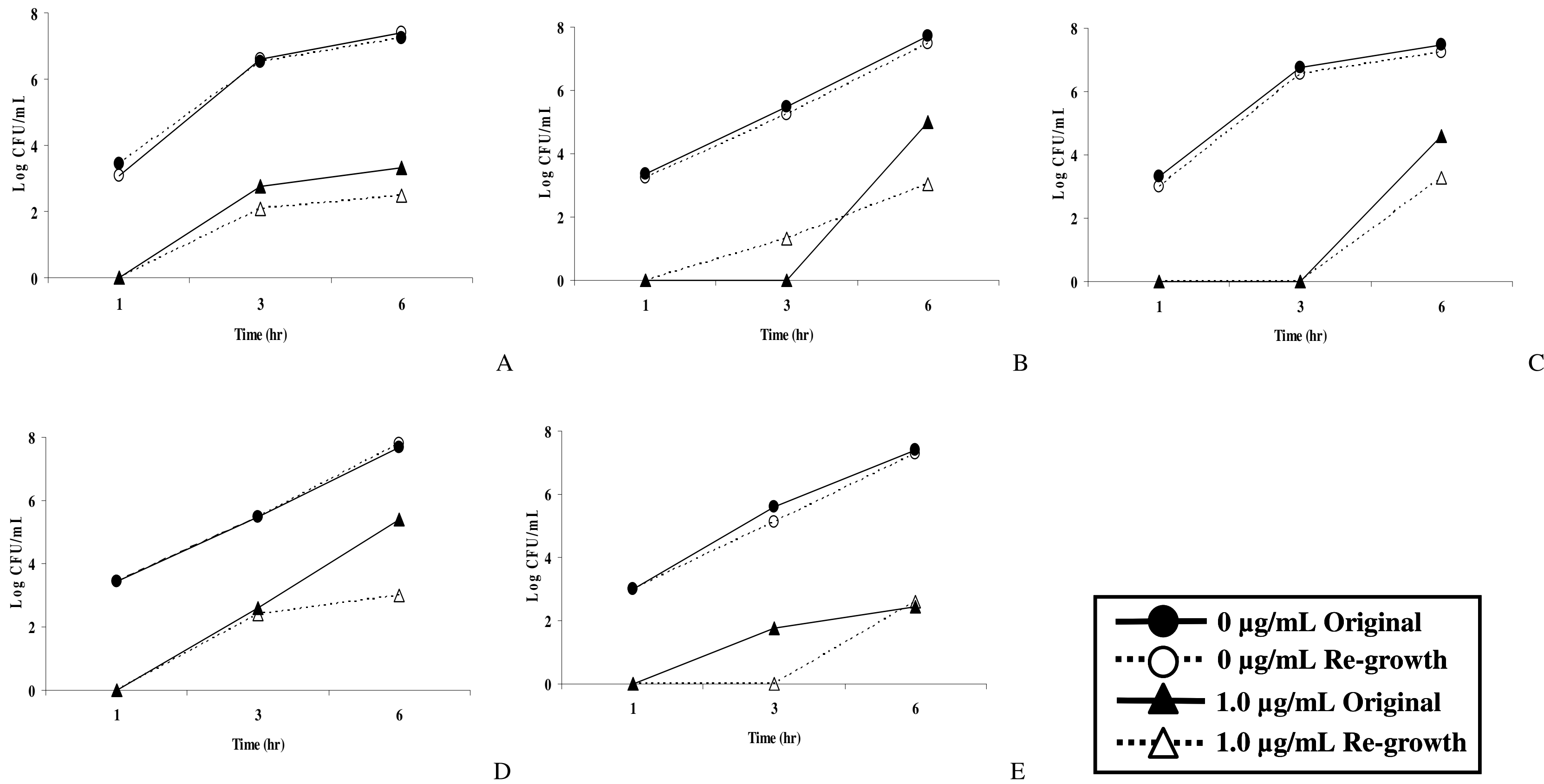


Figure 3. Acquired resistance evaluation of *Listeria monocytogenes* to ColE1.

المنارة للاستشارات
www.manaraa.com

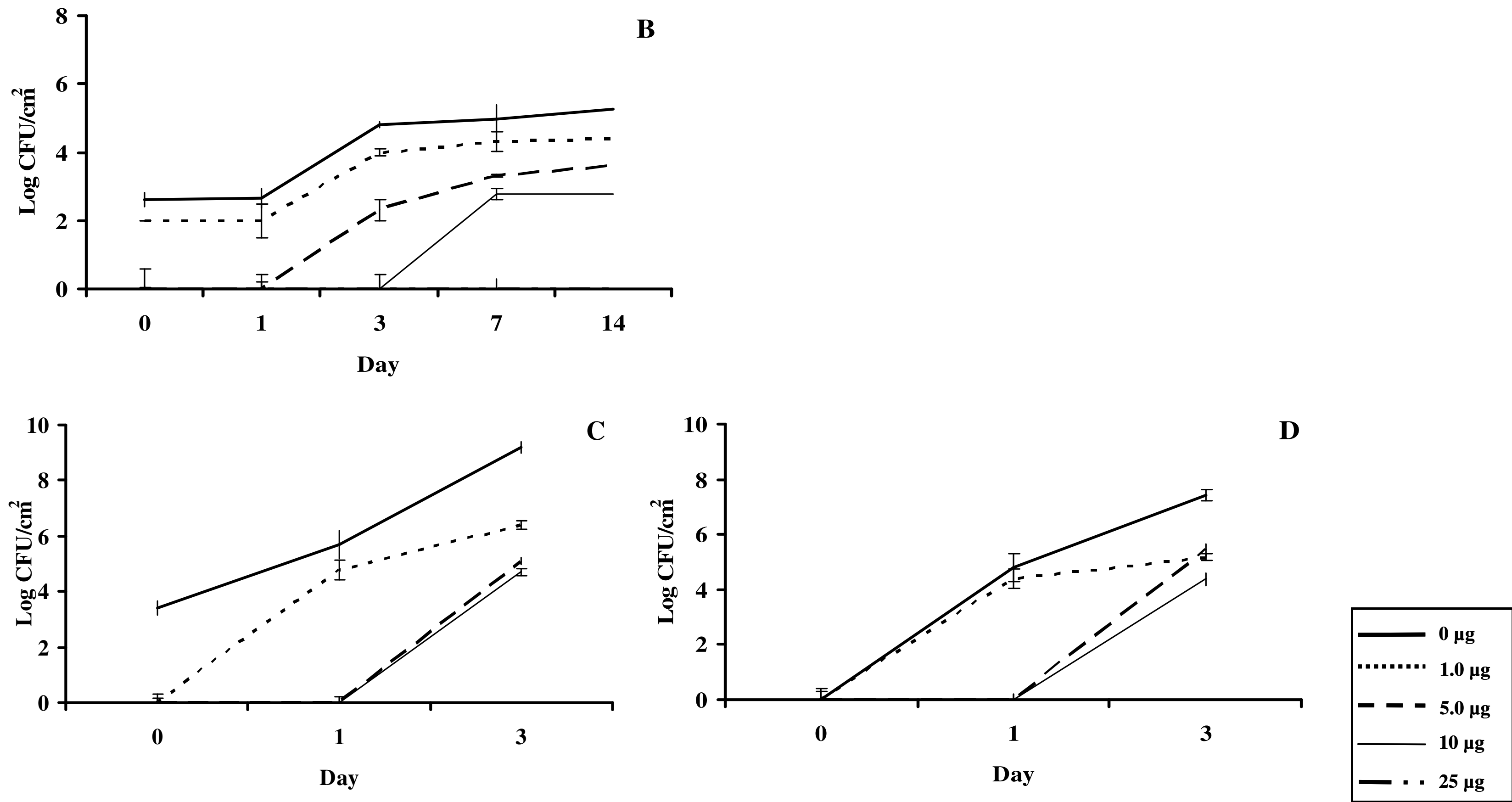


Figure 4. **The efficacy of ColE1 against *L. monocytogenes* in RTE products.**

المنارة للاستشارات
www.manaraa.com

CHAPTER 4. ON THE ROLE OF COLICIN E1 AGAINST *LISTERIA MONOCYTOGENES*

A paper to be submitted to Antimicrobial Agents and Chemotherapy

Brenda S. Patton[1,2], J. S. Dickson[2], T. M. Pepper[3], S. A. Cutler[4], and C. H. Stahl[4,*]

Abstract

Colicin E1 exerts its cytotoxic activity on gram-negative bacteria in a three-step process: (a) E1 binds to the outer membrane (OM) receptor, BtuB, (b) translocates across the OM using the Tol protein system, and (c) creates a lipid-dependent toroidal pore in the cytoplasmic membrane. These processes rely on proteins conserved to gram-negative species. Recent work has shown that E1 has inhibitory effects against the gram-positive pathogen *Listeria monocytogenes*. To elucidate the mechanism of lethality which E1 utilizes against *L. monocytogenes*, cytoplasmic membrane depolarization of *L. monocytogenes* was observed by flow cytometry using the membrane-potential sensitive dye DiBAC$_{(4)}$. Colicin E1 exerted 30% higher rate of membrane depolarization in *L. monocytogenes* compared to *E. coli* O157:H7. Cellular leakage was similar in *E. coli* and *L. monocytogenes* treated with 0.1 and 1.0µg/mL E1. Colicin E1 was visualized over time within *L. monocytogenes* cells using transmission electron microscopy, using immunocytochemical techniques and

---

[1]Graduate research assistant.
[2]Department of Food Science and Human Nutrition, Iowa State University, Ames, IA, 50011.
[3]Microscopy and NanoImaging Facility, Iowa State University, Ames, IA, 50011.
[4]Department of Animal Sciences, Iowa State University, Ames, IA, 50011.
*Author for correspondence, principal investigator.

www.manaraa.com

gold-conjugated secondary antibodies. Colicin-E1 was biotinylated, incubated with *L. monocytogenes* and eluted over a monomeric avidin column to capture interactive proteins. Fractions were collected, run on SDS-PAGE, and detected via silver staining. Four distinct proteins were analyzed by MALDI-TOF and identified as E1 of *E. coli,* DNA polymerase III, LepA, and Cell wall anchor family protein, of *L. monocytogenes*. Association with these proteins indicates E1 is acting independently of its recognized mechanism, and acting against *L. monocytogenes* in a novel manner.

Introduction

Bacteriocins are being extensively evaluated for antimicrobial activity and industrial applications (7, 9). Implementation of these bacterially-produced proteins are being used in food preservation, medical device sanitization, as well as livestock feed additives (6, 18, 25). Colicins are bacteriocins produced by, and effective against, *Escherichia coli* and similar species. Colicins can be subdivided by their mode of action on target bacteria, including pore-formation, nuclease activity against DNA, RNA, and tRNA, as well as protein synthesis inhibition (5). A common characteristic of all colicins is a narrow spectrum of activity, generally restricted to *E. coli* . Colicin E1 has long been known for its effectiveness against *E. coli* serovars (17). Few bacteriocins are effective across spectrums of bacteria. The mechanism is presence of specific receptors at the surface of the sensitive strains on which colicin binds before killing. Colicins must first bind to a preferential target on the outer membrane of *E. coli*, translocate across the outer membrane, and insert itself into the membrane to create a pore, or enter the cytoplasm for nucleic acid destruction. Mutation of the receptor can lead to the loss of sensitivity [resistance] to the corresponding colicin, proving the selectivity and necessity of colicin binding (8, 21). A specific pore-forming

المنارة للاستشارات
www.manaraa.com

colicin, E1 has an orchestrated and well-documented mode-of-action, including binding to BtuB, the vitamin B_{12} receptor of *E. coli*, translocating across the outer-membrane by the use of the Tol protein system, and forming a pore in the cytoplasmic membrane (3, 19, 20, 24). Target bacterial cells are killed by membrane depolarization and loss of cellular constituents (10).

Recent evidence has shown that a colicin E1 has lethal activity against the gram-positive pathogen *Listeria monocytogenes* in broth culture, as well as in packaged deli meats (15). The activity of colicin E1 against *Listeria monocytogenes* is interesting due to the fact that these bacteria lack BtuB, the preferred binding target of colicin E1, as well as Tol system of translocation proteins.

The objective of this research was to ascertain the mechanistic action of colicin E1 against the gram-positive pathogen *Listeria monocytogenes*. The ability to effectively reduce pathogens of a broad nature would be desirable to many uses, including medical, agricultural or cosmetic applications.

Materials and Methods

Colicin Production and Purification

Colicin E1 was produced and purified by the method of Stahl et al. (23). ColE1 was produced from an *E. coli* K-12 strain containing plasmid pColE1-K53 (National Collection of Type Cultures, Public Health Laboratory Service, London, UK). Colicin expression was induced with mitomycin C, and the ColE1 was purified from the cell-free supernatant by ion exchange chromatography using Q Sepharose (Amersham Biosciences, Piscataway, N.J.). This extract was then concentrated and desalted by ultra-filtration across a regenerated

www.manaraa.com

cellulose membrane in a stir cell apparatus (Amicon, Millipore, Bedford, Mass.). The purity of the ColE1 preparation used in these experiments was 98.3%.

Flow Cytometry

Cellular preparation. Frozen cultures of *Listeria monocytogenes* (FSIS Strain 1126) and *Escherichia coli* O157:H7 strain 933 (ATCC 43895) were suspended in 99 mL of tryptic soy broth containing 0.6% yeast extract (TSBYE), or tryptic soy broth (TSB), respectively. Cultures were grown overnight at 37°C, at which point 1mL of each culture was resuspended in 99 mL fresh media until reaching exponential growth levels (approximately 4.0 $\log_{10}$ CFU/mL). Cells were harvested by centrifugation (10 minutes at 10,000 x g), washed twice in 100mM phosphate buffer, pH 7.5, and then resuspended in 9 mL TSBYE or TSB. One mL of each culture was then added to culture tubes containing 0 (control), 2.5, 1.25, 1.0, 0.6, 0.3, and 0.1µg colicin E1/mL of PBS. Control and colicin E1-culture mixtures were placed in incubation at 37°C, and tested at 1, 5, 10, 15, 25, 30, 45, and 60 minutes. At each time interval, culture tubes were centrifuged for two minutes at 1400 x g, and the supernatant was removed by needle vacuum. Cells were resuspended in 200µL of 100mM filtered PBS, and tubes were covered with aluminum foil until cytometric analysis.

Cytometic analysis. Membrane and cytometric analyses were conducted following a modified method of Wickens et al. (2000). The fluorochrome bis-(1,3-dibutylbarbituric acid) trimethine-oxonol ($DiBAC_4$) was purchased from Molecular Probes Inc. (Eugene, OR). $DiBAC_4$ was dissolved in 70% ethanol at 1 mg/ml and further diluted in filtered phosphate-buffered saline (PBS) to a final concentration of 100 mg/ml. 2.0µL of the $DiBAC_4$ solutions were added to 180-µL aliquots of the treated or untreated cultures to give final dye concentrations of 1.0 mg/ml. The mixtures were held for one minute in the dark before

www.manaraa.com

analysis BD Biosciences FACSCanto™ (BD Biosciences, San Jose, CA) equipped with 17 mW HeNe 633 nm lasers. Cell concentration of each sample was approximately 10^4–10^5 CFU/mL. Dye excitation was achieved at 488nm, and 10,000 events were collected for each sample. Data acquisition and processing were performed using BD FACSDiva™ 4.0. (Software from BD Biosciences). The uptake of $DiBAC_4$ is equivalent to loss of membrane potential, and data are represented in flow cytometry histograms with gated zones representing permealized cells. These assays were performed a total of four times.

Cell Permeability

Potassium ion leakage. *L. monocytogenes* and *E. coli* O157:H7 cells were grown to 4 log_{10} CFU/mL, harvested by centrifugation (3 min at 3300 x g), and washed with 100 mM filtered PBS. Cells were resuspended in either TSBYE or TSB containing either 0 (control), 2.5, 1.25, 1.0, 0.6, 0.3, and 0.1µg colicin E1/mL. Culture tubes were held at 37°C and analyzed for potassium efflux every five minutes from one to 40 minutes in incubation. Colicin E1-induced potassium efflux was monitored using a pH-meter pH 213 (Hanna Instruments, Kehl am Rhein, Germany) with a MI-442 potassium electrode and MI-409F reference electrode following the method of Orlov et al. (14). Before each experiment, the electrodes were calibrated with standard solutions containing 0.01, 0.1, or 1 mM KCl in buffer. A linear relationship was seen between these concentrations of KCl and measured electrode voltage. Prior to treatment with colicin E1, both *L. monocytogenes* and *E. coli* O157:H7 cultures were tested for potassium disruption via sonification. The concentrations of K^+ in the medium initially (K_{init}) and after sonication (K_{total}) were calculated from the measured voltages by applying the linear equation ($V_{meas} = m\log_{10} [K+] + z$) and the potassium concentration equation ($[K+] = 10^{V_{meas} - z / m}$). Calculations of potassium-efflux in

www.manaraa.com

percent as % release = $([K^+]_{meas} - [K^+]_{init} / [K^+]_{total}) - [K^+]_{init} \times 100$ (14). Ion data was collected, and regression calculations were performed with the Sigma Plot graphics program (SPSS Science, Chicago, IL). These assays were performed a total of three times.

Cellular protein leakage. Bacterial suspensions were prepared and treated with colicin E1 as stated previously. Culture tubes were incubated at 37°C and measured spectrophotometrically (260nm) for protein leakage at 5, 10, 20, 30, 45, 60, 75, and 120 minutes. The extent of leaked material in the supernatant fluid was determined in a Unicam SP 825 spectrophotometer. These assays were performed a total of three times.

Visualization of Colicin E1 Against Listeria Monocytogenes

Cellular preparation. Cultures of *L. monocytogenes* (FSIS 1126) were grown, harvested, and washed as previously described. Cells were resuspended in 9 mL TSBYE. 1mL of each culture was added to a culture tube containing 2.5, 1.0, and 0.1µg colicin E1/mL TSBYE. Culture tubes were placed at 37°C. At 1, 10, 30, and 60 minutes, samples were centrifuged at 2500 x g for 20 minutes to ensure optimal pelleting. Pelleted cells were fixed with 2% glutaraldehyde (w/v) and 0.1% paraformaldehyde (w/v) in 0.1M cacodylate for two hours at 4°C. Samples were rinsed in deionized water and repelleted via microcentrifuge. Samples were dehydrated in a graded ethanol series and infiltrated and embedded using LR White resin (Electron Microscopy Sciences, Ft. Washington, PA). Resin blocks were polymerized for 48 hours at 4°C under UV light. Thick and ultrathin sections were made using a Reichert Ultracut S ultramicrotome (Leeds Precision Instruments, Minneapolis, MN). Ultrathin sections (60–70nm) were collected onto formvar coated nickel grids and held for immunocytochemical labeling.

المنارة للاستشارات

www.manaraa.com

Transmission electron microscopy. Grids were rinsed gently with deionized water and placed in Petri dishes containing moist whatman filter paper (Grade no. 43, Whatman plc, Middlesex, UK) and Perafilm (Alcan Packaging, Neenah, WI). Grids were blocked for two hours at room temperature with PBS, pH 7.4. Grids were then incubated for two hours at room temperature with a colicin E1-antibody diluted 1:50 in PBS (pH 7.4) supplemented with 2% BSA-c and 0.01% fish gel. Grids were washed 5 x 5 minutes in PBS incubation buffer, pH 7.4. Gold labeling was carried out by incubating the grids for two hours at room temperature with goat-anti-rabbit conjugated to 10nm gold particles (Aurion ImmunoGold, Costerwegs, Netherlands) diluted 1:40 in PBS incubation buffer, pH 7.4. Grids were washed 4 x 5 minutes in PBS incubation buffer (pH 7.4) and then washed 4 x 5 minutes in deionized water. Images were captured using a JEOL 1200EX scanning and transmission electron microscope (Japan Electron Optic Laboratories, Peabody, MA) with a Megaview III digital camera (OSIS Pro Software, Olympus Soft Imaging Solutions, Lakewood, CO).

Target Protein Analysis

Biotinylation of colicin E1. Colicin E1 was biotinylated using the EZ-Link® Sulfo-NHS-SS-Biotinylation Kit (Pierce, Rockford, IL). Ten mg of Colicin E1 in 0.1mM Tris-HCL, pH 7.5 was dialyzed into filtered 100mM PBS using Slide-A-Lyzer™ dialysis tubes (Pierce, Rockford, IL) for 24 hours. A 10 mM solution of Sulfo-NHS-SS-Biotin was prepared by dissolving 2.2 mg in 360 µl ultrapure water. To reach a desired level of 1:1 incorporation, 175µL of biotin was added to each 1mL of colicin E1. The tubes were incubated on ice for two hours, and the samples were then desalted to remove excess biotin using a Zeba™ Desalt Spin Column (Pierce, Rockford, IL) and then were assayed for biotin incorporation. Level of biotin incorporation was assessed using the 4´-hydroxyazobenzene-

المنارة للاستشارات
www.manaraa.com

2-carboxylic acid (HABA)/avidin cuvette assay. Briefly, 100µL of biotinylated colicin E1 were added to 900µL of HABA/avidin solution. The absorbance (500nm) was read for the HABA/avidin solution alone, as well as the mixed cuvette containing the biotinylated colicin E1. Following kit calculations, an incorporation value of 1.5 biotin molecules/colicin E1 molecule was achieved. Biotinylated colicin fractions were visualized using western dot-blot techniques onto PVDF membranes. Membranes were incubated with streptavidin-HRP, and biotinylated colicin E1 was detected using ECL Plus™ (GE Healthcare Life Sciences, Piscataway, NJ). To evaluate the activity of the biotinylated colicin E1, *Listeria monocytogenes* cultures were prepared as previously described in fresh TSBYE and treated with either 0 (control), 1µg/mL of colicin E1, or 1µg/Ml of biotinylated colicin E1. These cultures were plated to evaluate growth on MOX agar, incubated at 37°C, and sampled at 1, 3, and 6 hours post treatment.

Cellular preparation. *L. monocytogenes* (FSIS 1126) cells were grown, harvested, and resuspended in TSBYE containing 0 or 10µg of biotinylated colicin E1. Cultures were incubated for 45 minutes at 37°C. At 45 minutes, cells were centrifuged for 30 minutes at 2500 x g, washed twice with 100mM filtered PBS, and resuspended in a lysing buffer of 1mM Tris-HCl, pH 8.2, containing 1mg/mL of lysozyme and 1% trichloracetic acid (TCA). Cells were lysed for two hours at 37°C and centrifuged for one hour at 2500 x g. Lysed cells were resuspended in 100mM PBS.

Affinity chromotography. Two 2 ml monomeric avidin columns (Immunopure™ immobilized monomeric avidin column, Pierce, Rockford, IL) were packed according to the manufacturer's instructions. Each column contained 2ml of settled gel with an immobilized monomeric avidin support coupled to a 50% slurry of 4% beaded agarose. Each column was

المنارة للاستشارات
www.manaraa.com

washed with 2 x 4 ml of PBS, and then with 3 x 2 ml of biotin blocking and elution buffer (2 mM biotin in PBS) to block any nonreversible biotin binding sites on the column. Loosely bound biotin from the reversible biotin-binding sites was removed by washing with 3 x 4 ml regeneration buffer (0.1 M glycine, pH 2.8) and then with 2 x 4 ml of PBS. The cell lysate supernatant containing biotinylated colicin E1 was added onto one of the columns drop-wise, while the control cell lysate supernatant without biotinylated colicin E1 was loaded onto the other monomeric avidin column. After all of the sample solution had been added, 0.25 ml of PBS was added to wash the sample completely into the column. The sample was allowed to incubate on the column for 1 hour at room temperature. The column was washed with 6 x 2 ml of PBS and fractions collected. The absorbance of the fractions was monitored at 280 nm until all unbound protein was washed off the column. The bound, biotinylated protein was eluted from the column with 6 x 2 ml of the biotin blocking and elution buffer and the absorbance of the collected fractions monitored at 280 nm. In order to elute bound biotinylated peptide completely, the column was washed with at least 6 x 2 ml of regeneration buffer.

Target detection. Wash and elution samples obtained from the immobilized monomeric avidin column were run on 8% SDS-PAGE mini gels (Bio-Rad, Hercules, CA). Gels containing wash or elution fractions from each column were loaded and run for three hours at 120mV. Gels were then fixed and stained according to FOCUS Fast Silver™ detection kit instructions (G-Biosciences, St Louis, MO). These assays were completed a total of three times.

Protein identification. To identity the *L. monocytogenes* proteins associated with colicin E1, duplicate silver stained mini-gels (8%) were digested using Investigator

المنارة للاستشارات
www.manaraa.com

ProGest™ (Genomic Solutions, Ann Arbor, MI), and the resulting peptides from the digestion were analyzed by Matrix Assisted Laser Desorption/Ionization Time-of-Flight (MALDI-TOF) on a ThermoBioanalysis Dynamo (TBA, Santa Fe, NM). Peptide mass and peak data were collected using Voyager™ DE Pro 5.0 software. The calculated peptide masses for each experimental sample were compared with masses in the sequence database MS-Fit (ProteinProspector v. 4.0.8., UCSF Mass Spectrometry Facility, San Francisco, CA). Ms-Fit parameters included the use of NCBInr database and analyzed sequences with a mass tolerance of 50ppm. Experimental mass values higher than 900Da were compared to the masses of the microbiological database of NCBInr. Proteins with the highest percentage of masses matched were grouped and identified for each digested protein band. Cysteine carbamidomethylation was considered as fixed modification, and oxidation of Met and pyroGlu formation of N-terminal Gln as variable modification. Identification parameters included that the protein must rank at the top two hits with at least six matched peptides, a total coverage of over 15%, consideration of protein molecular weight search (MOWSE ≥ 66).

Results

Flow Cytometry

Fluorescence of DiBAC($_4$) is directly attributable to the depolarization of bacterial membranes. *E. coli* O157:H7 cells treated or untreated with colicin E1 are depicted in Figure 2a-b. After 60 minutes of incubation at 37°C, 57% of the initial sampling population *of E. coli* O157:H7 had been depolarized by 1.0µg/mL of colicin E1. Similar results were observed in *L. monocytogenes* (Figure 3a-f). At 30 minutes of incubation, 17 and 32% of the initial *L. monocytogenes* populations were depolarized by 0.1 and 1.0µg/mL of colicin E1,

المنارة للاستشارات
www.manaraa.com

respectively. At 60 minutes, 55 % of initial *L. monocytogenes* populations were depolarized by 0.1µg/mL colicin E1, and 89% were depolarized by 1.0µg/mL. There was a ~30% increase in depolarization of *L. monocytogenes* populations compared with *E. coli* O157:H7 at 60 minutes of incubation.

Cell Permeability

Potassium leakage. Cell permeability was monitored by two different methods to supplement cytometric data. The first method was monitoring the potassium ion leakage from cell cultures treated with colicin E1. Control cultures of both *E. coli* O157:H7 and *L. monocytogenes* remained at zero for the length of the experiment. Treatment with colicin E1 at 0.1µg/mL on both strains is shown in Figure 4a. Both strains exhibited similar potassium leakage patterns over the 40 minute period. At 40 minutes, it appeared that *L. monocytogenes* had loss slightly more potassium compared to *E. coli* O157:H7. Figure 4b represents treatment of cultures with colicin E1 at 1.0µg/mL. Treatment with a 10-fold higher amount of colicin E1 had very comparable results to the 0.1µg/mL dose, with the exception that the rate of potassium loss was higher with the higher dose (Figure 4b).

Protein leakage. The second method of cellular permeability analysis was spectrophotometric measurement of protein leakage (260nm). Figure 5a-c shows the changes in protein leakage of treated and untreated bacterial cultures at 5(a), 60(b), and 120(c) minutes incubation with 0.1, 10, and 100µg/mL colicin E1. These figures show clear cellular damage due to treatment with colicin E1 at all concentrations. Only slight differences were observed between *L. monocytogenes* and *E. coli* O157:H7 cultures at 60 minutes of incubation. Data indicate similar leakage rates and volumes for each treated cell culture over the course of the experiment.

المنارة للاستشارات

www.manaraa.com

Microscopic Visualization

Transmission electron microscopy. Figure 7a portrays untreated *L. monocytogenes* cells that were subsequently detected using the colicin-E1 antibody and the gold-conjugated secondary antibody. There was virtually no nonspecific binding to these cells. Figure 6b-d were taken at various incubation times at a colicin E1 dose of 1.0μg/mL. Colicin E1 appeared to migrate from the peptidoglycan layer into the cytoplasm as the time in incubation advanced. Untreated *L. monocytogenes* control cells remained viable and intact at 60 minutes of incubation (Figure 6a). After 10 minutes of incubation with 1.0μg colicin E1/mL, cells appear intact with a high concentration of colicin E1 localizing throughout the membrane and into the cytoplasm of *L. monocytogenes* (Figure 6b). After 30 minutes of incubation, some cells in the culture appeared to have lost complete membrane integrity, whereas others were similar to those seen at 10 minutes of incubation. In the intact cell, colicin E1 appears to have fully migrated into the cytoplasm and off of the membranous region. At 60 minutes, there was complete cellular loss of integrity and death (Figure 6d). Debris surrounding the dying cell was heavily coated with colicin E1.

Target Detection

A cell lysate containing biotinylated colicin E1 and *L. monocytogenes* (4 $\log_{10}$ CFU/mL) was incubated and eluted using an Immunopure™ immobilized monomeric avidin column. Column wash fractions were collected prior to protein elution, run on an 8% SDS-PAGE mini-gel, and silver stained for protein detection. Stained gels from the collected wash fractions (columns 2–7) held virtually no detectable protein (Figure 7a). Elution fractions were similarly collected and analyzed, as shown in Figure 7b. Four clear bands were detected in lanes 2–5 of the gel, decreasing in intensity as fraction number increased

المنارة للاستشارات
www.manaraa.com

from the first elution. Band #1 had an approximate molecular weight of ~100-130 kDa. Band #2 was slightly lower on the gel and had an approximate molecular weight of 90 kDa. Band #3 was presumably the loaded colicin E1, as the approximate molecular weight was ~50-60 kDa. Colicin E1 has a molecular weight of 56 kDA. Band #4 had the lowest intensity of all of the bands and had an approximate molecular weight of ~10-15 kDA.

Protein identification. Proteins identified from MALDI-TOF digestion are presented in Table 1. Digested protein masses were compared with the NCBInr microbiological database. For each band, five proteins with highest molecular weight score (MOWSE) and sequence homology were reported. The protein from that group with the highest homology to the experimental protein data was chosen. Band #1 had over 80% homology with the experiment protein and was identified as DNA Polymerase III of *L. monocytogenes*. DNA polymerase III is involved in bacterial protein synthesis. The second band was identified as LepA, a GTP-binding protein in the cytoplasmic membrane of *L. monocytogenes*. LepA belongs to the GTPase family of proteins and is involved in protein translation. The third protein band (MW ~50-60 kDa) was identified as colicin E1 from *E. coli*. The final protein band was identified as Cell wall surface anchor family protein of *L. monocytogenes*. This protein had the lowest homology with only 71% homology to the experimental data.

Discussion

Colicins are plasmid-produced antibacterial peptides produce by *Escherichia coli* species. All colicins exhibit the same structural organization in which three domains orchestrate the functions of each colicin. The central regions of each molecule make up the receptor binding domain that forms unique structures that bind to outer membrane receptor proteins (8). The amino-terminus acts in “unknown” ways during the transport of the toxin

المنارة للاستشارات
www.manaraa.com

through the outer membrane of gram-negative bacteria (1, 4) and has been shown to interact with either the Tol protein system (group A colicins) or the TonB system (group B colicins). The third domain is the "cytotoxic" domain and functions regardless of which colicin group it belongs to. For instance, colicin E3 is a group A colicin that functions as a cytotoxic ribonuclease that specifically cleaves 16S rRNA at the ribosomal A-site to abolish protein synthesis in sensitive *Escherichia coli* cells (26). Colicin E1, the colicin used in these experiments, is also a group A colicin but functions as a pore-former. The one characteristic that all colicins are noted for is the narrow range of activity against target bacteria. The reason behind this has been hypothesized that colicins are cytotoxic only against *E. coli* and closely related bacteria because more distantly related bacteria lack a suitable receptor and/or translocation system for uptake of the colicin (5).

Colicin E1 is clearly effective against both gram-negative and gram-positive species. When treated with a membrane-potential sensitive probe (DiBAC($_4$)), *L. monocytogenes* cells were rapidly depolarized by colicin E1. *L. monocytogenes* cells were 30% more depolarized at 60 minutes of incubation compared to *E. coli* O157:H7 cultures treated with the same colicin E1 concentration (Figures 2 and 3). Supplementing the flow cytometry data are the two permeability assays conducted on both cultures. Potassium and protein leakage from each culture was similar at each time point and with each colicin E1 concentration. One reason the further extent of depolarization was not detected in the permeability assays was the detection level of the assay. Flow cytometric analysis has a much lower detection capacity compared to spectrophotmetric methods. Also, over 10,000 events were recorded on the cytometer for each bacterial sample. The spectrophotometric assays were done in triplicate.

المنارة للاستشارات
www.manaraa.com

In order to visualize membrane depolarization, transmission electron microscopy (TEM) was used. The immunocytochemical techniques enabled viewing of colicin E1 directly acting on *L. monocytogenes* cells (Figure 6). Images showed a clear movement of colicin E1 from the peptidoglycan region of the cell inward to the cytoplasm as time progressed. After 30 minutes in incubation with colicin E1, *L. monocytogenes* cellular cytoplasm was completely filled with colicin, and cells began to lyse. If colicin E1 were truly creating pores in the membrane of *L. monocytogenes*, it is unclear why colicin concentrations move into the cytoplasm. In Figures 8a-b, there are clearly defined pore-like openings in the membrane that are labeled with colicin E1. Although pore-formation did occur in these particular cells, this observation was not noted on the majority of treated cells. No similar colicin microscopy work has been documented; however, similar electron images were observed when *Listeria innocua* cultures were treated with the gram-positive bacteriocin nisin (2). In that study, cells inhibited cytoplasmic clumping, leakage of cellular material, and rupture of the cell walls and cell membranes. Nisin efficiently inactivates bacteria through a unique mechanism which includes inhibition of cell wall biosynthesis and pore formation in cytoplasmic membranes. Both mechanisms are based on interaction with the cell wall precursor lipid II which is simultaneously used as target and pore constituent (11). Colicin E1 may be acting on lipid II in *L. monocytogenes* cells in a similar manner. Hyde et al. (12) provided electron micrograph images of the gram-positive bacteria *Bacillus subtilus* with or without treatment with nisin at 5µg/mL. In that study, cells treated with nisin had clearly visible zones of cell wall separation from the cytoplasmic membrane. In the current experiment, cell wall separation was not observed in any of the electron images. If colicin is acting on lipid II (similar to nisin), this molecule presumably would have been

المنارة للاستشارات
www.manaraa.com

detected during the protein target and identification assays utilized in these experiments. Elution fractions holding tightly bound colicin E1-protein complexes were analyzed by trypsin digestion using MALDI-TOF and were identified as DNA polymerase III, the GTP-binding protein LepA, and Cell wall surface anchor family protein, all of *L. monocytogenes* (Table 1). The fourth band was identified to be colicin E1 based on molecular weight and similarity to loaded colicin E1 on silver-stained SDS-PAGE mini-gels (Figures 8a-b).

DNA polymerase III is the primary enzyme of DNA replication, which functions by adding deoxynucleotides corresponding to the newly synthesized DNA strand. LepA is also associated with protein synthesis and has recently been described as the third elongation factor required for accurate and efficient protein synthesis in bacteria. LepA has the unique function of back-translocating post-translocational ribosomes. Due to this characteristic, the function of LepA is to recognize and fix translocation errors (16, 29). DNA polymerase III is a cytoplasmic protein, whereas LepA is situated on the cytoplasmic membrane.

Colicin E1, up to this point, was thought primarily to interact with surface lipids and proteins in a toroidal organization forming a channel pore (22, 27). There has been no literature explaining any interaction of the pore-forming colicin E1 with DNA, RNA, or tRNA related enzymes. The majority of enzymatic colicins that have been described target phosphodiester bonds in the bacterial cytoplasm, eliciting cell death as either hydrolases or transferases. This varied group of enzymes target genomic DNA (DNases), 16S rRNA (rRNases), or tRNAs (tRNases). Only two nuclease colicins, E5 and D, do not hydrolyze phosphodiester bonds, but rather act as phosphotransferases (5). These data indicate that

المنارة للاستشارات

www.manaraa.com

colicin E1 interacts with several proteins in *L. monocytogenes* that are not directly associated with the mechanism of any other colicin.

The last protein identified, Cell wall anchor family protein, is involved in cell wall stabilization and attachment to the cytoplasmic membrane (13). Although this protein has not been previously identified in colicin E1 mechanism research, it seems logical that a pore-forming protein would interact with a surface protein of this nature. Given these intriguing experimental outcomes, a possibility to the mechanism of colicin would be that it accumulates in the cytoplasm of the cell through passive diffusion over the peptidoglycan layer. Once situated along the inner membrane, the cytotoxic domain of colicin E1 may form an intermediate pore. This formation would explain the interaction with cell wall anchor family protein, as well as LepA, whom are associated with the cell membrane. It is premature to fully explain the mechanism of colicin E1 on *L. monocytogenes* at this point; however, the protein identification of DNA polymerase III, as well as LepA, provide intriguing insight to a novel mechanism taken by colicin E1 against gram-positive bacteria.

Conclusions

This research indicates a novel mechanism incorporated by colicin E1 against the gram-positive pathogen *Listeria monocytogenes*. While similar membrane depolarization was observed between *L. monocytogenes* and *E. coli* O157:H7 treated with colicin E1, immunocytochemical images followed the rapid movement of colicin E1 from the cytoplasmic membrane into the cytoplasm. Structural damage to *L. monocytogenes* cells was similar to what has been observed with the bacterioicn nisin against gram-positive bacteria, indicating a possible connection between colicin E1 and the cytotoxic mechanisms employed by nisin. Bacterial proteins having affinity to colicin E1 were identified as DNA polymerase

المنارة للاستشارات
www.manaraa.com

III, LepA, and a cell wall anchor family protein by use of monomeric avidin affinity chromatography and MADLI-TOF. Colicin E1 is the first colicin to have reported affinities with these proteins, which is a pivotal step in understanding the mode-of-action this bacteriocin implements against gram-positive bacteria.

Acknowledgements

This work could have not been accomplished without the expertise of Tracey Pepper, M.S., of the Iowa State University Microscopy and Nano-Imaging Facility, as well as Shawn Rigby, PhD., of the Iowa State University Flow Cytometry and Cell Hybridoma Facility. This research was funded in part by the Biotechnology Research and Development Corporation (BRDC), the Tri-State Food Safety Consortium, and the ISU Institute for Food Safety and Security.

References

1. **Bouveret, E., A. Rigal, C. Lazdunski, and H. Benedetti**. 1998. Distinct regions of the colicin A translocation domain are involved in the interaction with TolA and TolB proteins upon import into *Escherichia coli*. Mol. Microbiol. **27**:143–157.

2. **Calderón-Miranda, M. L., G. V. Barbosa-Cánovas, and B. G. Swanson**. 1999. Inactivation of Listeria innocua in skim milk by pulsed electric fields and nisin. Int J Food Microbiol. **51**:19–30.

3. **Cao, Z., and P. E. Klebba**. 2002. Mechanisms of colicin binding and transport through outer membrane porins. Biochimie**. 84**:399–412.

المنارة للاستشارات

www.manaraa.com

4. **Carr, S., C. N. Penfold, V. Bamford, R. James, A. M. Hemmings, J. K. Davies, and P. Reeves.** 2000. The structure of TolB, an essential component of the tol-dependent translocation system, and its protein–protein interaction with the translocation domain of colicin E9. Structure. **8**:57–66.

5. **Cascales, E., S. K. Buchanan, D. Duché, C. Kleanthous, R. Lloubès, K. Postle, M. Riley, S. Slatin, and D. Cavard**. 2007. Colicin biology. Microbiol. Mol. Biol. Rev. **71**:158–229.

6. **Cleveland, J., T. J. Montville, I. F. Nes, and M. L. Chikindas**. 2001. Bacteriocins: safe, natural antimicrobials for food preservation. Int. J. Food Microbiol. **71**:1–20.

7. **Cotter, P. D., C. Hill, and R. P. Ross**. 2005. Bacteriocins: developing innate immunity for food. Nat. Rev. Microbiol. **3**:777–788.

8. **Di Masi, D. R., J. C. White, C. A. Schnaitm, and C. Bradbeer**. 1973. Transport of vitamin B12 in Escherichi coli: common receptor sites for vitamin B12 and E. colicins on outer membrane of cell envelope. J. Bacteriol. **115**:506–513.

9. **Ennahar, S., K. Sonomoto, and A. Ishizaki**. 1999. Class IIa bacteriocins from lactic acid bacteria: antibacterial activity and food preservation. J. Biosci. Bioeng**. 87**:705–716.

10**. Gould, J. M. and W. A. Cramer.** 1977. Studies on depolarization of *Escherichia coli* cell-membrane by colicin E1. J. Biol. Chem. **252**:5491–5497.

11. **Hasper, H. E., N. E. Kramer, J. L. Smith, J. D. Hillman, C. Zachariah, O. P. Kuipers, B. de Kruijff, and E. Breukink**. 2006. An alternative bactericidal mechanism of action for lantibiotic peptides that target lipid II. Science. **313**:1636–1637.

المنارة للاستشارات

www.manaraa.com

12. **Hyde, A. J., J. Parisot, A. McNichol, and B. B. Bonev**. 2006. Nisin-induced changes in Bacillus morphology suggest a paradigm of antibiotic action. Proc. Natl. Acad. Sci. **103**:19896–19901.

13. **Nelson, K. E., D. E. Fouts, E. F. Monogodin, J. Ravel, R. T. DeBoy, J. F. Kolonay, D. A. Rasko, S. V. Angiuoli, S. R. Gill, I. T. Paulsen, J. Peterson, O. White, W. C. Nelson, W. Nierman, M. J. Beanan, L. M. Brinkac, S. Daugherty, R. J. Dodson, A. S. Durkin, R. Madupu, D. H. Haft, J. Selengut, S. Van Aken, H. Khouri, N. Federova, H. Forberger, B. Tran, S. Kathariou, L. D. Wonderling, G. A. Uhlich, D. O. Boyles, J. B. Luchansky, and C. M. Fraser.** 2004. Whole genome comparisons of serotype 4b and 1/2a strains of the food-borne pathogen *Listeria monocytogenes* reveal new insights into the core genome components of this species. Nucleic Acids Res. **32**:2386–2395.

14. **Orlov, D. S., T. Nguyen, and R. I. Lehrer**. 2002. Potassium release, a useful tool for studying antimicrobial peptides. J. Microbiol. Methods. **49**:325–328.

15. **Patton, B. S., J. S. Dickson, S. M. Lonergan, S. A.Cutler, and C. H. Stahl**. 2007. Inhibitory activity of colicin E1 against *Listeria monocytogenes*. J Food Prot. **70**:1256–1262.

16. **Qin, Y., N. Polacek, O. Vesper, E. Staub, E. Einfeldt, D. N. Wilson, and K. H. Nierhaus**. 2006. The highly conserved LepA is a ribosomal elongation factor that back-translocates the ribosome. Cell. **127**:721–733.

17**. Riley, M. A., and D. M. Gordon**. 1996. The ecology and evolution of bacteriocins. J. Ind. Microbiol. **17**:151–158.

18**. Schamberger, G. P., R. L. Phillips, J. L. Jacobs, and F. Diez-Gonzalez.** 2004. Reduction of *Escherichia coli* O157:H7 populations in cattle by addition of colicin E7-producing *E. coli* to feed. Appl. Environ. Microbiol. **70**:6053–6060.

المنارة للاستشارات
www.manaraa.com

19. **Schendel, S. L., E. M. Click, R. E. Webster, and W. A. Cramer**. 1997. The TolA protein interacts with colicin E1 differently than with other group A colicins. J. Bacteriol. **179**:3683–3690.

20. **Shin, Y. K., C. Levinthal, F. Levinthal, and W. L. Hubbell**. 1993. Colicin E1 binding to membranes—Time resolved studies of spin-labeled mutants. Science. **259**:960–963.

21**. Smarda, J., and L. Macholan**. 2000. Binding domains of colicins E1, E2 and E3 in the receptor protein BtuB of *Escherichia coli*. Folia Microbiol. **45**:379–385.

22**. Sobko, A. A., E. A. Kotova, Y. N. Antonenko, S. D. Zakharov, and W. A. Cramer**. 2006. Lipid dependence of the channel properties of a colicin E1-lipid toroidal pore. J Biol Chem. **281**:14408–14416.

23. **Stahl, C. H., T. R. Callaway, L. M. Lincoln, S. M. Lonergan, and K. J.Genovese**. 2004. Inhibitory activities of colicins against *Escherichia coli* strains responsible for postweaning diarrhea and edema disease in swine. Antimicrob Agents Chemother. **48**:3119–3121.

24. **Tilley, S. J, and H. R. Saibil**. 2006. The mechanism of pore formation by bacterial toxins. Curr. Opin. Struct. Biol. **16**:230–236.

25. **Trautner, B. W., R. A. Hull, and R. O. Darouiche**. 2005. Colicins prevent colonization of urinary catheters**.** J. Antimicrob. Chemother. **56**:413–415.

26. **Walker, D., L. Lancaster, R. James, and C. Kleanthous**. 2004. Identification of the catalytic motif of the microbial ribosome inactivating cytotoxin colicin E3. Protein Sci. **13**:1603–1611.

المنارة للاستشارات

www.manaraa.com

27. **White, D., A. A. Musse, J. Wange, E. London, and A. R. Merrill**. 2006. Toward elucidating the membrane topology of helix two of the colicin E1 channel domain. J. Biol. Chem. **281**:32375–32384.

28. **Wickens, H. J., R. J. Pinney, D. J. Mason, and V. A. Gant.** 2000. Flow cytometric investigation of filamentation, membrane patency, and membrane potential in *Escherichia coli* following ciprofloxacin exposure. Antimicrob. Agents Chemother. 2000. **44**:682–687.

29**. Youngman, E. M, and R. Green**. 2007. Ribosomal translocation: LepA does it backwards. Curr. Biol. **17**:R136–R139.

Figure Legends

Figure 1. Biotinylation of colicin E1. Colicin E1 concentrations were tested for successful biotinylation by western dot-blotting (1A). Colicin E1 concentrations of 5, 10, and 20µg/mL were added on left hand side on PVDF membrane. Biotinylated colicin E1 concentrations of equal concentrations were added on the right-and side. For reference, a 1:2000 dilution of biotin was placed in-between both sides on a drawn-line. Dots from the biotnylated colicin E1 were clearly detected using ECL plus™. Figure 1B shows growth and inhibition of *L. monocytogenes* over a 6 hour period. ■ = 0, Control; 0; ● = 1µg/mL colicin E1; ○ = 1µg/mL biotinylated colicin E1.

Figure 2. Colicin E1 activity against *Escherichia coli* O157:H7. Flow cytometric analysis of *E. coli* O157:H7 treated with colicin E1. Cell integrity histograms show a: untreated *E. coli* O157:H7 samples at 60 minutes of incubation at 37°C. b: *E. coli* O157:H7 samples treated with 1.0µg/mL colicin E1.

المنارة للاستشارات

www.manaraa.com

Figure 3. Colicin E1 activity against *Listeria monocytogenes*. Flow cytometric analysis of *L. monocytogenes* treated with colicin E1 using the membrane-potential sensitive probe DiBAC($_4$). a: Untreated *L. monocytogenes* at 30 minutes of incubation at 37°C. b: *L. monocytogenes* samples treated with 0.1µg/mL colicin E1, 30min. c: *L. monocytogenes* samples treated with 1.0µg/mL colicin E1, 30min. d: Untreated *L. monocytogenes* at 60 minutes of incubation at 37°C. e: *L. monocytogenes* samples treated with 0.1µg/mL colicin E1, 60min. f: *L. monocytogenes* samples treated with 1.0µg/mL colicin E1, 60min. Percentage of depolarized sample population is indicated above histogram (%).

Figure 4. Potassium leakage induced by colicin E1. *L. monocytogenes* and *E. coli* O157:H7 cultures were treated with 0 (control) 0.1 (a) or 1.0µg/mL (b) colicin E1, and potassium leakage was measured using a pH-meter pH 213 with a MI-442 potassium electrode and MI-409F reference electrode. Calculations of potassium-efflux in percent as % release = $([K^+]_{meas} - [K^+]_{init} / [K^+]_{total}) - [K^+]_{init} \times 100$. ■ = *L. monocytogenes* (FSIS 1126); □ = *E. coli* O157:H7 strain 933; X = Untreated *L. monocytogenes* (FSIS 1126); Δ = Untreated *E. coli* O157:H7.

Figure 5. Protein leakage induced by colicin E1. *L. monocytogenes* and *E. coli* O157:H7 were treated with either 0, 0.1 or 1.0µg/mL of colicin E1, and protein leakage was measured spectrophotometrically at 260nm at various time points up to 120 minutes of incubation at 37°C. 5a represents protein

المنارة للاستشارات
www.manaraa.com

leakage at five minutes of incubation, 5b at 60 minutes, and 5c at 120 minutes. ■ = *L. monocytogenes* (FSIS 1126); □ = *E. coli* O157:H7 strain 933; Δ = Untreated *L. monocytogenes* (FSIS 1126).

Figure 6. Colicin E1-treated *L. monocytogenes* cells over 60-min period. Transmission electron microscopic images validate the effects of colicin E1 against the gram-positive pathogen. Untreated *L. monocytogenes* cells appear healthy at 60 minutes of incubation at 37°C. Cells treated with 1.0µg/mL of colicin E1 are shown at 10 minutes (b), 30 minutes (c) and 60 minutes (d) of incubation.

Figure 7. *L. monocytogenes* proteins with affinity to colicin E1. Biotinylated colicin E1 was combined with *L. monocytogenes* cultures (4 $\log_{10}$ CFU/mL), incubated, and eluted using an Immunpure™ immobilized monomeric avidin column. Column wash fractions (8a) were loaded on an 8% SDS-PAGE mini-gel (lanes 2-7), as well as a load sample of biotinylated colicin E1 (lane 1) and a molecular weight standard (8), and detected using silver staining. Column elution fractions (8b) were loaded and ran in similar manner (lanes 2-8) with a molecular weight standard (lane 1). Four visible protein bands were detected as indicated by arrows.

Figure 8. Pore-formation by colicin E1 in *Listeria monocytogenes*. Cell walls of *L. monocytogenes* with large openings enveloped with gold-labeled colicin E1. Arrows indicate areas of pore-formation.

المنارة للاستشارات
www.manaraa.com

Table 1. Identification of elution fraction protein fragments associated with colicin E1.

Band Number	Identification	Species	MOWSE[a]	PC[b]	SC%[c]	Accession Number[d]
1	DNA Polymerase III, alpha subunit	*Listeria monocytogenes*	171	20/24	34	AAT 04371
2	LepA, GTP-binding protein	*Listeria monocytogenes*	115	15/20	29	CAC99557
3	Colicin E1	*Escherichia coli*	98	22/23	18	AAY68489
4	Cell wall surface anchor family protein	*Listeria monocytogenes*	113	12/17	32	AAT04978

[a] MOWSE score: statistical probability of true positive identification of predicted proteins calculated by MS-fit with 50 ppm masses tolerance and one allowed missed cleavage (MOWSE score $\geq$ 66).

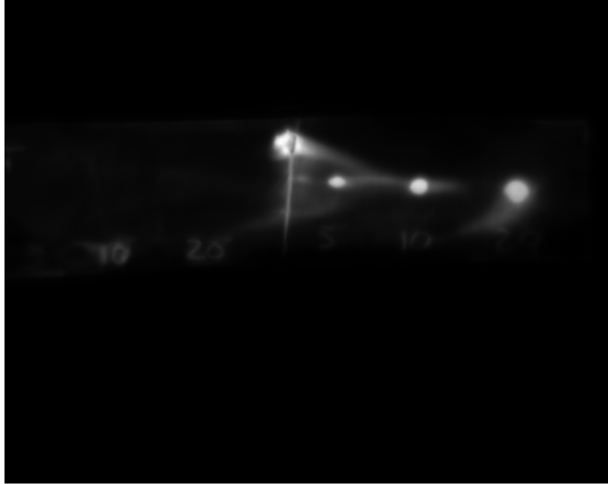
[b] PC: number of peptides matching predicted protein sequences.

[c] SC%: percentage of predicted protein sequence covered by matched sequences.

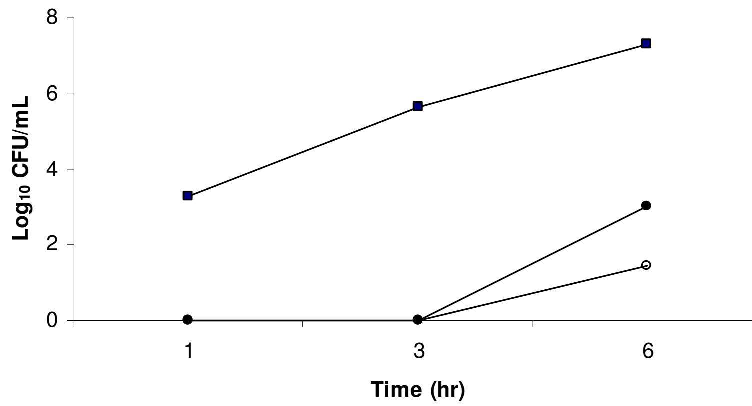
[d] Accession no.: the identification of predicted protein in NCBInr.

المنارة للاستشارات

www.manaraa.com



A



B

Figure 1. Biotinylation of colicin E1.

المنارة للاستشارات
www.manaraa.com

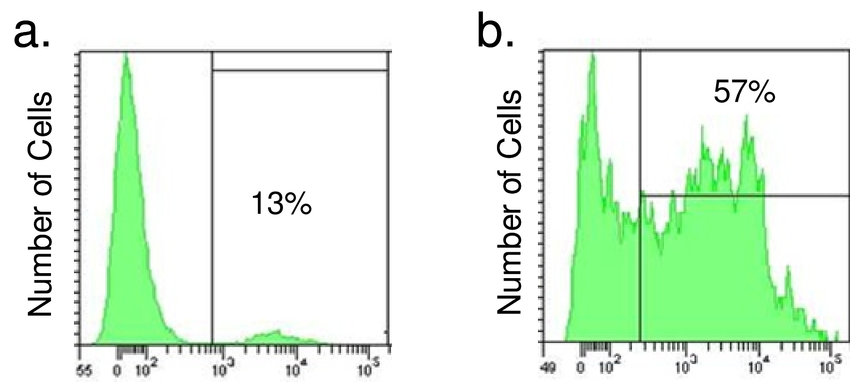


Figure 2. Colicin E1 activity against *Escherichia coli* O157:H7.

www.manaraa.com

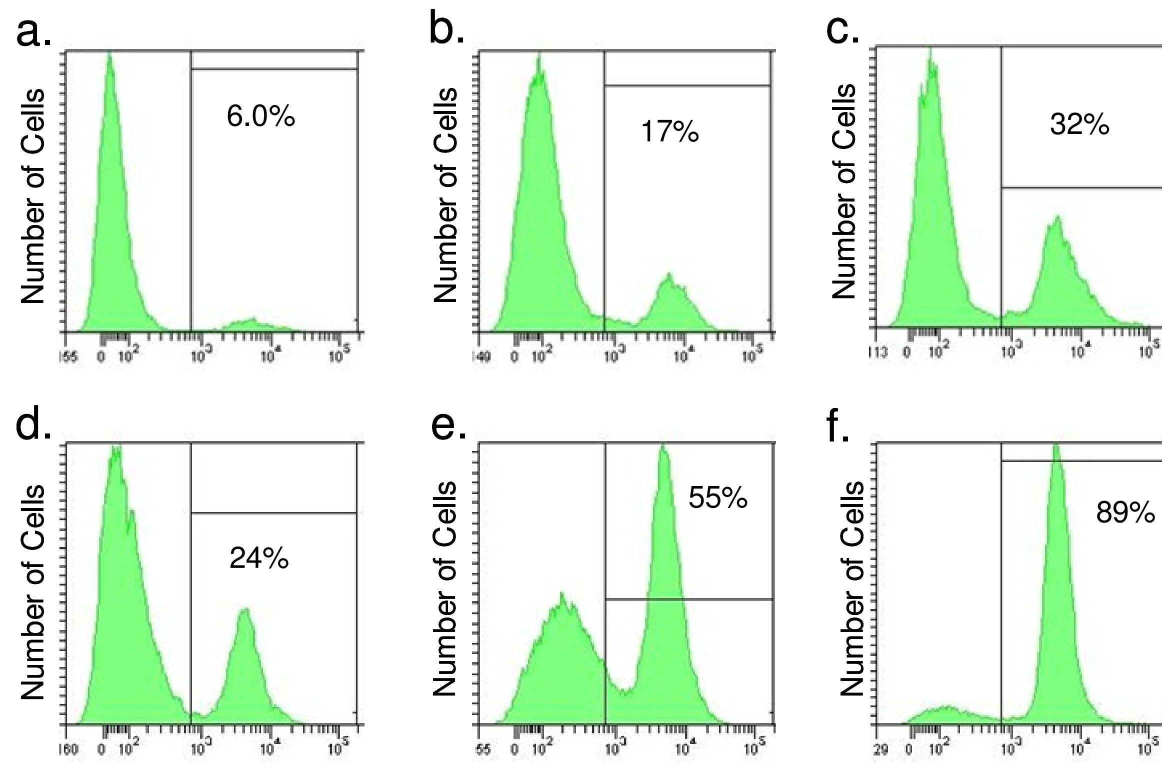
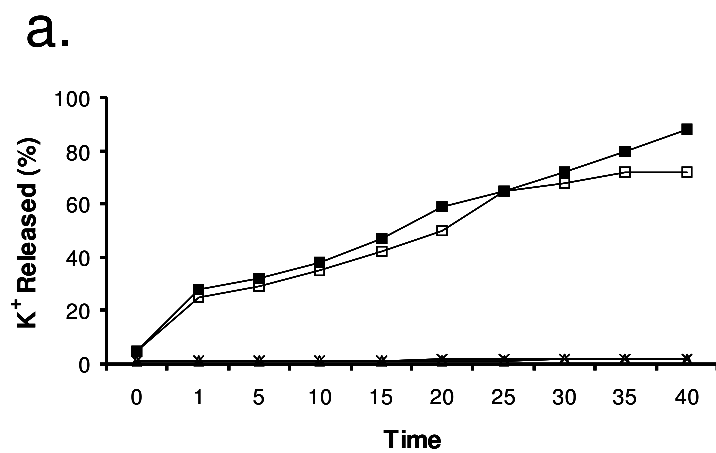


Figure 3. Colicin E1 activity against *Listeria monocytogenes*.

المنارة للاستشارات
www.manaraa.com

a.



b.

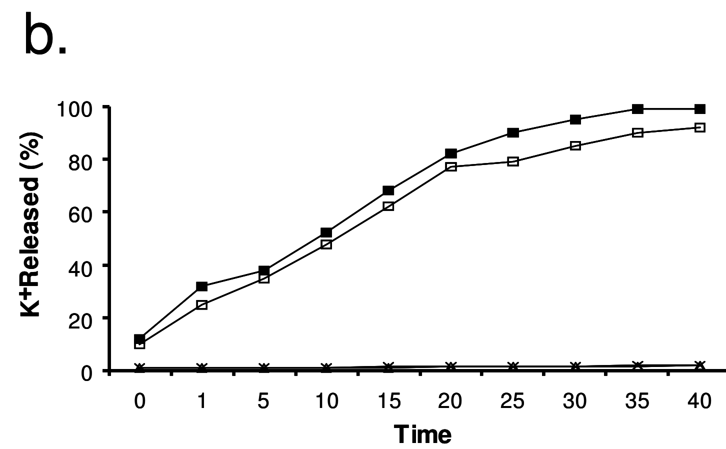
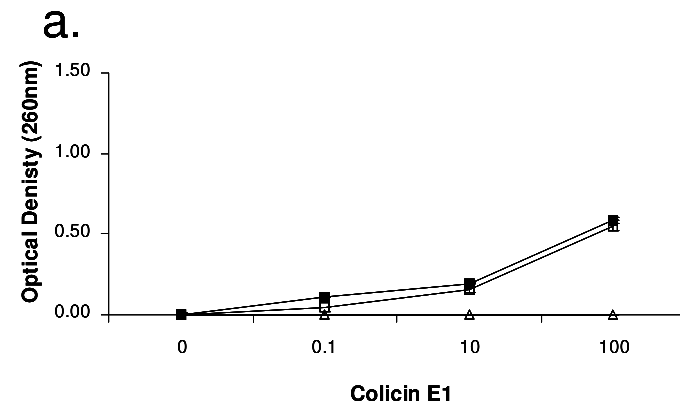
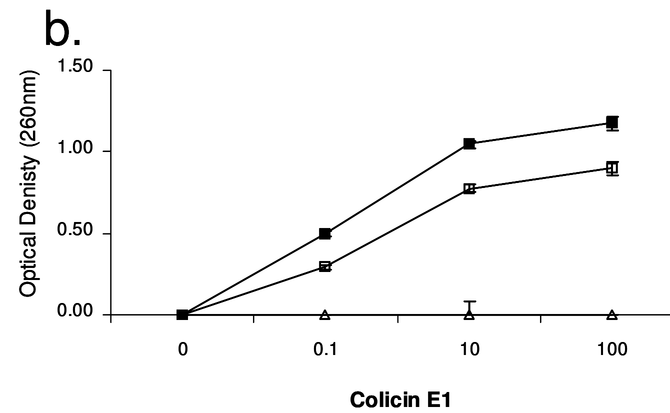


Figure 4. Potassium leakage induced by colicin E1.

المنارة للاستشارات

www.manaraa.com





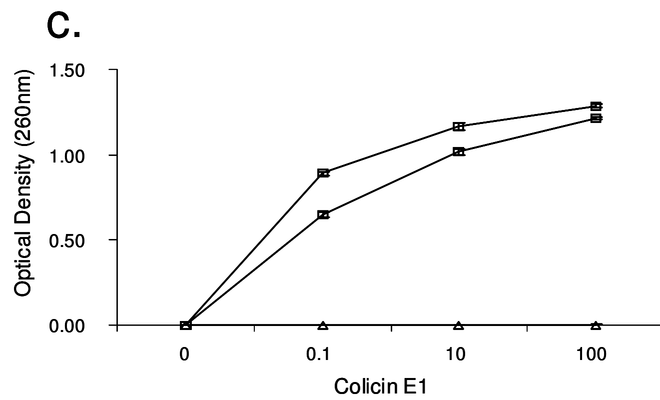


Figure 5. Protein leakage induced by colicin E1.

www.manaraa.com

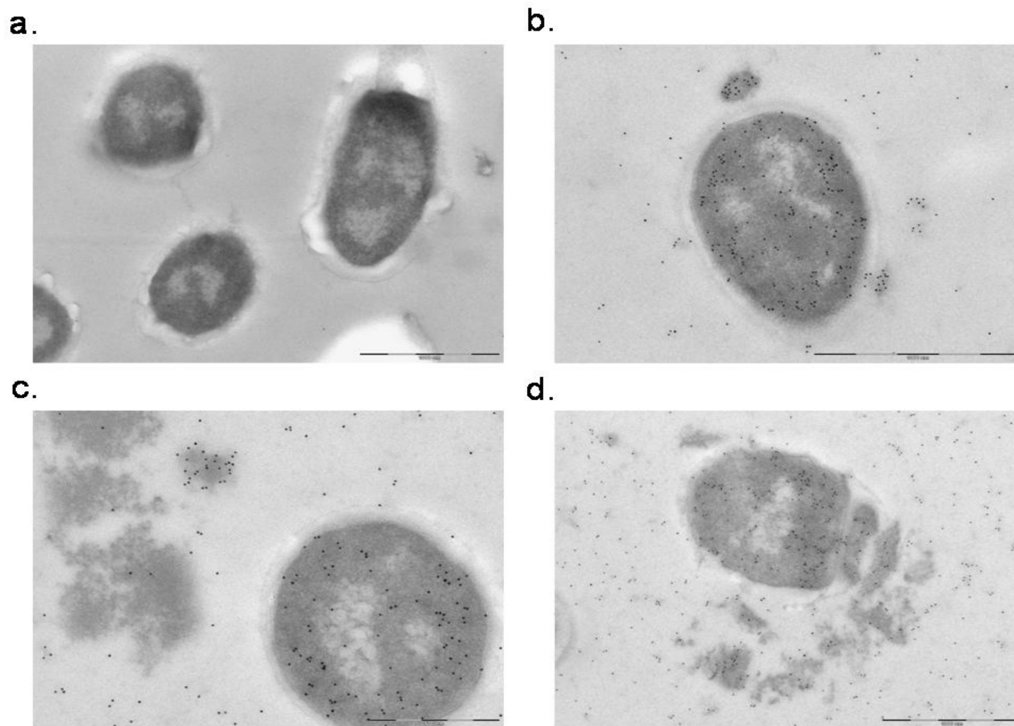


Figure 6. Colicin E1-treated *L. monocytogenes* cells over 60-min period.

www.manaraa.com

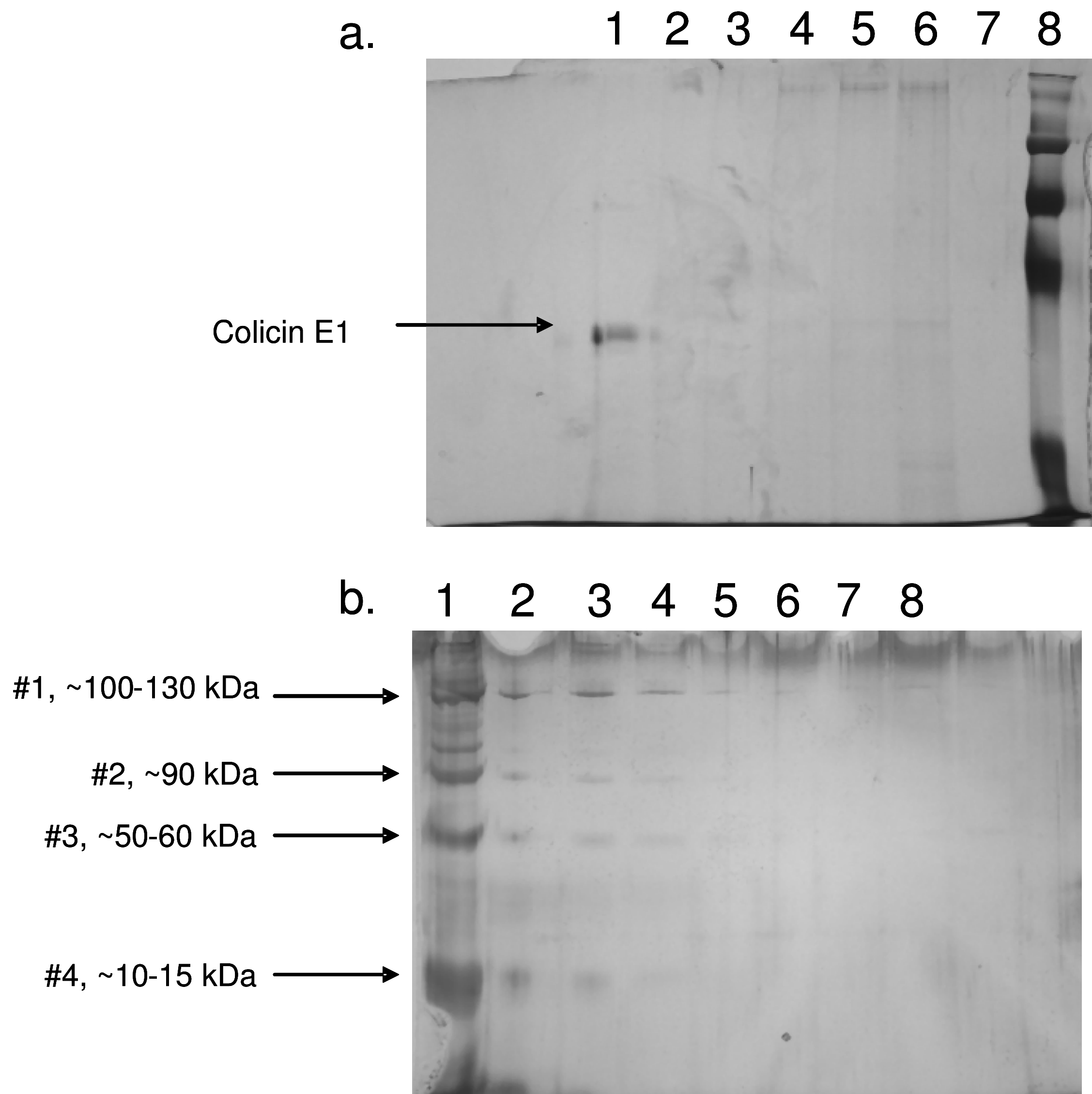


Figure 7. *L. monocytogenes* proteins with affinity to colicin E1.

www.manaraa.com

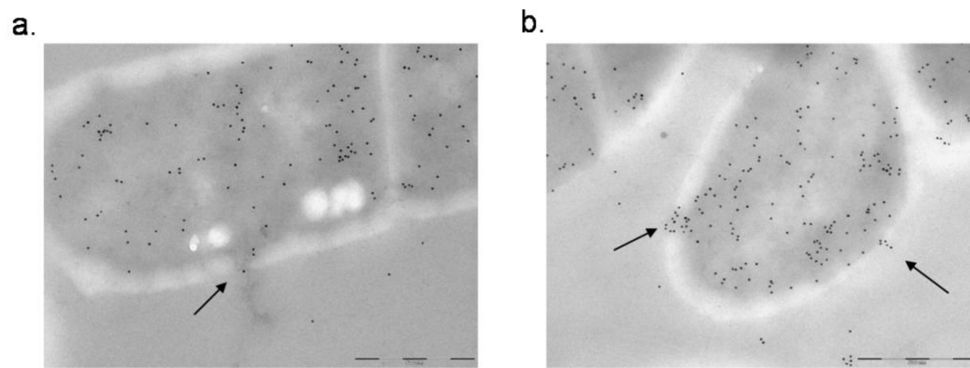


Figure 8. Pore-formation by colicin E1 in *Listeria monocytogenes*.

www.manaraa.com

CHAPTER 5. GENERAL CONCLUSIONS

General Discussion

Antimicrobial intervention strategies are rapidly being optimized to replace previous antibiotic strategies. Concerns surrounding antibiotic resistance and overall bacterial fitness strategies are driving these efforts towards natural, non-synthetic compounds with little to no toxicity towards animals and humans. Controlling bacterial contamination in livestock production and transport, animal slaughter and meat processing facilities, as well as dips, sanitizers, and food packaging systems is imperative in preventing catastrophic outbreaks of pathogenic microorganisms. An intervention strategy, as noted in this dissertation, is the use of the bacteriocins colicin E1, produced by *Escherichia coli*. In a 98% pure form, concentrations as low as 0.1µg/mL of colicin E1 was able to inhibit growth of *L. monocytogenes* for a duration of 6 hours at 37°C. Colicin E1 appears to be more effective against the gram-positive *L. monocytogenes* compared to *E. coli*, which was observed using flow cytometry, potassium and nucleic acid leakage assays, as well as broth culture inhibition studies. Colicin E1 appears to be acting upon these gram-positive pathogens in a novel mechanism compared to the well orchestrated and documented pore-formation against *E. coli* (1, 2). Recovered proteins with high affinity to colicin E1 using an immobilized monomeric avidin column and biotinylated colicin E1 included DNA polymerase III, LepA, Cell wall anchor family protein of *L. monocytogenes*. No other colicin has been reported to interact with these proteins, suggesting that colicin E1 is acting atypically on *L. monocytogenes*. While pore-formation did occur in treated few *L. monocytogenes* cells, colicin E1 appeared to localized in the cytoplasm of infected cells until lysis. It is obvious that this bacteriocins

المنارة للاستشارات
www.manaraa.com

is effective at inhibiting pathogenic bacteria, however, the mechanism by which this bacteriocins is causing the fate of gram-positive bacteria remains unknown.

Recommendations for Future Research

We have successfully biotinylated the colicin E1 molecule without impacting its antimicrobial activity. Also, a polyclonal antibody was produced in rabbits that showed little to no non-specific binding when used in various immunological assays. Using this antibody could be used in certain activity assays of colicin E1 on purified DNA polymerase III-mutant bacteria. Staining and localization methods of these proteins within bacterial cells to negotiate binding within cell culture to colicin E1 would also be beneficial. Also, if direct activity against these enzymes was noted, colicin-E1 could be prepared and purified to over-express factors capable of recognizing these enzymes, making the colicins more highly active against *L. monocytogenes*. Lastly, immunoprecipitation with membrane proteins of *L. monocytogenes* might give an insight into other protein systems or localization of the interaction that colicin E1 has with these proteins in terms of cellular constituents, including the cell membrane and peptidoglycan later. Other possible experiments could be to test nuclease activity on L. monocytogenes RNA or DNA-containing agar. This would allow us to evaluate if colicin E1 did indeed have nuclease activity against *L. monocytogenes* at all. Also, truncating the colicin E1 molecule to the c-terminus, and determining the location of import along the inner membrane.

In more applied terms, to evaluate the efficacy and possibility of this bacteriocin to be used as a food bio-preservative, a more thorough characterization of shelf-life extension made possible by colicin E1 treatment needs to be conducted. An accurate dose-response

المنارة للاستشارات
www.manaraa.com

relationship and subsequent impact on surviving cells would be an important step in determining the amount required to provide static or toxic control in an industrial setting. Lastly, *L. monocytogenes* is the only gram-positive organism known to contain trace quantities of LPS. It may be beneficial to denote affinity of colicin E1 to *L. monocytogenes* LPS.

In conclusion, we have only begun to understand colicins themselves; their actions against their common host, Escherichia coli, and how differing colicins employ opposing strategies upon reaching and infiltrating a target cell. Now, we are faced with further knowledge that these bacteriocins indeed have a broader spectrum of activity than originally anticipated, and interestingly, a novel mode-of-action to initiate this activity. Given this first chapter of exploratory research, much more work needs to conducted to examine the possibilities of colicins, both in nature, and applied uses to benefit people and animals.

References

1. Cao, Z., and P. E. Klebba. 2002. Mechanisms of colicin binding and transport through outer membrane porins. *Biochimie*. 84:399–412.

2. Cascales, E., S. K. Buchanan, D. Duche, C. Kleanthous, R. Lloubes, K. Postle, M. Riley, S. Slatin, and D. Cavard. 2007. Colicin biology. *Microbiol. Mol. Biol. Rev*. 71:158–229.

المنارة للاستشارات

www.manaraa.com

ACKNOWLEDGEMENTS

I would like to start by thanking my major professor, Dr. James Dickson, for giving me a chance and letting me "steer my own ship" throughout my graduate program at Iowa State. I would also like to thank and recognize Dr. Byron Brehm-Stecher for not getting too cloudy or irritated over my rambling conversations of antimicrobial peptides and how I planned to take on the world, one point mutation at a time. Also, I would like to thank Dr. Aubrey Mendonca, Dr. Terri Boylston, and Dr. Nancy Cornick for serving patiently on my committee.

This work would have been a twinkle in my eye if it hadn't been for the diligence, patience, and determination of Dr. Chad H. Stahl. Thank you for being an irreplaceable link in the foundation of this research, as well as in my life. Also, much gratitude and thankfulness goes to Dr. Steven M. Lonergan, who was a friend, confidant, and colicin enthusiast with the rest of us "microbiologists."

Lastly, I would like to thank Dr. Manpreet Singh for being a close personal friend and the only person to whom I could complain about my lack of understanding in pore-formation, colicin kinetics, and the ways of the world.

للاستشارات المنارة
www.manaraa.com