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Use Of Genetically Modified *Carnobacterium piscicola* To Control *Escherichia coli* O157:H7 In Vacuum-Packaged Ground Beef

by

Douglas John Watters

A THESIS

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ABSTRACT

The genetically modified colicin V-producing *Carnobacterium piscicola* UAL26 pCV22 was evaluated for its ability to inhibit the growth and potentially kill *E. coli* O157:H7 in broth and meat systems. The pCV22 plasmid was stable in *C. piscicola* UAL26 and colicin V was produced during exponential growth phase. *C. piscicola* UAL26 pCV22 was bacteriocidal against *E. coli* in a broth system; however, it was bacteriostatic in a ground beef system. The results of this study have shown that *C. piscicola* UAL26 pCV22 possesses characteristics suitable as a prototype for controlling pathogens in meat systems.

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LIST OF ABBREVIATIONS

ABC	-ATP-binding cassette
ADP	-adenosine diphosphate
Amp^r	-ampicillin resistant
APT	-All Purpose Tween
APT-Cm	-APT containing chloramphenicol
APT-Em	-APT containing erythromycin
ATP	-adenosine triphosphate
AU	-arbitrary activity unit
BHI	-Brain Heart Infusion
CFU	-colony forming unit
Cm	-chloramphenicol
CMM	-Cooked Meat Medium
Cm^r	-chloramphenicol resistant
CO_2	-carbon dioxide
EDTA	-ethylenediaminetetraacetic acid
EHEC	-enterohemorrhagic *E. coli*
Em	-erythromycin
Em^r	-erythromycin resistant
H_2O_2	-hydrogen peroxide
HC	-hemorrhagic colitis
HCl	-hydrochloric acid
HUS	-hemolytic uremic syndrome
KOH	-potassium hydroxide
LAB	-lactic acid bacteria
MALDI-TOF MS	-matrix assisted laser desorption/ionization time-of-flight mass spectrometry
MAP	-modified atmosphere packaging
MRS	-Lactobacilli MRS (de Man, Rogosa and Sharp)
NA	-Nutrient Agar

NaOH	-sodium hydroxide
NB	-Nutrient Broth
O_2	-oxygen
PBS	-Phosphate Buffered Saline
PCA	-Plate Count Agar
PMF	-proton motive force
SDS-PAGE	-sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SLTEC	-shiga-like toxin producing *E. coli*
Sm^r	-streptomycin resistant
STEC	-shigatoxin producing *E. coli*
TCA	-trichloroacetic acid
TFA	-trifluoroacetic acid
TSB	-Tryptic Soy Broth
TTP	-thrombotic thromocytopenic purpura
VP	-vacuum-packaging
VTEC	-verotoxin-producing *E. coli*

1. INTRODUCTION

The microbial safety of fresh meats is of increasing concern to consumers and the food industry. In spite of good sanitation practices, spoilage and pathogenic microorganisms will be inadvertently introduced onto the surface of meat and into ground meat during processing. A number of foodborne illness outbreaks have been associated with the consumption of undercooked ground beef contaminated with *Escherichia coli* O157:H7. Infection with *E. coli* O157:H7 is serious because of its low infective dose and the severe consequences of infection (Buchanan and Doyle, 1997). To prevent foodborne illness it is important to control pathogenic microorganisms in the food product. Presently the primary method for control of contaminating bacteria on fresh meat is refrigeration and cooking.

Lactic acid bacteria (LAB) have been used extensively as starter cultures in the manufacture of fermented dairy and meat products. LAB contribute to both the sensory quality and the preservation and safety of fermented foods by the production of metabolic substances, primarily lactic and acetic acids and bacteriocins. Bacteriocins are proteinaceous antibacterial substances that are generally active against closely related bacteria (Klaenhammer, 1993). Bacteriocins or the organisms that produce them in food products has the potential for food preservation (Stiles, 1996). There are three different ways in which bacteriocins can be used in food: the addition of purified bacteriocins such as nisin, the addition of a concentrated fermentation liquor containing bacteriocins such as Microgard® (Wesman Foods Inc., USA) and ALTA™2341 (Quest International, Netherlands), or the use of a starter culture, which produces bacteriocin(s) *in situ* such as *Pediococcus acidilactici* in

fermented sausage (Stiles, 1996).

Over the past 20 years there has been a great deal of interest in bacteriocins produced by LAB and their potential for application in food systems. Research has focused on the purification and characterization of bacteriocins and the elucidation of the genetic determinants responsible for bacteriocin production (McMullen and Stiles, 1996). Studies involving the application of a genetically modified LAB to control *E. coli* or other gram-negative organisms in a meat system have not been reported. A focus of the research group at the University of Alberta is to purify and characterize bacteriocins from LAB with the intent of developing strategies for increasing their antibacterial spectrum through site-directed mutagenesis or the production of multiple bacteriocins within a gene cassette. Another research goal is to genetically engineer LAB to specifically reduce spoilage and/or improve safety of vacuum-packaged, chill-stored meats. This study, evaluates the effectiveness of a genetically modified LAB designed to heterologously produce bacteriocins to control the growth of *E. coli* O157:H7 in vacuum-packaged, chill-stored meats.

2. LITERATURE REVIEW

2.1. *Escherichia coli* O157:H7 – A Concern for Food Safety

E. coli serotype O157:H7 is a gram-negative, mesophilic, facultatively anaerobic, non-sporeforming short rod, which does not ferment sorbitol. *E. coli* O157:H7 was first reported as a human pathogen by Johnson *et al.* (1983) when it was implicated in an outbreak of hemorrhagic colitis at a Canadian institution for elderly patients in November, 1982. Riley *et al.* (1983), also reported E. coli O157:H7 when it was implicated in two outbreaks of hemorrhagic colitis, which were linked to a fast food restaurant. *E. coli* O157:H7 infections are generally classified as enterohemorrhagic *E. coli* (EHEC). EHEC is a subgroup of verocytotoxigenic *E. coli* (VTEC) also called verotoxin producing *E. coli*. VTECs produce verotoxins, or shiga-like toxins that are similar to toxins produced by *Shigella dysenteriae* (Chart, 2000). Shiga-like toxin producing *E. coli* (SLTEC) and shiga toxin producing *E. coli* (STEC) are also commonly used designations for these organisms. Even though *E. coli* O157:H7 is the most renowned SLTEC, there are over 200 different types of SLTEC that have been found in food and animals, of which 60 serotypes have been linked with human disease (Acheson, 2000).

Individuals infected with this class of pathogen develop hemorrhagic colitis and may display the following clinical symptoms: severe cramps, abdominal pain, grossly bloody diarrhea, vomiting and little or no fever. In 5 to 10 percent of EHEC infections a more serious secondary condition called hemolytic uremic syndrome (HUS) develops. HUS occurs mostly in children and may result in acute renal failure and death. Further complications may result in thrombotic thrombocytopenic purpura

(TTP), which is similar to HUS but more typical in older individuals and involves fever and nervous system disorder (Dundas and Todd, 2000). Serious complications from EHEC infections most often occur in the very young, the elderly, and immunocompromised individuals.

Based on the low number of *E. coli* O157:H7 found in food linked to outbreaks, it was established that the infective dose of this organism is very low. It was estimated to be less than 50 cells (Armstrong *et al.*, 1996). An investigation of an outbreak of *E. coli* O157:H7 linked to dry salami also established the infective dose to be about 50 organisms, and some of the cases may have resulted from the consumption of only 5 organisms (Tilden and Young, 1995). In an investigation by Davis *et al.* (1993) beef burger patties implicated in a major outbreak had contamination levels ranging from 1 to 15 organisms per gram. A single raw patty would have contained 40 to 600 organisms and the number remaining after cooking would have been very low.

The primary reservoir for *E. coli* O157:H7 is cattle, particularly calves (Chapman, 2000). Other animals that can be transient carriers of *E. coli* O157:H7 include deer, dogs, sheep, poultry, horses and swine. Feces from these animals and humans may contaminate food or water and pose a risk of causing disease in humans. A food source commonly implicated in *E. coli* O157:H7 outbreaks is undercooked ground beef. Meat becomes contaminated at the time of slaughter and grinding mixes the pathogen into the interior of the meat, where it is more likely to survive cooking. Doyle and Schoeni (1987) studied the prevalence of *E. coli* O157:H7 in different meat types and found 1 of 147 (0.7%) beef samples, 3 of 250 (1.2%) pork samples, 4

of 257 (1.6%) poultry samples and 4 of 205 (2.0%) lamb samples obtained from retail outlets in Madison, Wisconsin to be contaminated with *E. coli* O157:H7. Since this study, other surveys of retail ground beef have found similar results to those in Madison. A survey in Manitoba in 1989 found 4 of 165 (2.4%) ground beef samples were positive for *E. coli* O157:H7 (Sekla *et al.*, 1990). In two other surveys in Wisconsin in 1990 and 1991, 3 of 107 (2.8%) (Padhye and Doyle, 1991) and 1 of 76 (1.3%) (Kim and Doyle, 1992) ground beef samples were positive for *E. coli* O157:H7. In contrast, a number of studies failed to find *E. coli* O157:H7 in ground beef (Read *et al.*, 1990; Willshaw *et al.*, 1993; Samadpour *et al.*, 1994). Armstrong *et al.* (1996) speculated that the difference in the prevalence seen in different studies is an artifact of the sensitivity of the method used to detect *E. coli* O157:H7.

Enteric pathogens must survive the acidity of the stomach prior to reaching the intestine and cause illness. Therefore, bacterial responses to an acidic environment are important in the pathogenicity of *E. coli* O157:H7 (Tsai and Ingham, 1997). A number of acidic foods such as unpasteurized apple cider (Besser *et al.*, 1993), mayonnaise (Weagant *et al.*, 1994), and yogurt (Morgan *et al.*, 1993) have also been implicated in outbreaks. Glass *et al.* (1992) reported that *E. coli* O157:H7 can grow in the pH range of 4.5 to 9.0, with optimum growth at pH 7.0 and can survive extended storage at low pH. Zhao *et al.* (1993) reported that *E. coli* O157:H7 survived in unpasteurized apple cider (pH 3.6 to 4.0) for up to 31 days at 8°C. Other foods suspected or implicated as vehicles in *E. coli* O157:H7 outbreaks are unpasteurized milk, sandwiches, potatoes, cantaloupe, lettuce, radish sprouts and contaminated bovine food products (Griffin, 1995).

Public interest in *E. coli* O157:H7 grew in the wake of a major outbreak of hemorrhagic colitis and HUS in the pacific northwest of the United States in early 1993. The source of the outbreak was traced to ground beef burger patties from a single restaurant chain and resulted in four deaths and more than 700 illnesses (Bell *et al.*, 1994). There have been a number of other outbreaks of illness attributed to *E. coli* O157:H7 that have been linked to the consumption of ground beef or beef burgers (Wells *et al.*, 1983; Belongia *et al.*, 1991; Willshaw *et al.*, 1994). A recent waterborne outbreak in Walkerton, Ontario in 2000 claimed 7 lives and caused 2,300 illnesses. The town's water supply was contaminated with *E. coli* O157:H7. It is estimated that the pathogen causes 250 deaths and 20,000 cases of illness each year in the United States alone (Griffin, 1995). Economic value associated with productivity losses and medical costs due to *E. coli* O157:H7 has been estimated to be $229 to $610 million per year (Murinda *et al.*, 1996).

Cassin *et al.* (1998) applied risk models to make a quantitative risk assessment of *E. coli* O157:H7 in cooked hamburgers. The model predicted the probability of a child contracting HUS to be 3.7×10^{-6} and the probability of mortality 1.9×10^{-7} per meal. Cassin *et al.* (1998) indicated that the estimates are probably high for all hamburger meals, but they are probably reasonable for home-prepared hamburgers.

As a result of recent outbreaks and poor prognoses for severe cases of *E. coli* O157:H7 infection there has been intensive research targeting the elimination of this pathogen from its sources. There are many approaches that can be used to eliminate or reduce the number of *E. coli* O157:H7 from both the pre- and post-harvest stages of meat production. The expectations of pre-harvest reduction strategies are to

prevent the introduction of pathogens into the food chain. Pre-harvest strategies considered include the use of special diets, probiotics and vaccines for cattle, the primary reservoir (Kudva *et al.*, 1999). Post-harvest strategies include: carcass pasteurization, irradiation, preservatives, organic acids, bacteriocins and live cultures of LAB in the meat products. The increasing consumer demand for fresh, natural, and preservative-free foods makes some strategies more acceptable than others (Stiles, 1996). The antimicrobial activity of bacteriocins against undesirable spoilage agents or foodborne pathogens makes these compounds candidates for use as biopreservatives in conjunction with, or in place of, traditional chemical preservatives (Stiles, 1996).

Cassin *et al.* (1998) used risk modeling to evaluate the efficacy of three risk mitigation strategies: 1) reducing microbial growth during retail storage through reduction in storage temperature; 2) reducing concentration of *E. coli* O157:H7 in the feces of cattle; and 3) convincing consumers to cook hamburgers more thoroughly. The temperature reduction strategy was predicted to be the most effective, the average probability of illness was predicted to be reduced by 80%. Palumbo *et al.* (1995) evaluated the minimum and maximum temperatures for growth by hemorrhagic strains of *E. coli* in Brain Heart Infusion broth and found that all 16 strains tested grew from at least 10 to 45°C, with some strains growing at 7.4°C, measured by optical density and plate count. Shaw *et al.* (1971) reported similar results, the minimum temperature for growth of nonpathogenic *E. coli* ML30 in glucose minimal medium was 7.5°C, measured by optical density.

In a final fermented dry sausage product, *E. coli* O157:H7 cannot grow; however, they survive under conditions of low water activity, low pH and high sodium nitrite and sodium chloride concentrations that are typically used in the fermentation and drying of meat (Lahti *et al.*, 2001). The concern is that the infective dose is very low and this is a ready-to-eat product. Therefore a LAB starter culture with the ability to reduce the *E. coli* O157:H7 would be desirable.

Vold *et al.* (2000) showed that high levels of background bacteria inhibited growth of *E. coli* O157:H7 in ground beef stored aerobically and anaerobically at 12°C. The results indicated the importance of natural LAB background flora in meat for the inhibition of *E. coli* O157:H7. Jay (1996) also speculated that the increase in the incidence of hemorrhagic colitis caused by *E. coli* is because current pathogen-reduction strategies applied to animal carcasses also remove the protective bacterial population including LAB. Jay (1996) hypothesized that the raw ground beef is so "clean" that the low numbers of pathogens have no antagonists; however, if the background microflora existed at levels of 5 to 6 log CFU/g the extremely low numbers of *E. coli* O157:H7 would be eliminated. Jay (1996) suggested that immediately following the application of a pathogen reduction strategy an appropriate mixture of harmless LAB bacteria could be sprayed onto the carcass to protect against subsequent growth of pathogens.

2.2. Lactic Acid Bacteria – Potential Biopreservatives

LAB are a group of gram-positive, non-motile, non-sporeforming, rod- and coccus-shaped organisms that produce lactic acid as the primary end-product of

fermentation (Kandler, 1983). LAB are classified in the *Clostridium* branch of the gram-positive bacteria because of their low DNA content of guanosine and cytosine (G+C). All strains classified as LAB have a G+C molar percentage less than 50. The metabolism of LAB is either homofermentative or heterofermentative. Homofermentative LAB degrade hexoses to lactic acid while heterofermentative LAB produce additional products such as acetate, formate, succinate, ethanol and CO_2. The main genera of LAB are, *Lactobacillus, Leuconostoc, Carnobacterium, Lactococcus, Pediococcus, Enterococcus, Streptococcus* and *Weissella* (Stiles and Holzapfel, 1997). Since ancient time, LAB have been used in the fermentation of food to extend storage life, to enhance product safety, and to impart desirable flavor characteristics. Traditional foods fermented with LAB include yogurt, sauerkraut, salami and cheese. Common LAB starter cultures include *Streptococcus thermophilus, Lactococcus delbrueckii* subsp. *bulgaricus*, *Lactococcus lactis* and *Pediococcus acidilactici*. It has been estimated that 25% of the European diet and 60% of the diet in many developing countries is comprised of fermented foods (Holzapfel *et al.*, 1995). LAB can control the growth of spoilage and pathogenic bacteria by competitive exclusion and by production of antibacterial compounds. Some of the antibacterial metabolites produced by LAB are: organic acids, diacetyl, H_2O_2, CO_2 and antimicrobial peptides termed bacteriocins (Klaenhammer, 1993). H_2O_2 and diacetyl are not produced to any great extent under anaerobic conditions and therefore would not be a factor under anaerobic storage conditions. Organic acids such as lactic and acetic acids lower the pH of food, which inhibits the growth of acid-sensitive organisms. The main focus of our research group is the application

of bacteriocins produced by LAB to control the growth of spoilage or pathogenic microorganisms.

2.2.1. *Carnobacterium* - an Important Meat Lactic Acid Bacterium

Carnobacterium is a recently recognized genus (Collins et al., 1987). The genus consists of six species: C. piscicola, C. divergens, C. gallinarium, C. funditum, C. alterfunditum and C. mobile. Collins et al. (1987) proposed the reclassification of Lactobacillus divergens and Lactobacillus piscicola to this new genus as C. divergens and C. piscicola, respectively. Carnobacteria are gram-positive, rod-shaped, non-motile, psychrotrophic, facultatively anaerobic, and heterofermentative. Carnobacteria produce only L(+)-lactate and are nonaciduric lactics, which means that they do not grow on acetate agar at pH 5.6 (Collins et al., 1987). Most strains of carnobacteria have been isolated from vacuum-packaged, chill-stored meat (Hitchener et al., 1982; Holzapfel and Gerber, 1983; Shaw and Harding, 1984; Burns, 1987), fish (Stoffels et al., 1992) and soft cheese (Mathieu et al., 1994). The fermentation by carnobacteria results in a minimal change to organoleptic properties of the meat, making these bacteria potential candidates for biopreservation of chill-stored, vacuum-packaged meat (McMullen and Stiles, 1996). Generally carnobacteria grow well at 15°C, but do not grow at temperatures below 0°C or above 40°C (Holzapfel and Gerber, 1983). Growth of carnobacteria is inhibited below pH 4.5, whereas most lactobacilli grow at a lower pH. Unlike lactobacilli, carnobacteria grow well at a basic pH, as high as pH 9.0.

Well characterized bacteriocins produced by carnobacteria include: divergicin A produced by *C. divergens* LV13 (Worobo *et al.* 1995) and carnobacteriocin A

produced by *C. piscicola* LV17 (Shaw and Harding, 1984). Bacteriocins produced by carnobacteria are generally active against other LAB and strains of *Listeria* and *Enterococcus* (Ahn and Stiles, 1992; McMullen and Stiles, 1996). Burns (1987) isolated *C. piscicola* UAL26 from vacuum-packaged, chill-stored ground meat. She determined that *C. piscicola* UAL26 produces a chromosomally mediated bacteriocin that is active against a broad spectrum of gram-positive organisms, including *Listeria monocytogenes* and *Clostridium perfringens*, but it is not active against gram-negative organisms. Rosario (2001) attempted to purify the bacteriocin produced by *C. piscicola* UAL26 but he was unsuccessful. It is speculated that the active component may be a two-peptide nonlantibiotic bacteriocin.

Leisner *et al.* (1995) evaluated the effect of growth of *C. piscicola* LV17 and UAL26 on storage life of beef slices stored under vacuum. They reported that growth of *C. piscicola* LV17 and UAL26 was unpredictable, achieving maximum population in 2 to 8 weeks. Both *C. piscicola* LV17 and UAL26 produced off-odors after 8 weeks of storage at 2°C; however, a limited amount of acid was produced, reflected by the fact that the surface pH did not change.

2.3. Bacteriocins – Produced by Lactic Acid Bacteria

Tagg *et al.* (1976) was the first to describe bacteriocins of LAB as a heterogeneous group of plasmid-mediated proteinaceous compounds, produced by lethal biosynthesis that kill closely related bacteria that have specific binding sites for that bacteriocin. The heterogeneity of bacteriocins, pointed out by Tagg *et al.* (1976) has become more apparent and it has become clear that very few bacteriocins of LAB

meet all of the criteria of this classical definition. Klaenhammer (1993) classified bacteriocins of LAB into four classes: class I bacteriocins are known as lantibiotics, which contain post-translationally modified amino acids such as lanthionine and β-methyllanthionine (Jung, 1991); the class II bacteriocins are small heat-stable nonlantibiotics, which do not contain post-translationally modified amino acids and undergo only minimal post-translational modification, usually involving the cleavage of a leader peptide; the class III bacteriocins are large heat-liable proteins; and the class IV bacteriocins are complex compounds, with protein and with lipid and/or carbohydrate moieties. Klaenhammer (1993) divided the class II bacteriocins into three subclasses; class IIa, pediocin-like bacteriocins with strong antilisterial activity; class IIb, two-peptide bacteriocins; and class IIc, thiol-activated peptides. A review by Nes *et al.* (1996) questioned the existence of the class IV bacteriocins and speculated that the complex bacteriocinogenic activity may be artifacts caused by interaction of the bacteriocin with cell or media components. Nes *et al.* (1996) suggested that the thiol-activated bacteriocin class IIc should be excluded because research had shown that oxidation of the sulfydryl group with other chemicals did not interfere with activity. Nes *et al.* (1996) proposed a new class IIc subgroup, which included the sec-dependent secreted bacteriocins, such as divergicin A (Worobo *et al.*, 1995).

The most extensively studied bacteriocin is nisin. Nisin is a lantibiotic that contains 34 amino acids and is produced by *L. lactis*. It has been licensed for use as a food additive in over 45 countries, but not in Canada (Delves-Broughton, 1990). Nisin was first permitted for use as a food preservative in the United Kingdom in

1959. In 1969, the World Health Organization approved the use of nisin as a food additive. Nisin is sold under the trade name Nisaplin and is manufactured by Aplin & Barrett Ltd., UK. In the United States, nisin is defined as an antimicrobial agent and it is used in pasteurized processed cheese spreads, fruits, and vegetables to inhibit the outgrowth of *Clostridium botulinum* spores and toxin formation (Vandenbergh, 1993). The bacteriocin is primarily active against a wide range of gram-positive bacteria but it has been shown to be active against gram-negative organisms when used in combination with chelating agents (Cutter and Siragusa, 1995). Cutter and Siragusa (1995) found that nisin in combination with EDTA reduced *E. coli* O157:H7 by 4 log CFU/cm^2 *in vitro*; however, such reductions were not observed in a beef model *in situ*. Only 0.4 log CFU/cm^2 reduction was observed. Nisin is very effective in dairy products; however, it is inactivated in meat products (Rayman *et al.*, 1983). Nisin inactivation in meat was attributed to poor solubility, proteases, binding or reaction with proteins or phospholipids, high bacterial loads and poor distribution throughout the meat product (Bell and De Lacy, 1986; Henning *et al.*, 1986; Cutter and Siragusa, 1994; Stringer *et al.*, 1995), but Rose *et al.* (1999b) determined that the inactivation of the nisin in fresh meat was caused by an enzymatic reaction with glutathione. Even though nisin is known to be inactivated in fresh meat this is not the rule for all bacteriocins. Some class II bacteriocins are known to be effective against pathogens and spoilage bacteria in meat (Daeschel, 1989; Leisner *et al.* 1996).

The production of leucocin A by *L. gelidum* UAL187 was shown to significantly delay the spoilage by a sulfide-producing *Lactobacillus sakei* on chill-stored, vacuum-packaged beef disks (Leisner *et al.*, 1996). The nonbacteriocinogenic

variant did not delay the onset of spoilage. In a study by Panayach (1998) the number of *L. monocytogenes* was lowered to a greater extent with the leucocin A-producing *L. gelidum* UAL187 compared with the isogenic nonbacteriocinogenic variant in vacuum-packaged, chill-stored ground beef.

A limitation and concern with the use of bacteriocins in raw meats is the development of resistant populations of bacteria. The development of bacteriocin resistant pathogens in food products affects the safety or quality of the food product. However, it is unlikely that bacteriocin resistant variants will have a route to reenter the food supply. Spontaneous sub-populations of variants of *L. monocytogenes* resistant to bacteriocins have been reported, including: mesenterocin 52, plantaricin C19 (Rekhif *et al.*, 1994), divercin V41 (Duffes *et al.*, 2001) and nisin (Davies and Adams, 1994; Crandall and Montville, 1998).

It is not intended that bacteriocins would be used as the only food safety "hurdle" but it is intended that it would be used in combination with other inhibitory factors. Applying the Leistner (2000) "hurdle" concept, bacteriocins can be used in combination with low water activity (a_w), pH, temperature and other preservatives. The use of chelating agents or hydrostatic pressure in combination with bacteriocins is known to increase the inhibitory spectrum of bacteriocins (Stevens *et al.*, 1991; Cutter and Siragusa, 1995; Padgett *et al.*, 1998).

2.4. Colicins

In general colicins are a large group of ribosomally synthesized antibacterial proteins produced by *E. coli* and other members of the *Enterobacteriaceae* (Waters

and Crosa, 1991). They are relatively large proteins with molecular weights in the range of 30 to 80 KDa. (Konisky, 1982).

2.4.1. Colicin V

Gratia (1925) reported studies on an antibacterial substance that was subsequently named colicin V. The substance was given the name "colicin" because it was produced by *E. coli* and called "V" because it was a virulent strain. Based on the mode of action, colicin V is classified within the group B colicins that include colicin E1, A, K, Ia and Ib (Hardy *et al.*, 1973). Colicin V displays many properties that distinguish it from most other colicins. Colicin V is a relatively small size, with a molecular weight of 8733 Da. Because colicin V is not a typical colicin and it is a small bacteriocin, it has been suggested that colicin V may be considered a microcin (Yang and Konisky, 1984; Nissen-Meyer and Nes, 1997).

Even though colicin V is produced by a gram-negative organism, it is similar to class IIa nonlantibiotic bacteriocins produced by gram-positive LAB (Håvarstein *et al.*, 1994; Fath *et al.*, 1994; Nes *et al.*, 1996). Colicin V is a heat stable, ribosomally synthesized protein that undergoes minimal post-translational modification and the amino acid sequence of the leader peptide is similar to other class IIa bacteriocins (Håvarstein *et al.*, 1994; Fath *et al.*, 1994; Nes *et al.*, 1996).

Colicin V is encoded on a large, low copy number plasmid (Gilson *et al.*, 1987). The plasmid-encoded genetically linked genes, *cvaA* and *cvaB* encode CvaA and CvaB proteins respectively, which mediate the secretion of colicin V (Gilson *et al.*, 1987). The protein encoded by *cvaA* belongs to the family of ATP-binding

cassette (ABC) transporter proteins and *cvaB* encodes an accessory protein (Higgins, 1992). Both export proteins are anchored in the inner membrane. The accessory protein has a single transmembrane domain near its N-terminus with the C-terminus extending into the periplasmic space (Franke *et al.*, 1996). TolC is a chromosomally encoded outer membrane protein that is involved in the secretion of colicin V (Zhang *et al.*, 1995). The structural gene *cvaC* encodes a 108 amino acid prepeptide. The prepeptide has a 15 amino acid double-glycine-type leader peptide that is cleaved by the ABC transporter protein, which simultaneously transports the mature 88 amino acid colicin V across the cell membrane (Fath *et al.*, 1994; Håvarstein *et al.*, 1994). The protein responsible for immunity is encoded by the *cvi* gene, which is located upstream of the colicin V structural gene.

Colicin V is produced by a number of strains of *E. coli* and it is active against closely related bacteria. Colicin V acts on target cells by permeabilization of the cytoplasmic membrane followed by ion leakage and depletion of the proton motive force (Yang and Konisky, 1984; Montville and Bruno, 1994). The proton motive force (PMF) drives the phosphorylation of ADP for ATP and the accumulation of ions, hence the depletion of PMF leads to cell death due to cessation of energy requiring biosynthesis processes.

Inhibitory activity of colicin V against strains of *E. coli* O157:H7 has been reported. Murinda *et al.* (1996) determined using deferred inhibition assays that colicin V inhibited only 2 of 11 (18%) strains of diarrheagenic *E. coli* including serotype O157:H7. In contrast, Bradley and Howard (1990) reported 18 of 20 (90%) *E. coli* O157:H7 strains were sensitive to colicin V.

2. 5. Lactic Acid Bacteria Antagonism Against Pathogens

Bacteriocin production among LAB is a widespread phenomenon (Klaenhammer, 1988) and bacteriocins are believed to have great potential as natural food preservatives (Stiles, 1996). There have been numerous studies investigating the use of bacteriocins to control growth of *L. monocytogenes* and *C. botulinum*. Control of growth of *L. monocytogenes* by bacteriocins has been demonstrated in meat products, including frankfurters (Berry *et al.*, 1991), dry fermented sausage (Foegeding *et al.*, 1992) and fresh meat (Motlagh, *et al.*, 1992). The control of outgrowth of spores of *C. botulinum* by bacteriocins has also been demonstrated in chicken salad (Hutton *et al.*, 1991) and processed cheese (Okereke and Montville, 1991).

2.5.1. Activity of Lactic Acid Bacteria Against *E. coli*

Lahti *et al.* (2001) examined the survival of *E. coli* O157:H7 and *L. monocytogenes* during the manufacture of dry sausage using two different starter cultures, (a) *Staphylococcus xylosus* with bacteriocin-producing *Pediococcus acidilactici* and *Lactobacillus bavaricus*, and (b) *Staphylococcus carnosus* and *Lactobacillus curvatus*. Numbers of *E. coli* O157:H7 decreased by 2.5 and 4.5 log CFU/g during the fermentation, with starter cultures a and b, respectively, but they were not eliminated after 49 days of storage at 15 to 17°C. The number of *L. monocytogenes* decreased faster than the number of *E. coli* O157:H7, and they were eliminated from the fermented sausage after 49 days of storage at 15 to 17°C. The reason for the greater reduction in numbers of *E. coli* O157:H7 cells using starter b is not fully understood. In other studies, fermentation and drying of sausage pre-

inoculated with similar levels of *E. coli* O157:H7 have been reported to reduce the numbers of *E. coli* by only 1 to 2 log units (Glass *et al.*, 1992; Hinkens *et al.*, 1996; Riordan *et al.*, 1998). The comparison of these studies is difficult because fermentation and drying procedures varied greatly between the studies.

Zhang and Mustapha (1999) studied the effect of nisin and nisin combined with EDTA in reducing growth of *E. coli* O157:H7 in vacuum-packaged fresh beef. The reduction was marginal, only a decrease of 1.02 and 0.8 log CFU/cm^2 was observed, respectively for nisin and nisin and EDTA. Brashears and Durre (1999) studied antagonism of *L. lactis* towards *E. coli* O157:H7 during growth in TSB at 37°C. Growth of *L. lactis* resulted in a significant reduction in pH, inhibiting the growth of *E. coli* O157:H7, which was not detectable after 24 h of incubation. Interaction studies with neutralized broth indicated that the acid production by *L. lactis* was primarily responsible for inhibition. Brashears *et al.* (1998) investigated the inhibitory action of a strain of *L. lactis* (selected for its ability to produce H_2O_2), against *E. coli* O157:H7 on chicken meat incubated at 5°C in Whirl Pak bags. *E. coli* O157:H7 populations decreased significantly compared with controls. Interaction experiments in the presence of catalase indicated that H_2O_2 was primarily responsible for inhibition.

2.6. Microbiology of Ground Beef

E. coli is not naturally present in the musculature; however, they are present as a direct result of contamination of the carcass at one or many points between slaughter and packaging. It is virtually impossible to avoid contamination of the beef

carcass with *E. coli* during processing. Contamination of the carcass may occur during the removal of the hide because contaminants on the hide and aerosols may come in contact with the exposed meat (Worobo, 1997). Handling during evisceration, trimming and processing spreads contamination over the carcass surface. The preparation of ground beef mixes surface contamination throughout the meat. Beef is a medium that favors the growth of many bacteria, it has high water activity, it has a pH of 5.6 to 5.7, and it contains nutrients, minerals and other factors necessary for growth.

Ground beef is traditionally prepared by grinding beef trim from in-store preparation of retail cuts and centrally-produced, vacuum-packaged (VP) beef trimmings (Worobo, 1997). A more recent method of ground beef production is to prepare it at a centralized facility and to transport it to the retail outlet. The centrally-prepared ground beef results in products with a lower microbial load (Shoup and Oblinger, 1976; Prokuda, 1999). Centralized preparation minimizes product handling at the retail level, which further prevents cross-contamination with pathogens, which has the potential to improve product safety.

Once the ground beef is put on display for retail sale, the control of the temperature of the meat is often lost and growth of mesophilic microbes may occur, resulting in large numbers of spoilage and pathogenic bacteria in the meat. The temperature of the retail display cabinets varies considerably. Greer *et al.* (1994) reported that meat surface temperatures can range from 1.7 to 10°C. A study by Palumbo *et al.* (1995) indicated that *E. coli* can grow above 7.4°C and therefore it has the potential for growth in temperature-abused refrigerated foods. Even though *E.*

coli does not grow below 7.4°C, its presence is still a concern because pathogenic serotypes such as O157:H7 have a very low infective dose and can cause severe life-threatening diseases.

The total bacterial load of ground beef is extremely variable and it depends on a number of factors, primarily the initial bacterial load, process sanitation and hygienic standards, type of packaging, storage time and temperature. The bacterial load of ground beef ranges from 3 to more than 7 log CFU/g at the retail level (Duitschaever *et al.*, 1973; Gill and McGinnis, 1993; Prokuda, 1999). Gill *et al.* (1996) reported the hygienic condition of beef dispatched to hamburger patty manufacturing plants ranged from 3.5 to 4.9, 0.7 to 3.0 and 0.2 to 2.6 log CFU/g for total, coliform and *E. coli* counts, respectively.

Initially, many different species of bacteria are present in the ground beef and their growth is affected by storage conditions. For instance, the growth of mesophilic bacteria would be suppressed by chill-storage (4°C); however, psychrotrophic bacteria would continue to grow.

Storage of meat under aerobic or anaerobic refrigerated conditions significantly affects the type and the number of bacteria that grow, which affect the storage life and safety of the product. Psychrotrophic gram-negative rod-shaped aerobic bacteria grow most rapidly under aerobic chill-storage conditions and dominate the spoilage microflora that develops (Ingram, 1962; Gill and Newton, 1977; Dainty and Mackey, 1992). The principal genera are *Pseudomonas, Moraxella and Acinetobacter* (Ingram, 1962; Gill and Newton, 1977; Dainty and Mackey, 1992). The pseudomonads usually predominate on meat at chill temperatures.

Aerobically stored products spoil quickly, within "days" because of the production of amines, sulfites and esters produced by these putrefactive aerobic organisms (Gill, 1983). Anaerobic conditions can be produced by vacuum-packaging (VP) or modified-atmosphere packaging (MAP) of the meat product prior to storage. This can be accomplished by removing the air in the package or by flushing with a mixture of gases that do not contain O_2. In VP the accumulation of CO_2 and the absence of O_2 restricts the growth of aerobic pseudomonads giving rise to a microflora that is dominated by LAB (Ingram, 1962; Beebe *et al.*, 1976; Dainty and Mackey, 1992). The LAB genera most frequently identified on vacuum-packaged, chill-stored meat are *Lactobacillus, Leuconostoc* and *Carnobacterium* (Hitchener *et al.* 1982; Shaw and Harding, 1984; Schillinger and Lücke, 1987; McMullen and Stiles, 1993). The dominance of LAB in vacuum-packaged , chill-stored meat can extend the shelf life from "days" to "weeks" (Leisner *et al.*, 1995, 1996; Worobo, 1997). Worobo (1997) reported that under chill-stored (2°C) aerobic conditions, meat has a storage life of only one week, whereas under vacuum the storage life can be extended to 8 weeks. VP/MAP chill-storage environments readily support the growth of psychrotrophic pathogenic bacteria, such as *L. monocytogenes, Yersinia entercolitica* and *Aeromonas hydrophila* (Gill and Reichel, 1989; Farber, 1991). At higher temperatures, species of *Enterobacteriaciae* can dominate the microflora (Gill and Newton, 1980). Another safety concern with VP/MAP is that the aerobic spoilage bacteria, which usually warn consumers of spoilage, are inhibited, while the growth of pathogens may be allowed or even stimulated (Genigeorgis, 1985; Farber, 1991).

2.7. Production of Colicin V by Lactic Acid Bacteria

Van Belkum *et al.* (1997) investigated the role of leader peptides in the recognition of the prepeptide by the dedicated ABC transporter system and the subsequent export in homologous and heterologous hosts. When colicin V was fused behind the leader peptide of leucocin A, production of colicin V was achieved in *L. lactis*, using the lactococcin A dedicated secretion apparatus.

Another important pathway used for the secretion of most prokaryotic proteins is the general protein secretory pathway (Pugsley, 1993). Proteins that are exported via the general secretory pathway contain an N-terminal signal peptide, which is cleaved when the bacteriocin is exported. Worobo *et al.* (1995) characterized divergicin A and showed that it utilized the general secretory pathway for its export from the cell. The bacteriocin, divergicin A is produced by *C. divergens* LV13 (Worobo *et al.*, 1995). There have been a number of studies utilizing the divergicin A signal peptide and the general protein secretion pathway to export bacteriocins in heterologous hosts (Worobo *et al.*, 1995; van Belkum *et al.*, 1997 ; McCormick *et al.*, 1996, 1998, 1999; Franz *et al.*, 2000).

The export of colicin V from LAB was accomplished by replacing the double-glycine-type leader peptide of colicin V with the divergicin A signal peptide (McCormick *et al.*, 1999). McCormick *et al.* (1999) constructed the colicin V expression plasmid pJKM37 and successfully electrotransformed the plasmid into *C. piscicola* UAL26. McCormick *et al.* (1999) demonstrated that colicin V was secreted utilizing the general protein secretion pathway.

Jiang (unpublished data) constructed and electrotransformed plasmid pCV22 into *C. piscicola* UAL26. The pCV22 (Jiang, unpublished data) contains many of the components of pJKM37 (McCormick *et al.*, 1999) including: P32 promoter from *L. lactis* (van der Vossen *et al.*, 1987); divergicin A signal peptide from *C. divergens* LV13 (Worobo *et al.*, 1995) and colicin V structural gene from pHK22 isolated from *E. coli* ZK514 (Gilson *et al.*, 1987). The pCD3.4 vector used in pJKM37 was replaced with pCaT, which was isolated from *Lactobacillus plantarum* (Jewell and Collins-Thompson, 1989). The colicin V immunity gene is not required because the host organism *C. piscicola* UAL26 is naturally resistant to colicin V. The fusion construct was introduced into *C. piscicola* UAL26, which was originally isolated from vacuum-packaged, chill-stored ground beef (Burns, 1987).

2.8. Research Objectives

The *in situ* production of bacteriocins by LAB was shown to be effective at extending storage life and enhancing the control of specific foodborne pathogens in vacuum-packaged, chill stored ground beef (Leisner *et al.*, 1996; Panayach, 1998). An additional level of safety may be achieved by utilizing a genetically modified LAB that produces a bacteriocin that is specifically active against the gram-negative pathogen *E. coli* O157:H7.

The objective of this study was to evaluate the ability of partially purified colicin V and colicin V-producing *C. piscicola* UAL26 pCV22 to inhibit and kill *E. coli* O157:H7 in a broth and meat system, with a goal to enhance the safety of the food product.

3. MATERIALS AND METHODS

3.1. Bacterial Cultures and Plasmids

The bacterial cultures and plasmids used in this study are listed in Table 3.1. *C. piscicola* UAL26 was isolated from vacuum-packaged ground beef (Burns, 1987). *C. piscicola* UAL26 is plasmidless and produces an uncharacterized, chromosomally-mediated, broad-spectrum bacteriocin. The colicin V-producing *C. piscicola* UAL26 pCV22 was genetically engineered by Jiang (unpublished data). The parent strain, *C. piscicola* UAL26, and therefore *C. piscicola* UAL26 pCV22 are resistant to the action of colicin V. *C. piscicola* UAL26 is sensitive to both chloramphenicol and streptomycin, whereas *C. piscicola* UAL26 pCV22 is resistant to both antibiotics. The *C. piscicola* and *E. coli* used in this study were checked for bacteriocin production against one another by direct and deferred inhibition tests as described by Ahn and Stiles (1990).

The plasmid pCV22, as shown in Figure 3.1., was engineered and electrotransformed into *C. piscicola* UAL26 by Jiang (unpublished data). The plasmid pCV22 contains a number of genetic components including P32 promoter, divergicin A signal peptide gene (*dvn*) and colicin V structural gene (*cvaC*). The divergicin A signal peptide was originally isolated from *C. divergens* LV13 and was characterized by Worobo *et al.* (1995). The colicin V structural gene was obtained from pHK22 that was isolated from *E. coli* ZK514 and was characterized by Gilson *et al.* (1987). The P32 promoter from *L. lactis* (containing pMG36e) was characterized

Table 3.1. Bacterial strains and plasmids used in this study.

Strains or plasmids	Relevant characteristics	Source/Reference
Strains		
Escherichia coli DH5α	F⁻ ϕ80d*lacZ*ΔM15 Δ(*lac*ZYA-*arg*F) U169 *deo*R *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *pho*A *sup*E44 λ- *thi*-1 *gyr*A96 *rel*A1, colicin V sensitive	BRL Life Technologies Inc.
E. coli O157:H7		
43895		ATCC[a]
12096		Waters[b]
12902		
13025		
98.E3093		Tyrrell[c]
98.E3139		
98.E3708		
98.E3744		
98.R967		
98.R993		
98.R1298		
98.R1303		
98.R1389		
98.R1719		
E. coli ZK514	colicin V producer, Cm^r contains pHK22	Gilson *et al.*, (1987)
Carnobacterium piscicola UAL26	plasmidless, produces uncharacterized bacteriocin, resistant to colicin V	Burns, (1987)
C. piscicola LV17 NCFB2852		Shaw[d, e]
C. divergens LV13 NCFB2855		Shaw[d, e]

Strains or plasmids	Relevant characteristics	Source/Reference
Enterococcus faecalis 7080		ATCC
Lactobacillus plantarum 4008		ATCC
Pediococcus acidilactici 8042		ATCC
Brochothrix campestris 43754		ATCC
Lactococcus lactis 11454		ATCC
Leuconstoc mesenteroides 23368		ATCC
Leuconostoc gelidum UAL187		Hastings *et al.*, (1991)
Listeria monocytogenes 15313		ATCC
Listeria innocua 33090		ATCC
Plasmids		
pCV22	pCaT derivative containing *dvn-cvaC*, Cm^r, Sm^r, 7.9 kb	Jiang[f]
pJKM37	292-bp *Hin*dIII - *Kpn*I fragment from pJKM42-1 cloned in pRW19e, *dvn-cvaC* , Em^r, 4.0 kb	McCormick *et al.* (1999)
pCaT	Cm^r, Sm^r, 8.9 kb	Jewel and Collins-Thompson (1989)
pHK22	pACYC184 derivative containing 9.4-kb colicin V gene cluster, *cvaA*, *cvaB*, *cvaC*, *cvi*, Cm^r	Gilson *et al.* (1987)
pMG36e	expression vector, Em^r , 3.6 kb	van de Guchte *et al*, (1989)

Strains or plasmids	Relevant characteristics	Source/Reference
pRW19e	514-bp EcoRV - AccI fragment from pCD3.4 cloned in pMG36e; *dvn*, *dvi*, Em^r, 4.1 kb	Worobo *et al.* (1995)
pJKM42-1	292-bp *Hin*dIII-*Kpn*I PCR product from pHK22 cloned in pUC118; Amp^r, 3.5 kb	McCormick *et al.* (1999)

a. ATCC, American Type Culture Collection, USA.
b. Supplied by Dr. J. Waters, Clinical Isolates, 1983, Provincial Laboratory of Public Health, Edmonton, Alberta.
c. Supplied by Dr. G. Tyrrell, Clinical Isolates, 1998, Provincial Laboratory of Public Health, Edmonton, Alberta.
d. Supplied by Dr. B. G. Shaw, Institute of Food Research, Langford, Bristol. UK.
e. NCFB, National Collection of Food Bacteria, Reading, United Kingdom.
f. L. Jiang, unpublished data, CanBiocin Inc., Edmonton, Alberta.

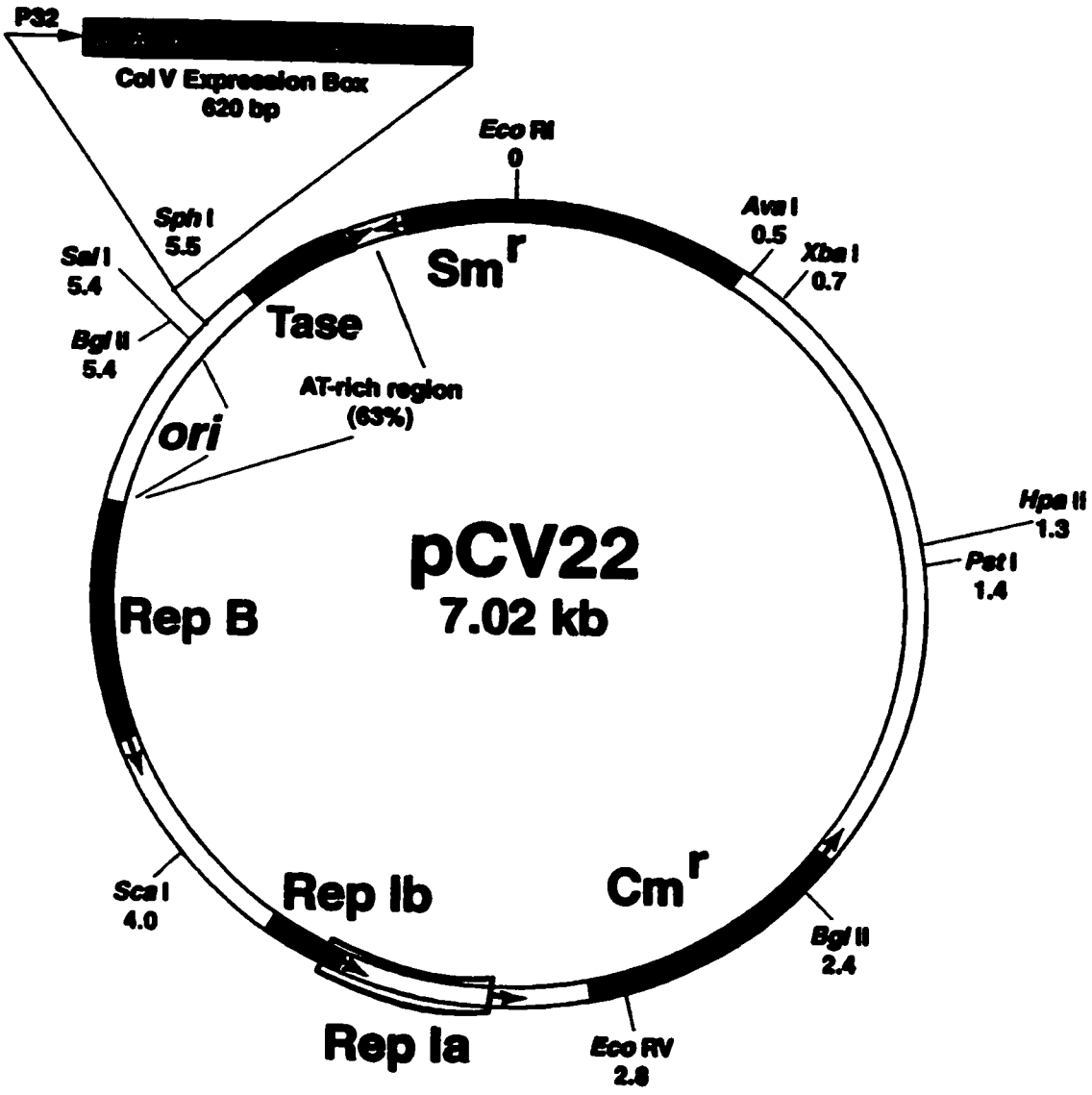

Figure 3.1. Physical map of the plasmid pCV22 showing the location of unique cleavage sites and colicin V expression box. Streptomycin resistant (Sm^r) and chloramphenicol resistant (Cm^r) genes; origin of replication (ori); truncated part of a transposase gene (Tase); replication protein genes (Rep). The black boxes represent the genes. The arrows show the direction of transcription. (Jiang, unpublished data).

by van der Vossen *et al.* (1987). The plasmid pCaT from *Lactobacillus plantarum* caTC2 was characterized by Jewell and Collins-Thompson (1989). pCaT contains chloramphenicol resistant (Cm^r) and streptomycin resistant (Sm^r) genes. The colicin V immunity gene is not required because the host organism *C. piscicola* UAL26 is naturally resistant to colicin V. The plasmid pJKM37 was engineered and electrotransformed into *C. piscicola* UAL26 by McCormick *et al.* (1999).

3.2. Media and Reagents

C. piscicola cultures were grown in APT broth (All Purpose Tween, Difco Laboratories Inc., Detroit, Mich.) at 25°C without agitation. *E. coli* cultures were grown in Nutrient Broth (NB; Becton Dickinson, BBL, Cockeysville, MD) at 37°C on a rotary shaker. Stock cultures were stored at -70°C in the appropriate broth containing 20% glycerol. Cultures were subcultured (0.1% inoculum) at least twice and not more than 5 times in the appropriate broth before use in experiments. Antibiotics were added as selective agents when appropriate: chloramphenicol (Cm; 5 µg/ml, Sigma) and erythromycin (Em; 5 µg/ml, Sigma). Agar plates were made by addition of 1.5% (wt/vol) agar to broth media. For the bacteriocin activity assay a 7 ml soft agar (0.75% wt/vol agar) overlay was used. Other media used in the study include: Plate Count Agar (PCA; Difco), Nutrient Agar (NA, Difco), MacConkey Sorbitol Agar (Difco), Brain Heart Infusion Broth (BHI; Difco), Lactobacilli MRS Broth (Difco), Tryptic Soy Broth (TSB; BBL), Cooked Meat Medium (CMM; Difco) and Phosphate Buffer Saline (PBS). Where applicable the pH of media was adjusted with either 1 N NaOH or 2 N HCl.

Culture identity was confirmed by determining Gram reaction, cellular morphology, oxidase and catalase reactions, carbohydrate fermentation and serological analyses. To differentiate between gram-negative and gram-positive bacteria the traditional gram stain test or the rapid gram test (Gregersen, 1978) were done. For the rapid gram test method, a colony from the surface of a solid medium was mixed with two drops of 3% KOH on a glass slide. After 10 sec the inoculation loop or toothpick was raised from the drop, if the emulsion was viscous and a thread of slime follows the loop the reaction is positive indicating a gram-negative bacterium. If there was no slime but a watery suspension the reaction is negative indicating a gram-positive bacterium. For the oxidase test, two drops of oxidase test reagent (N-N-N- Tetramethylparaphenylenediamine dihydrochloride, BDH) were placed on a strip of filter paper and cells from a colony were smeared onto the oxidase-saturated paper. Formation of a purple color within 10 sec indicates a positive oxidase reaction, no change in color indicates a negative reaction. For the catalase test, two drops of 3% H_2O_2 (Fisher Scientific) were placed on a glass slide and cells from a colony were mixed with the H_2O_2. Formation of bubbles indicates a positive catalase reaction, no bubbles indicates a negative reaction. The API 20 E System (bioMérieux Vitek Inc., Hazelwood, MO, USA) was used to confirm the identity of *E. coli* cultures. The *E. coli* O157:H7 serotype was confirmed using the RIM® *E. coli* O157:H7 Latex Test (Remel, Lenexa, KS, USA). The API 50 CHL System (bioMérieux Vitek Inc.) was used to confirm the identity of the *C. piscicola* cultures.

3.3. Partial Purification of Colicin V

Partial purification of colicin V was done using a modified method of Fath *et al.* (1994). A 1% inoculum of an overnight culture of *E. coli* ZK514 (colicin V producer) was grown, in 100 ml of NB containing 20 μg Cm per ml and 0.1 mM 2,2' dipyridyl at 37°C on a rotary shaker. Colicin V production was induced by the addition of the dipyridyl iron chelator because the expression of colicin V genes is repressed by excess iron (Fath *et al.*, 1994). The culture was diluted 1:10 into fresh medium and grown for 3.5 h to late log phase. The culture was heated (70°C, 20 min) and cells were removed by centrifugation (16,000 x *g*, 4°C, 5 min). Supernatant was removed and treated with trichloroacetic acid (TCA) to a final concentration of 10%. The sample was chilled on ice for 45 min and then centrifuged at 30,000 x *g*, 4°C for 15 min. The supernatant was removed and the precipitate was washed 2 x with acetone at -20°C. The precipitate was suspended in 1/500th of the original volume in 0.1% trifluoroacetic acid (TFA). The suspension was filter-sterilized and loaded onto a Sephadex G-25 column (1.5 x 6 cm; Pharmacia LKB Biotechnology, Uppsala, Sweden) equilibrated with 0.1% TFA. The column was run with a 0.1% TFA running buffer at room temperature (22°C) at a constant flow rate of 1 ml/min. The fractions with inhibitory activity were collected, pooled, filter sterilized and stored at -70°C. The level of colicin activity of the purification solution was quantified by spot-on-lawn technique (Ahn and Stiles, 1990) using *E. coli* DH5α as the indicator strain.

3.4. Sodium Dodecyl Sulfate-Polyacrylamide Gel Electrophoresis (SDS-PAGE) Analysis

Approximately 100 arbitrary activity units (AU) of partially purified colicin V was mixed with Laemmli gel-loading buffer, boiled for 2 min and run on SDS-PAGE (Laemmli, 1970). The reducing agent dithiothreitol was not added to the Laemmli gel-loading buffer because it is known to inactivate colicin V (Håvarstein *et al.*, 1994). Electrophoresis was done on a 15% SDS-polyacrylamide gel for 50 min at 200 V (Mini-PROTEAN II apparatus, Bio-Rad Laboratories, Richmond, Calif.). A 5% SDS-polyacrylamide stacking gel was used. The separating gel was fixed in 50% methanol for one hour. Colicin V and low-range protein molecular weight markers (Bio-Rad Laboratories, CA) were visualized by staining the fixed gel with silver salts (Sambrook *et al.*, 1989). A duplicate SDS-polyacrylamide gel was fixed in 50% methanol for one hour and washed with water for two hours. This gel was placed on a NA plate and overlayered with soft 15 ml of NA containing a 1% inoculum of *E. coli* DH5α and incubated at 37°C for 18 h. The location of colicin V activity in the gel was visualized by the location of zones of inhibition.

3.5. Matrix Assisted Laser Desorption/Ionization – Time of Flight Mass Spectrometry (MALDI-TOF MS) Analysis

MALDI-TOF MS was used to detect the presence of colicin V in the TCA purification product and in meat. Partially purified colicin V was analyzed on a Bruker Proflex III MALDI-TOF mass spectrometer (Bellerica, Mass.). The mass spectra were acquired using 337 nm radiation from a nitrogen laser with a 125-cm

flight tube. The mass spectrometer was operated in positive ion linear mode. A saturated solution of sinapinic acid (3,5-dimethoxy-4-hydroxycinnamic acid, Sigma) in a 2:1 mixture of 0.1% TFA:acetonitrile was mixed with an equal volume (5 µl) of partially purified colicin V. Solutions (1 µl) were deposited onto the stainless steel probe and allowed to air dry. Mass spectra were averaged over 100 consecutive laser shots. External mass calibration was performed with bovine insulin, MH^+ = 5734.6 (Sigma). To determine the bacteriocin presence in meat a 0.5 g sample of meat was mixed with 0.5 ml of distilled water by spatula and mixed on a vortex shaker for 1 min. The mixed sample was heat-treated (70°C for 20 min), centrifuged (16,000 x *g*, 4°C for 5 min) and analyzed for molecular size of its components by MALDI-TOF MS.

3.6. Assay for Bacteriocin Activity

Inhibitory activity of colicin V was determined using the spot-on-lawn and deferred inhibition assays described by Ahn and Stiles (1990). For deferred inhibition assays to screen for bacteriocin activity a Cathra Replicator was used (Cathra International Inc., St. Paul, Minneapolis). For spot on lawn assays, bacteriocin activity was expressed in arbitrary activity units (AU) per ml calculated as the reciprocal of the highest of a series of doubling dilutions that displays inhibition of the indicator organism. *E. coli* DH5α was used as the indicator organism because of its sensitivity to colicin V. The indicator organism (1% inoculum) was mixed with 7 ml of soft NA (0.75% agar) and overlayered onto a prepoured NA plate (1.5% agar). A sample of culture supernatant was centrifuged at 16,000 x *g*, 4°C for 5 min

to remove cells. The cell-free supernatant was heated at 70°C for 20 min to kill any remaining cells and to destroy native proteases. The heat-treated, cell-free supernatant was serially two-fold diluted and 10 µl amounts of each dilution were spotted onto a preseeded agar plate by overlaying a prepoured NA plate (1.5% agar) with the indicator organism (1% inoculum) mixed with soft nutrient agar (0.75% agar). Following the spotting of dilutions onto the preseeded plate the plate was incubated at 37°C for 18 h and observed for clear zones of inhibition. To confirm that a proteinaceous compound caused the zones of inhibition, the bacteriocin was inactivated by spotting 5 µl of Pronase E solution (1 mg/ml, Sigma) adjacent to one of the dried sample spots prior to incubation. To determine the bacteriocin activity in meat a 0.5 g sample of meat was mixed with 0.5 ml of distilled water by spatula and mixed on a vortex shaker for 1 min. The mixed sample was centrifuged (16,000 x *g*, 4°C for 5 min) and the supernatant solution was analyzed for activity by spot-on-lawn assay. In the deferred inhibition assay the test organism was first grown on an agar plate for 24 h, then a 1% inoculum of the indicator strain in soft agar was overlayered and the plate was incubated.

3.7. Plasmid Stability

Cultures of *C. piscicola* UAL26 pCV22 or pJKM37 for use in experiments were started from frozen stock, transferred to APT-Cm (5 µg/ml) or APT-Em (5 µg/ml) broth, subcultured twice into fresh APT-Cm or APT-Em broth then transferred to APT broth. *C. piscicola* UAL26 or UAL26 pCaT were used as the negative control. The culture was diluted in sterile, 0.1% peptone water and a 100 µl

sample was transferred into 10 ml of APT broth to give a final concentration of 3 log CFU/ml. Cultures were incubated at 4, 10, 25 and 37°C for 10 days, 96, 48 and 96 h, respectively. *C. piscicola* UAL26 pCV22 is a psychrotroph and grows slowly at 37°C therefore the longer incubation period is required. These incubation periods allowed the culture to grow to 9 log CFU/ml, which represents about 20 generations. Cultures were subcultured in APT broth for a total of 100 generations. After each of the six incubation periods the culture was diluted, plated onto APT agar and incubated at 22°C for 48 h. Sixty colonies were aseptically transferred to both APT and APT-Cm or APT-Em agar plates in a grid pattern. After incubating at 22°C for 24 h the colonies were enumerated. The number of colonies that grow on the APT-Cm or APT-Em as a percentage of colonies that grow in APT, represent the percent stability of the plasmid. To confirm plasmid stability, the incubated APT agar plate was overlayered with NA soft agar seeded with 1% *E. coli* indicator organism and incubated at 37°C for 18 h. After incubation the colonies were checked for zones of inhibition.

3.8. Growth of *C. piscicola* and *E. coli* in Broth

Overnight cultures of *C. piscicola* UAL26 and UAL26 pCV22 and *E. coli* DH5α were centrifuged at 16,000 x *g*, at 4°C for 5 min. The bacterial pellet was suspended in sterile, 0.1 % peptone water and serially diluted to yield a 0.5 ml inoculum to produce an initial inoculum level of approximately 5 log CFU/ml for both *C. piscicola* and *E. coli*. Pure and mixed culture experiments were done in BHI, NB, APT broth or PBS. Incubation temperatures were 4, 10, 25 and/or 37°C.

Partially purified colicin V was added to BHI, NB, APT broth or PBS to give a final concentration of 100 to 1600 AU/ml, depending on the experiment. The number of *C. piscicola* in the samples was enumerated by plating samples diluted in 0.1% peptone water onto APT agar and incubated at 15°C for 48 h. *E. coli* in the samples was enumerated by plating samples diluted in 0.1% peptone water onto NA and incubated at 37°C for 18 h. The samples were plated in duplicate and the mean log CFU/ml for each sample was determined. The pH of the samples was measured using a pH meter (Accumet Model 915, Fisher Scientific) and a combined electrode (Accu pHast, Fisher Scientific).

3.9. Growth of *C. piscicola* and *E. coli* in Ground Beef Samples

Vacuum-packaged beef was obtained from XL Meats, Calgary, Alberta and stored at –25°C until used. Prior to grinding, the meat was thawed at 4°C for 48 h. Approximately 10 kg of meat was dipped in 100% ethanol, suspended from a metal rack and ignited. The flaming process was repeated three times. The flamed meat was transferred to a Clean Air Bench and the outer section of the meat was aseptically removed. The meat was aseptically ground first through a 10 mm coarse plate and then through a 3 mm fine plate (Model 84185 Hobart, Troy, Ohio). The grinder parts that came in contact with the meat were autoclaved prior to grinding. Samples of the ground meat were used for enumeration of indigenous LAB on APT and total aerobic plate count on NA. Ground meat was divided into approximately 500 g portions, vacuum-packaged and stored at –25°C. When required, the ground meat was thawed under running water or at 4°C for 18 h.

To determine the ability of the genetically modified *C. piscicola* to inhibit the growth of *E. coli*, aseptically prepared ground beef was inoculated with *C. piscicola* UAL26 pCV22 and *E. coli* DH5α. Prior to inoculation cultures were centrifuged at 16,000 x *g*, at 4°C for 5 min and the bacterial pellet was suspended in sterile, 0.1 % peptone water and serially diluted to yield 0.5 ml inoculum to produce an initial inoculum level of approximately 5 log CFU/g for both *C. piscicola* and *E. coli*. Ground beef was weighed in 10 g portions and placed in low O_2 permeable vacuum package bags (mylar/PVDC/polyethylene vinyl alcohol film; O_2 transmission rate 7.7cc/m^2/24 h; moisture vapour transmission rate, 8.7 g/m^2/24 h; Unipac Packaging Products Ltd., Edmonton, Alberta). Inoculum was distributed over the surface of the ground meat and was mixed into the ground meat by massaging the outside of the package by hand. An equal amount of sterile 0.1% peptone water was distributed and mixed into the ground meat and used as an uninoculated control. Samples were maintained on ice until vacuum packaging. Each sample was vacuum-packaged (Fresh Vac Model A300, CVP Systems Inc., Downers Grove, Illinois) and stored at either 4 or 10°C. The temperature of storage was monitored with a Delphi temperature data logger (Model 861, Delphi Industries Ltd., Auckland, New Zealand). The data logger was calibrated as outlined in the manufacturer's manual. The accuracy of the data logger was also checked using a calibrated alcohol thermometer. Partially purified colicin V was added to meat samples to give a final concentration of 100 to 1600 AU/g.

After each incubation interval, 2 and 4 days at 10 and 4°C, respectively, two of the 10 g vacuum-packaged samples were blended separately with 90 ml of sterile,

0.1% peptone water for 1 min in a Colworth Stomacher (Model 400, Seward and Co., London, England). Serial dilutions of the homogenate were prepared with 0.1% peptone water and 0.1 ml of the appropriate dilutions was spread on the surface of prepoured agar plates. *C. piscicola* and indigenous LAB were plated on to APT agar and enumerated after aerobic incubation at 15°C for 48 h. *E. coli* and indigenous total aerobic plate count were determined on NA that was incubated at 37°C for 18 h. Identity of the colonies was confirmed by randomly selecting 20 colonies and analyzing for catalase and gram reaction. The mean log CFU/g for each sample was determined. The pH of the meat sample homogenates prepared for plate count was measured at the time of sampling using a Fisher Accumet pH meter.

4. RESULTS

4.1. Purification of Colicin V

The colicin V bacteriocin produced by *E. coli* ZK514 was partially purified to concentrate the bacteriocin and to exclude nonspecific inhibitors. The trichloroacetic acid (TCA) precipitation method used to partially purify colicin V from *E. coli* culture supernatant yielded 6,400 to 12,800 AU/ml depending on the preparation batch. The bacteriocin activity of the cell-free supernatant before purification ranged from 100 to 400 AU/ml. Prior to use in experiments the partially purified colicin V was analyzed by SDS-PAGE and MALDI-TOF MS. The silver stained SDS-PAGE gel showed a prominent band with a molecular mass of about 7.5 kDa (Figure 4.1), which corresponds, to the molecular weight of colicin V. The lack of other protein bands indicated that the sample is relatively pure. A duplicate washed polyacrylamide gel was overlayered with soft agar inoculated with *E. coli* DH5α to detect inhibitory activity. A band of inhibitory activity, was evident at the same location of the protein band on the silver stained gel. As seen in the MALDI-TOF mass spectrum (Figure 4.2) there is one prominent peak with an observed molecular mass of 8761 Da, which corresponds with colicin V that has a predicted molecular mass of 8733 Da for the colicin V peptide (Fath *et al.*, 1994).

4.2. Bacterial Identity and Confirmation of *E. coli* and *C. piscicola*

C. piscicola UAL26 pCV22 and *C. piscicola* UAL26 have the same sugar fermentation profiles determined by API 50 CHL System, and both strains were

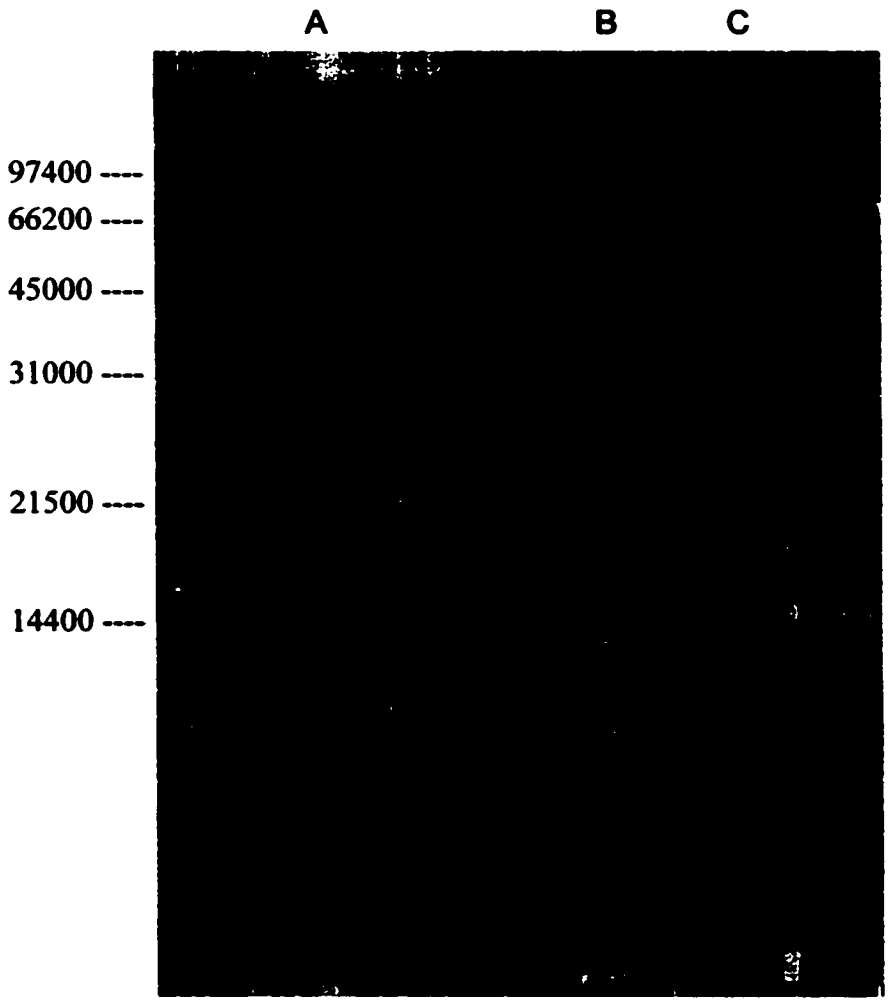

Figure 4.1. Polyacrylamide (15%) gel electrophoresis of partially purified colicin V. Lane (A) low range molecular mass protein standards of 97,400, 66,200, 45,000, 31,000, 21,500 and 14,400 Da. (Bio-Rad Laboratories, CA); (B) 2 µl sample of partially purified colicin V; (C) 5 µl sample of partially purified colicin V.

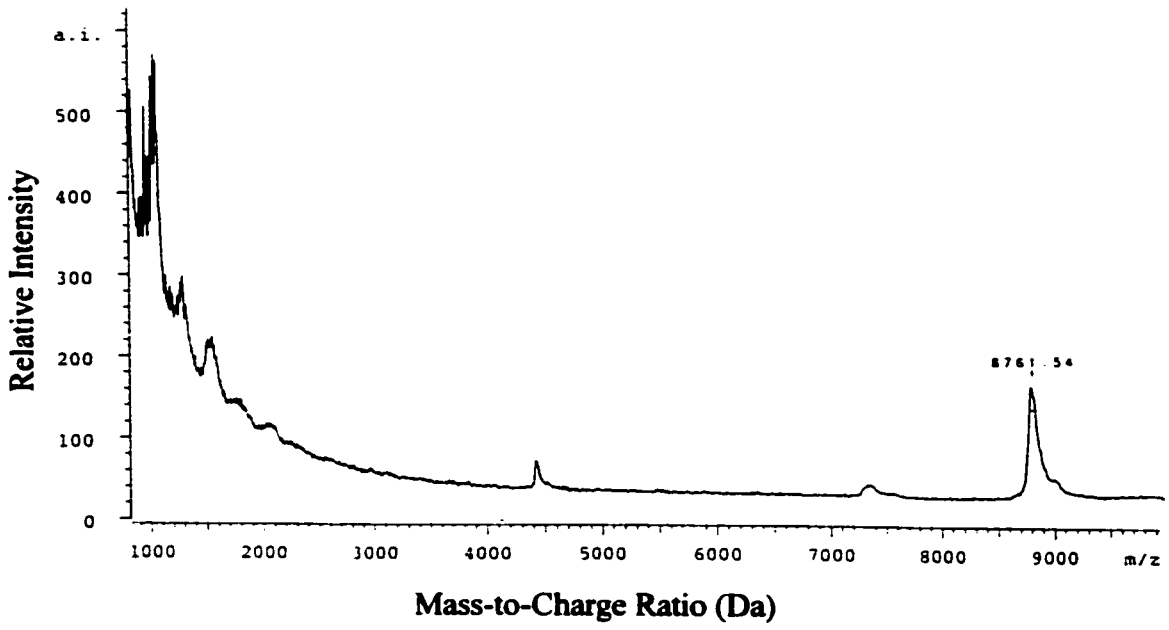

Figure 4.2. MALDI-TOF mass spectrum of partially purified protein extract from cell free supernatant, from a 18 h culture of colicin V producing *Escherichia coli* ZK514 grown in nutrient broth. Matrix: sinapinic acid; laser wavelength: 337 nm.

confirmed to be *C. piscicola*. *E. coli* DH5α, ZK514 and O157:H7 cultures were tested on API 20E System, as expected all were identified as *E. coli*. The *E. coli* O157:H7 cultures were tested for O and H antigen using the Remel Serotype Agglutination assay, and as expected cultures were O157 and H7 positive.

4.3. Plasmid Stability

Plasmid stability of pJKM37 and pCV22 in *C. piscicola* UAL26 was determined. The plasmid pJKM37 was not stable in *C. piscicola* UAL26, as measured by loss of erythromycin resistance and bacteriocin production after approximately 28 generations in APT broth at 25°C. After approximately 20 generations only 52% of the cells grew in APT agar containing erythromycin. After 40 generations none of the cells grew. In contrast, pCV22 was stable for 100 generations after growing at 4, 10 and 25°C in APT broth without added antibiotic to provide selective pressure (Figure 4.3). There was less than 15% decrease in stability after 100 generations at each incubation temperature. The plasmid pCV22 was not stable in *C. piscicola* UAL26 in ATP at 37°C. The producer organism *C. piscicola* UAL26 pCV22 grew poorly at 37°C, reaching a maximum population of 8 log CFU/ml after 96 h.

4.4. Spectrum of Activity

The inhibitory spectra of *C. piscicola* UAL26, UAL26 pCV22 and UAL26 pCaT are shown in Table 4.1. As expected the *C. piscicola* strains had similar activity spectra, the only difference observed was that UAL26 pCV22 was active

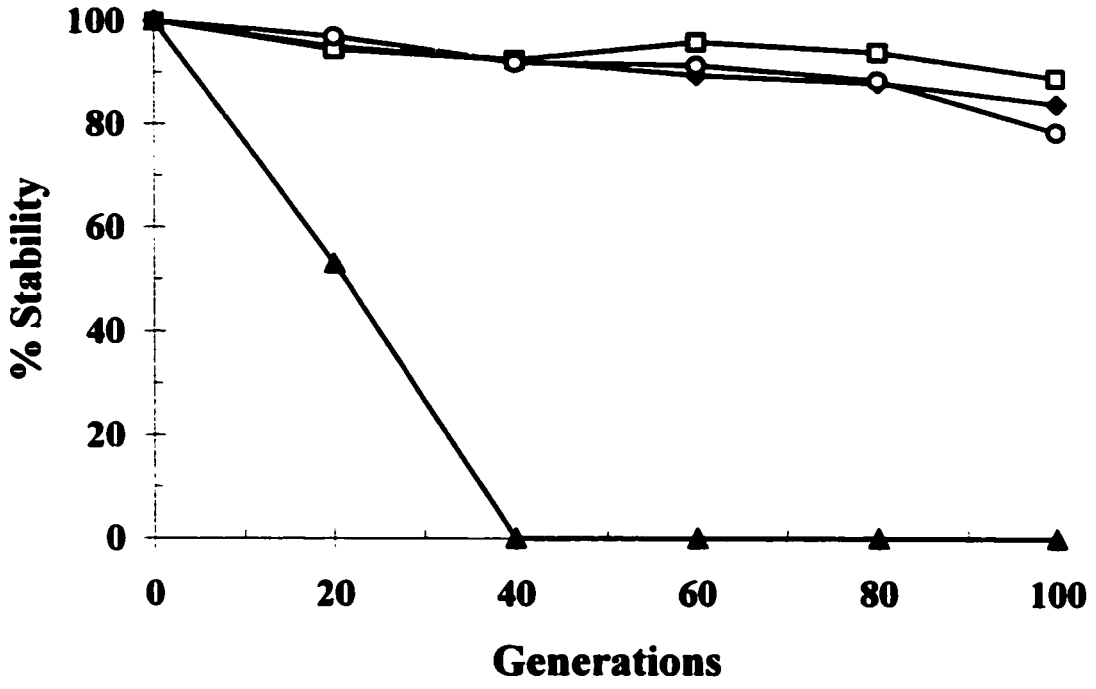

Figure 4.3. Stability of pCV22 in *C. piscicola* UAL26 incubated at 4°C (◆), 10°C (□), 25°C (○), and 37°C (▲) in APT broth. Each data point is the mean of three experiments.

Table 4.1. Activity spectra of *C. piscicola* UAL26 and a colicin V producing clone (UAL26 pCV22) and a colicin negative clone (UAL26 pCaT) determined by deferred inhibition on APT agar at 25°C for 24 h.

Indicator strain		Inhibitory activity*		
		C. piscicola		
		UAL26	UAL26 pCV22	UAL26 pCaT
E. coli	DH5α	-	++	-
C. piscicola	UAL26	-	-	-
	UAL26 pCV22	-	-	-
	UAL26 pCaT	-	-	-
	LV17	+	+	+
C. divergens	LV13	+	+	+
E. faecalis	ATCC 7080	+	+	+
L. plantarum	ATCC 4008	++	++	++
P. acidilactici	ATCC 8042	+	+	+
B. campestris	ATCC 43754	+	+	+
L. lactis	ATCC 11454	+	++	++
L. mesenteroides	ATCC 23368	+	+	+
L. gelidum	UAL187	+	+	+
L. monocytogenes	ATCC 15313	++	++	++
L. innocua	ATCC 33090	-	-	-

* Diameter of the zone of inhibition appearing in the indicator overlay
10 to 14 mm = ++, 5 to 9 mm = +, <5 mm = -

against *E. coli*, whereas *C. piscicola* UAL26 and *C. piscicola* UAL26 pCaT were not active against *E. coli*. The production of colicin V by *C. piscicola* UAL26 pCV22 does not inhibit the growth of UAL26 and UAL26 pCaT. Some strains, including *L. plantarum, L. mesenteroides, L. lactis and L. monocytogenes* were particularly susceptible to the inhibitory substance(s) produced by *C. piscicola*. *C. piscicola* UAL26, UAL26 pCV22 and UAL26 pCaT were effective at inhibiting most of the gram-positive indicator organisms tested, only *L. innocua* was not inhibited.

4.5. Sensitivity of *E. coli* O157:H7 to Colicin V

Fourteen different isolates of *E. coli* serotype O157:H7 were assayed for their sensitivity to partially purified colicin V compared with that of *E. coli* DH5α. The indicator *E. coli* DH5α was 32 times more sensitive to colicin V than all of the *E. coli* O157:H7 cultures tested, as determined by spot-on-lawn assay. In the deferred inhibition test, using the colicin V producer *C. piscicola* UAL26 pCV22, *E. coli* DH5α was strongly inhibited, indicated by large zones of inhibition, whereas all of the zones of inhibition against *E. coli* O157:H7 were smaller (Table 4.2.). Activity of colicin V against *E. coli* O157:H7 was confirmed by spot-on-lawn and broth tests with partially purified colicin V (100AU/ml).

4.6. Bacterial Growth and Bacteriocin Activity

Growth and bacteriocin activity studies were done in APT broth with approximately 5 log CFU/ml concentrations of colicinogenic *C. piscicola* UAL26 pCV22 and noncolicinogenic *C. piscicola* UAL26. Similar growth rates for *C.*

Table 4.2. Activity of colicin V producing *C. piscicola* UAL26 pCV22 against *E. coli* O157:H7 determined by deferred inhibition on APT agar at 25°C for 24 h.

Indicator Strain		Inhibitory activity*
		C. piscicola UAL26 pCV22
E. coli	DH5α	++
E. coli O157:H7	43895	+
	12096	+
	12902	+
	13025	+
	98.E3093	+
	98.E3139	+
	98.E3708	+
	98.E3744	+
	98.R967	+
	98.R993	+
	98.R1298	+
	98.R1303	+
	98.R1389	+
	98.R1719	+

* Diameter of the zone of inhibition appearing in the indicator overlay
10 to 14 mm = ++, 5 to 9 mm = +, <5 mm = -

piscicola UAL26 pCV22 and UAL26 were observed at 25°C (Figure 4.4). Both cultures reached maximum population of 9 log CFU/ml within 18 h. Colicin V was detected in all of the broth culture experiments containing the colicinogenic *C. piscicola* strain at all temperatures tested, 4, 7 and 25°C (Figures 4.5, 4.6, 4.7, respectively). Colicin V was detected by the middle to late exponential phase of growth and reached a maximum activity of 800 AU/ml at each of the incubation temperatures tested. Maximum population of 9 log CFU/ml was reached within 6 days, 4 days and 18 h at 4, 7 and 25°C, respectively. As expected, inhibitory activity was not detected in the control cultures containing noncolicinogenic *C. piscicola* UAL26.

4.7. Bacterial Growth in Different Broth Media

Different broth media were tested to determine which medium supported the growth of both *E. coli* indicator and *C. piscicola* colicin V producer organism. The media tested included: BHI, APT, MRS, TSB, CMM and NB. *C. piscicola* grew well in APT, BHI and TSB (data not shown) at 25°C and at 10°C. Colicin V activity levels after 24 h of growth in APT, BHI and TSB at 25°C were 400, 200 and 100 AU/ml, respectively. *C. piscicola* did not grow well in MRS, CMM and NB. *E. coli* DH5α grew well in BHI, CMM, TSB and NB at 25°C, but it did not grow well in APT or MRS. Both *E. coli* and *C. piscicola* grew well in BHI and this medium was selected for mixed culture experiments. BHI has a low glucose content (0.2%), which is close to that of ground beef (0.1%). *C. piscicola* UAL26 is sensitive to chloramphenicol and does not grow in broth or agar that contains greater than 5 mg of chloramphenicol

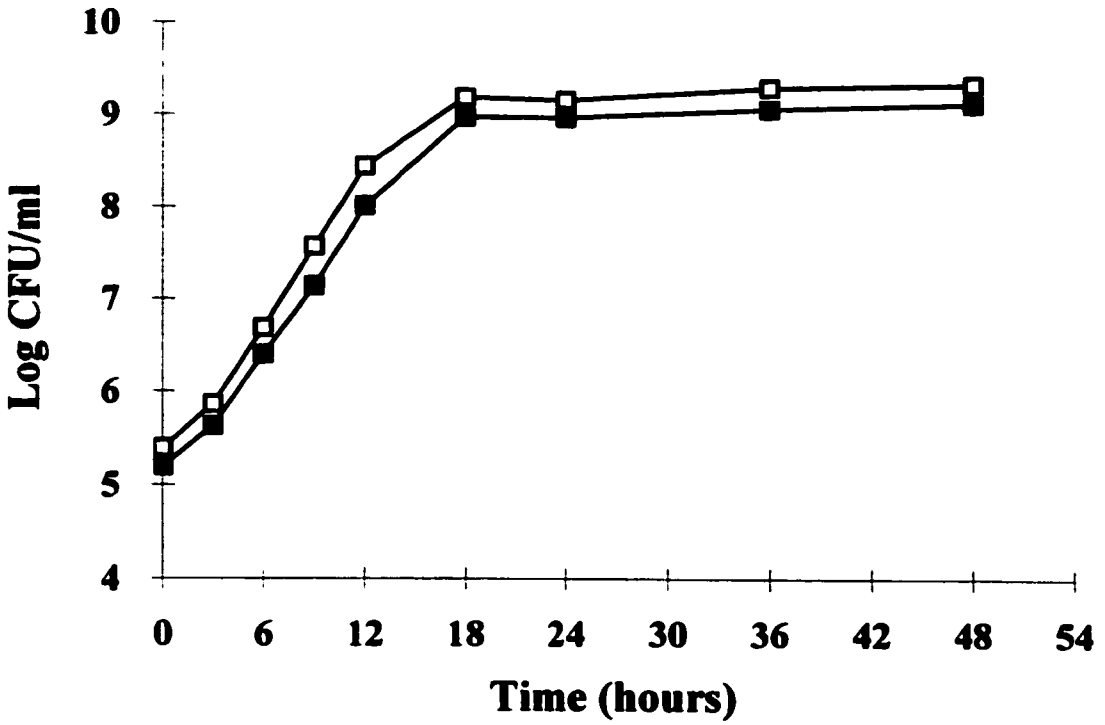

Figure 4.4. Typical growth curves for *C. piscicola* UAL26 (□) and *C. piscicola* UAL26 pCV22 (■) grown at 25°C in APT broth. Data based on two experiments.

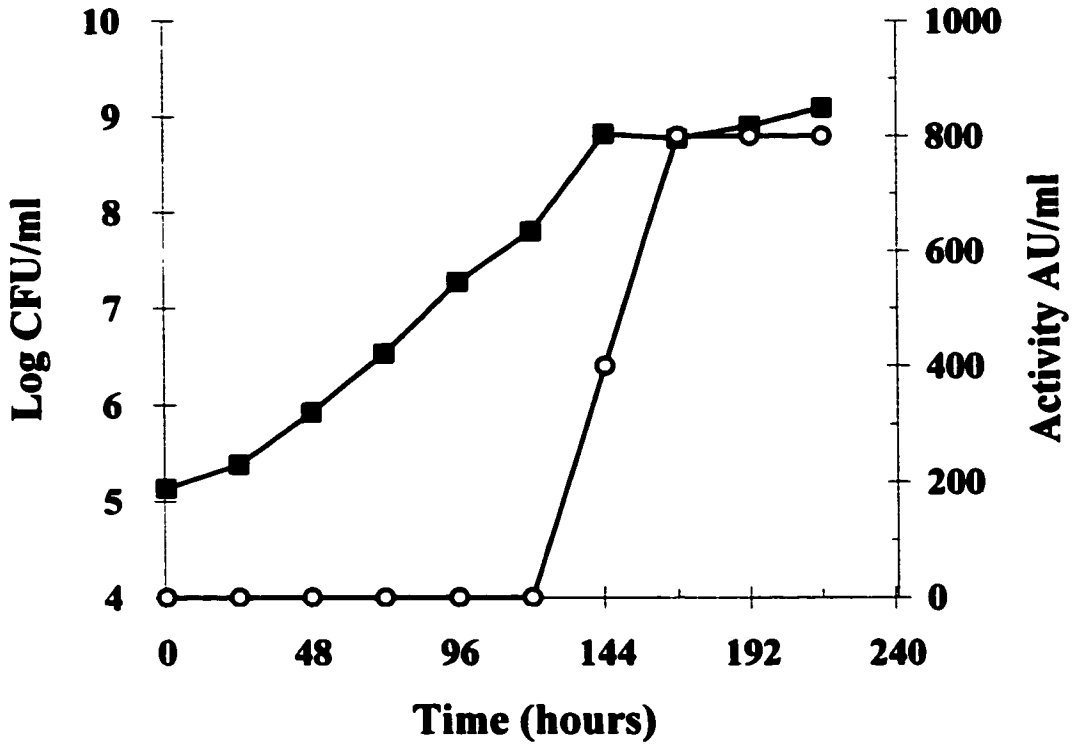

Figure 4.5. Representative data for colicin V activity and growth of *C. piscicola* UAL26 pCV22 grown at 4°C in APT broth. *C. piscicola* UAL26 pCV22 growth (■), colicin V activity (○) determined by spot-on-lawn with cell free supernatant against *E. coli* DH5α indicator. Data based on two experiments.

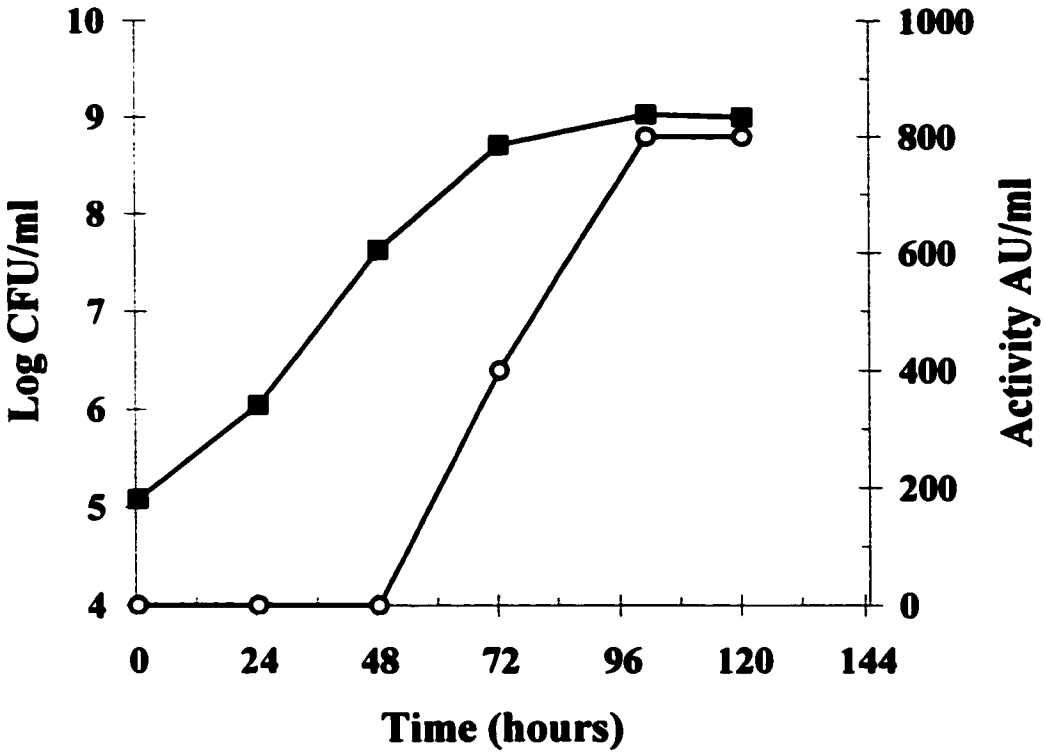

Figure 4.6. Representative data for colicin V activity and growth of *C. piscicola* UAL26 pCV22 grown at 10°C in APT broth. *C. piscicola* UAL26 pCV22 growth (■), colicin V activity (○) determined by spot-on-lawn with cell free supernatant against *E. coli* DH5α indicator. Data based on two experiments.

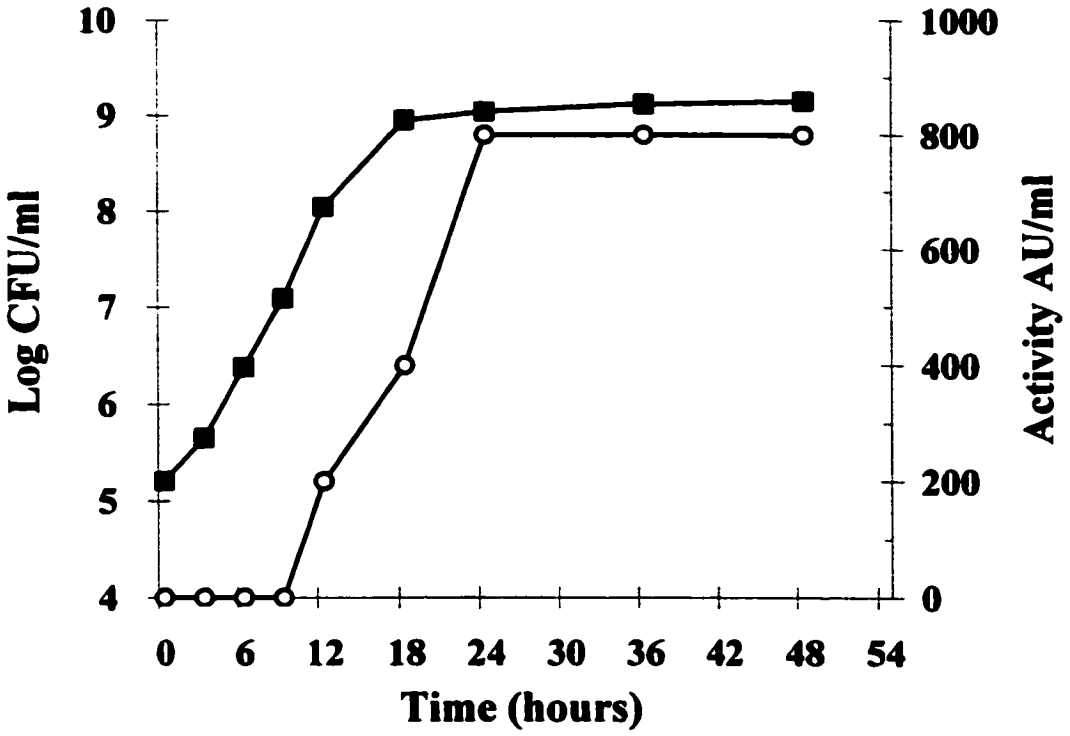

Figure 4.7. Representative data for colicin V activity and growth of *C. piscicola* UAL26 pCV22 grown at 25°C in APT broth. *C. piscicola* UAL26 pCV22 growth (■), colicin V activity (○) determined by spot-on-lawn with cell free supernatant against *E. coli* DH5α indicator. Data based on two experiments.

per ml, whereas *C. piscicola* UAL26 pCV22 was resistant to chloramphenicol. *C. piscicola* UAL26 pCV22 was inhibited by chloramphenicol at high concentrations (> 50 mg/ml).

4.8. Inhibition of *E. coli* by Partially Purified Colicin V in Broth

Growth and survival of *E. coli* DH5α in NB and PBS with or without partially purified colicin V treatment at 37°C is shown in Figure 4.8. Experiments were initiated by inoculating approximately 5 log *E. coli* CFU/ml. In NB the *E. coli* showed typical exponential growth and reached maximum population of 9 log CFU/ml within 24 h. The addition of 100 AU of colicin V per ml of NB caused the population to decrease to 2 log CFU/ml within 12 h. After 12 h, exponential growth resumed, reaching 9 log CFU/ml by 48 h. Cultures from this colicin V treatment were reinoculated (0.1%) into fresh NB with colicin V (100 AU/ml). In contrast to the first exposure to colicin V, the growth of *E. coli* was not affected by the colicin V and the growth curve was similar to that found in NB alone, indicating that the strain was colicin V resistant. As well, the colicin V activity in the cell-free supernatant before and after incubation had remained the same, indicating that activity is not lost during the incubation period. At sublethal concentrations of colicin V, resistant cells developed; however, at high colicin V concentration (1600 AU/ml) no cells survived and the development of resistant cells was not observed. As expected, *E. coli* did not grow in PBS. The addition of colicin V to resulted in a slow die off of *E. coli*, slowly reaching a minimum population of 2 log CFU/ml in 24 h.

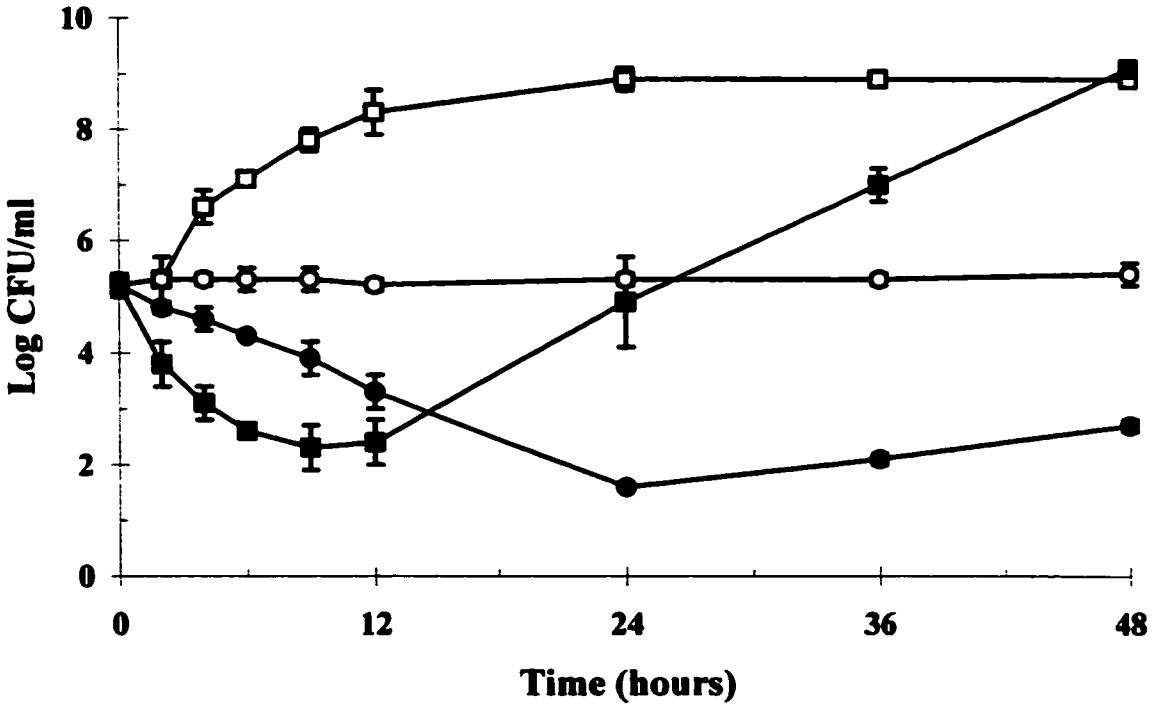

Figure 4.8. Growth and survival of *E. coli* DH5α in NB or in PBS with or without addition of partially purified colicin V. Cultures were incubated at 37°C with shaking. Growth in NB (□), in NB with 100 AU/ml colicin V (■), in PBS (○), in PBS with 100 AU/ml colicin V (●). Each data point is the mean of two experiments. Error bars represent the standard error of the mean log count.

The growth of a cocktail of four *E. coli* O157:H7 isolates (ATCC 12096, 12902, 13025, 43895) in NB or PBS with or without colicin V is shown in Figure 4.9. *E. coli* O157:H7 cocktail cultures in NB at 37°C reached maximum population of 9 log CFU/ml within 12 h. In contrast to *E. coli* DH5α, the *E. coli* O157:H7 strains were not as sensitive to the colicin V treatment. Within 6 h the population had decreased two log cycles to about 3 log CFU/ml. Similar to *E. coli* DH5α, the *E. coli* O157:H7 resistant cells resumed exponential growth, reaching maximum population within 24 h. *E. coli* O157:H7 did not grow in PBS and there was a 1 log decrease in viable count by 48 h. The PBS colicin V treatment did not have the apparent bactericidal effect on *E. coli* O157:H7 as seen on DH5α. To determine if one or all of the O157:H7 strains was responsible for the colicin V resistance the experiments were repeated using individual *E. coli* O157:H7 strains used in the cocktail. The results were similar for each of the four strains. Resistant cells were detected for each strain.

Experiments were done to determine if growth or colicin V production by *C. piscicola* UAL26 pCV22 was affected by the pH of the APT broth. Growth was monitored by absorbance and the cell-free supernatant was used to determine colicin activity with a spot-on-lawn assay. Maximum absorbance of 0.87 was measured after 48 h, as shown in Table 4.3. After 24 h of incubation, no growth was detected in APT at pH 5.0; however, a small amount of growth was detected after 48 h. The higher the pH the greater amount of activity was measured. Maximum activity of 800 AU/ml was detected in broth with initial pH 7.0 and 7.5 after 48 h of incubation.

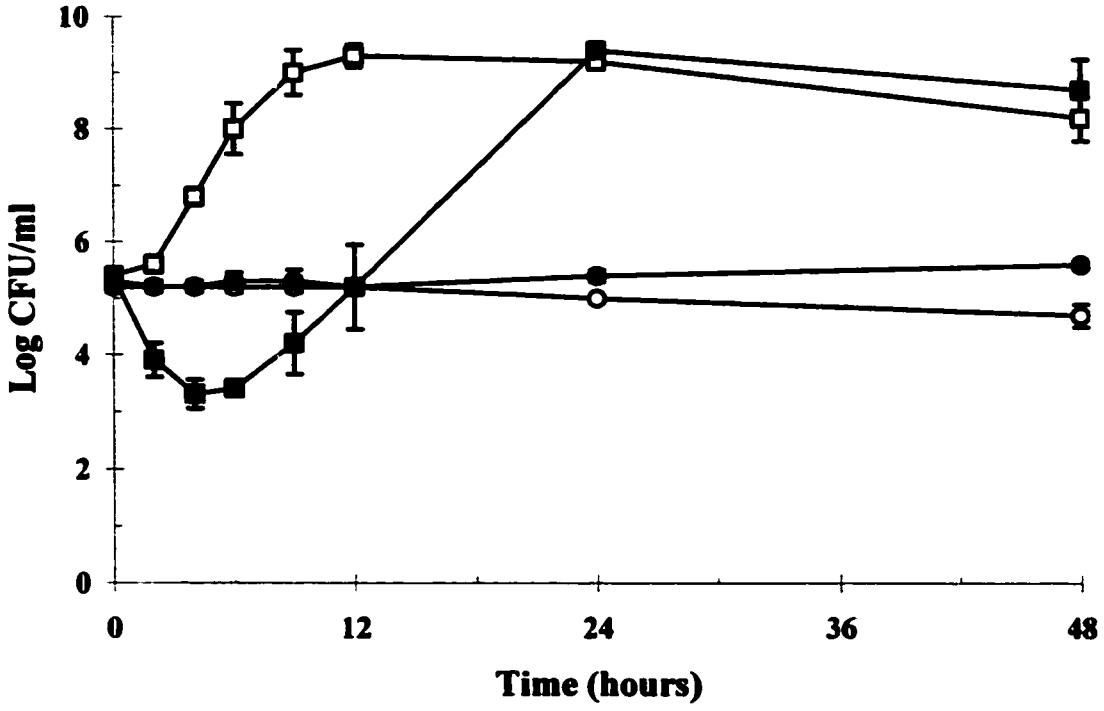

Figure 4.9. Growth and survival of *E. coli* O157:H7 cocktail in NB or in PBS with or without addition of partially purified colicin V. Cultures were incubated at 37°C with shaking. Growth in NB (□), in NB with 100 AU/ml colicin V (■), in PBS (○), in PBS with 100 AU/ml colicin V (●). Each data point is the mean of two experiments. Error bars represent the standard error of the mean log count.

Table 4.3. Growth and colicin V production by *C. piscicola* UAL26 pCV22 at different initial pH levels, in APT broth at 25°C. Absorbance was measured at 600 nm. Activity was measured by spot-on-lawn test using *E. coli* DH5α as the indicator organism.

pH	24 hours		48 hours*	
	Absorbance (600nm)	Activity (AU/ml)	Absorbance (600nm)	Activity (AU/ml)
7.5	0.84	400	0.87	800
7.0	0.74	400	0.70	800
6.5	0.75	400	0.78	400
6.0	0.61	400	0.65	400
5.5	0.31	200	0.70	200
5.0	0.00	0	0.24	200

4.9. Inhibition of *E. coli* by Colicin V producing *C. piscicola* UAL26 in Broth

Results of experiments done in BHI broth with or without added colicin V (200 AU/ml) with approximately equal (5 log CFU/ml) concentrations of *C. piscicola* and *E. coli* DH5α are shown in Figure 4.10. Colicin V was detected by the middle to late exponential phase of growth in all of the mixed culture experiments containing the colicinogenic *C. piscicola* Growth rates in BHI broth were similar for colicinogenic and noncolicinogenic *C. piscicola* when grown alone or in mixed culture with *E. coli*. After 12 h of incubation the colicinogenic *C. piscicola* had reached the late exponential phase of growth and colicin V activity (100 AU/ml) was detected in the culture medium (data not shown). After 48 h the activity had increased to 400 AU/ml. The *E. coli* in mixed culture with the colicinogenic *C. piscicola* initially increased about 1 log then slowly decreased to about 3 log CFU/ml. The death of *E. coli* was the result of colicin V activity, because population decrease was not observed in the presence of the noncolicinogenic *C. piscicola*. As observed in previous experiments, the addition of partially purified colicin V to a culture of *E. coli* resulted in an initial sharp decrease in viability followed by exponential growth. The concentration of colicin V added to the *E. coli* culture was the same at the end of the experiment (200 AU/ml) indicating that the growing cells are colicin V resistant. The numbers of *E. coli* treated with partially purified colicin V in mixed culture initially decreased by 3 log CFU/ml then the population remained constant. Both colicinogenic and noncolicinogenic *C. piscicola* inhibited the growth of colicin V resistant cells. The colicinogenic *C. piscicola* did not reduce the *E. coli* population any more than the noncolicinogenic *C. piscicola*.

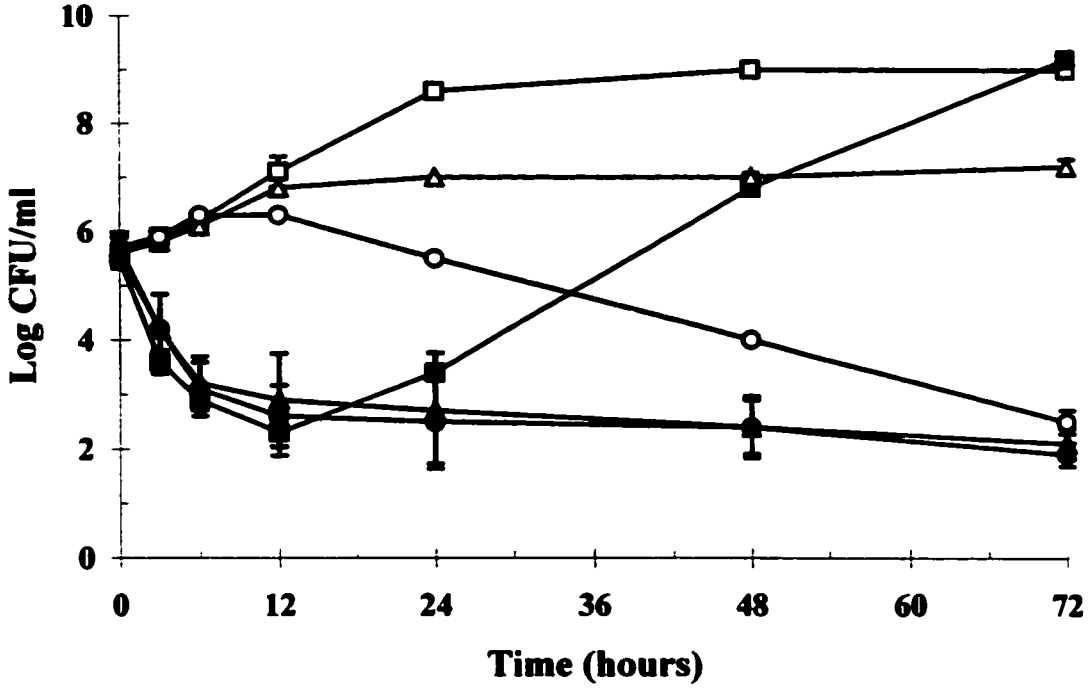

Figure 4.10. Growth and survival of *E. coli* DH5α in BHI broth at 25° C. Without addition of colicin V(□), with 200 AU/ml colicin V (■), with *C. piscicola* UAL26 (△), with *C. piscicola* UAL26 and 200 AU/ml colicin V (▲), with *C. piscicola* UAL26 pCV22 (○), with *C. piscicola* UAL26 pCV22 and 200 AU/ml colicin V (●). Each data point is the mean of two experiments. Error bars represent the standard error of the mean log count.

Experiments were also performed in meat modified BHI (10% aseptically prepared raw ground beef) and in BHI with or without the addition of colicin V or *C. piscicola* UAL26 pCV22. The activity level measured in BHI inoculated with *C. piscicola* UAL26 pCV22 was 400 AU/ml, whereas in meat modified BHI, the activity was 100 AU/ml after 24 h incubation, as shown in Table 4.4. Partially purified colicin V was added to BHI and meat modified BHI at a concentration of 200 AU/ml. Prior to incubation the concentration in BHI colicin V broth was determined to be 200 AU/ml, whereas in the meat modified BHI broth, the colicin V activity level was 800 AU/ml, which was much higher than expected. After 24 h of incubation the colicin V activity in the BHI colicin V broth had not changed, whereas in the meat modified BHI broth the colicin V activity had decreased from 800 to 100 AU/ml.

4.10. Inhibition of *E. coli* by Colicin V producing *C. piscicola* UAL26 in Ground Beef

The aseptically prepared ground beef used in the experiment was assayed for the number of indigenous LAB and *E. coli* on APT and NA, respectively. The same media and incubation conditions were used to enumerate pure and mixed cultures of *C. piscicola* and *E. coli* inoculated onto the ground beef. *C. piscicola* grew much better than *E. coli* on APT agar incubated at 15°C. *C. piscicola* formed large (3 mm) circular, cream-colored, opaque colonies whereas *E. coli* formed small (1 mm) circular, dull white, transparent colonies. *E. coli* grew well on NA incubated at 37°C, but *C. piscicola* failed to grow. *E. coli* formed large (2 mm) circular, dull white, opaque colonies on NA. Bacterial identity of randomly selected colonies was

Table 4.4. Effect of the addition of ground beef, *C. piscicola* UAL26 pCV22 and colicin V to BHI on pH and colicin V activity after incubation at 25°C for 24 h.

Sample	0 hour		24 hours	
	pH	Activity (AU/ml)	pH	Activity (AU/ml)
BHI broth (control)	7.6	0	7.6	0
Ground Beef [a]	7.2	0	6.5	0
Colicin V [b]	7.6	200	7.5	200
UAL26 pCV22 [c]	7.6	0	5.4	400
Ground Beef [a] + Colicin V [b]	7.2	800	6.5	100
Ground Beef [a] + UAL26 pCV22 [c]	7.2	0	6.2	100

a. 10% aseptically prepared raw ground beef added to BHI broth
b. 200 AU/ml colicin V added to BHI broth
c. *C. piscicola* UAL26 pCV22 grown in BHI broth

confirmed by gram (KOH method) and catalase reactions. The population of indigenous LAB and *E. coli* in the aseptically prepared ground beef was below the detection limits of the enumeration methods (<1 log CFU/g).

Aseptically-prepared ground beef was inoculated with approximately equal (5 log CFU/ml) concentrations of *C. piscicola* and *E. coli*. The indigenous LAB population was less than 1 log CFU/g in the control samples at time zero and grew to a maximum population of 8 log CFU/g after 7 days of storage at 10°C and 17 days of storage at 4°C. An indigenous *E. coli* population was not detected throughout the storage period. The growth of indigenous LAB is not likely to affect the counts of *C. piscicola* because there was a 4 log difference between them at time zero. Colicin V was not detected by spot-on-lawn and MALDI-TOF MS in any of the pure or mixed culture experiments containing the colicinogenic *C. piscicola*. It is not known if colicin V can be produced by *C. piscicola* UAL26 pCV22 in ground meat, if it is inactivated in the meat or if the levels are below the detection threshold. Growth rates of colicinogenic and noncolicinogenic *C. piscicola* when grown alone or in mixed culture with *E. coli* were similar. There was no reduction in numbers of *E. coli* at 4 or 10°C with the addition of either colicinogenic or noncolicinogenic *C. piscicola* (Figures 4.11 and 4.12, respectively). As expected, *E. coli* did not grow at 4°C. The addition of *C. piscicola* to ground beef inhibits the growth of *E. coli* at 10°C (Figure 4.12). The pure culture of *E. coli* increased by about 2 log CFU/g at 10°C by day 10 of storage and did not increase in numbers for 15 days of storage. The initial pH of the meat was 5.6 and the pH decreased to 4.9 by day 15 of storage at 10°C. The samples inoculated with *C. piscicola* had a slightly higher final pH 5.1 to 5.2.

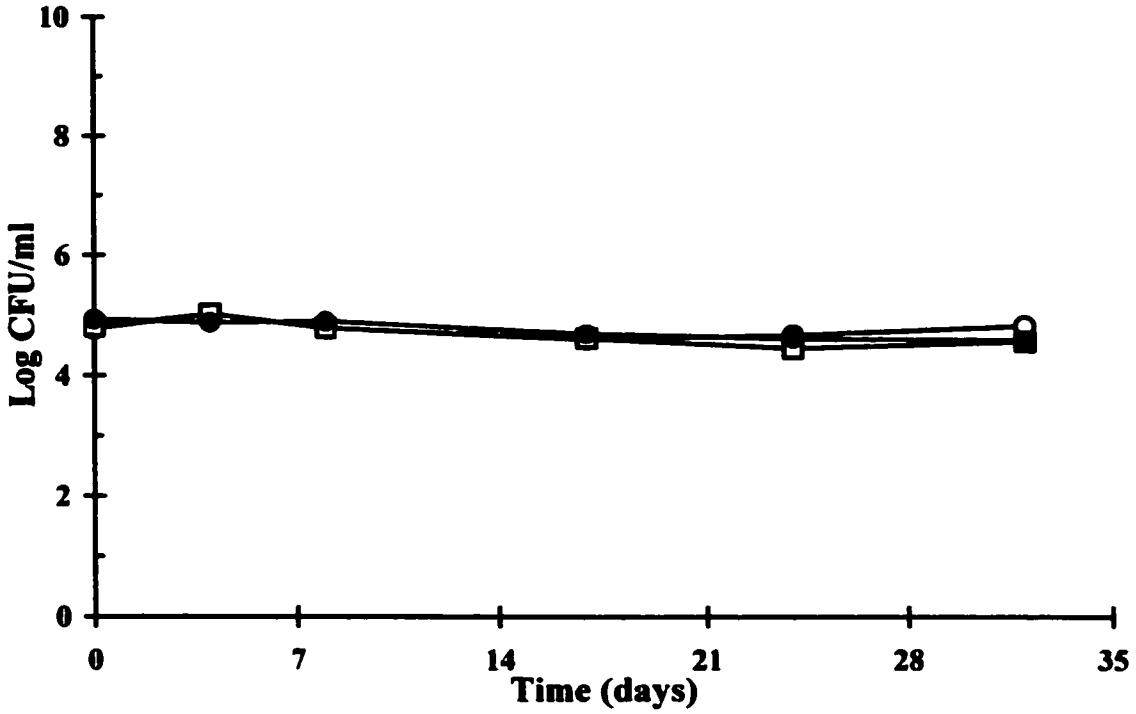

Figure 4.11. Survival of *E. coli* DH5α in aseptically prepared raw ground beef that was vacuum packaged and stored at 4°C, without addition (□), with *C. piscicola* UAL26 (○), with *C. piscicola* UAL26 pCV22 (●). Each data point is the mean of two samples.

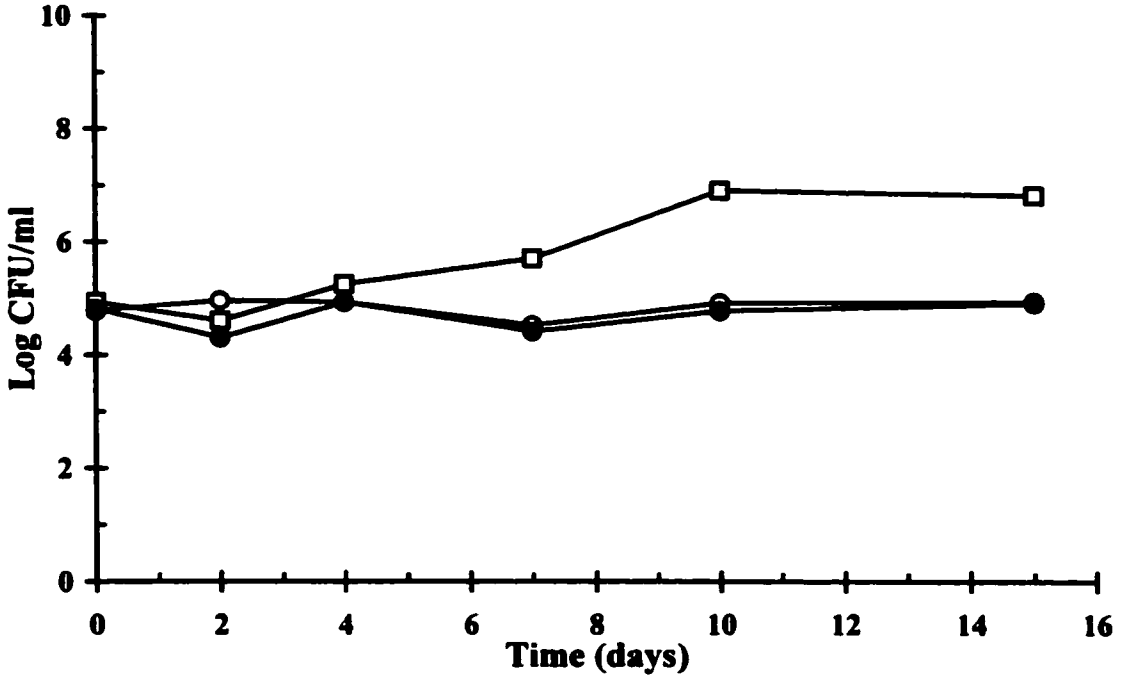

Figure 4.12. Growth and survival of *E. coli* DH5α in aseptically prepared raw ground beef that was vacuum packaged and stored at 10°C, without addition (□), with *C. piscicola* UAL26 (○), with *C. piscicola* UAL26 pCV22 (●). Each data point is the mean of two samples.

Experiments were done with the addition of partially purified colicin V and colicinogenic *C. piscicola* to raw ground beef. The addition of 400 AU/ml partially purified colicin V had no observable effect on the indicator organism at 4 or 10°C (data not shown). After the addition of partially purified colicin V the bacteriocin activity was initially detected in the meat; however, the bacteriocin activity decreased rapidly and it was not detected after 4 days of storage. Growth and survival of the indicator organism was the same when inoculated with colicinogenic or noncolicinogenic *C. piscicola.* As expected, there was no increase in the number of *E. coli* at 4°C. As observed previously, growth of colicinogenic *C. piscicola* did not decrease the number of *E. coli* surviving at either 4 or 10°C. Colicin activity was not detected in the meat samples that had been inoculated with colicinogenic *C. piscicola.*

5. DISCUSSION AND CONCLUSIONS

This study was done to investigate a novel method for controlling pathogenic *E. coli* in vacuum-packaged chill-stored meat. Meat can become contaminated with *E. coli* during slaughtering and processing. Preventing contamination of ground meat is difficult if not impossible, even under the strictest conditions of sanitation and hygiene. The consumption of only 10 to 100 cells of pathogenic *E. coli* O157:H7 can cause serious illness in the form of bloody diarrhea (hemorrhagic colitis) and hemolytic uremic syndrome (Armstrong *et al.*, 1996). The food industry relies on a number of safety "hurdles" to control growth of bacteria on raw meat, primarily refrigeration and modified atmosphere packaging including vacuum-packaging. Additional "hurdles" to control pathogenic *E. coli* in ground meat would enhance food safety and prevent foodborne disease.

The growth and dominance of indigenous LAB in vacuum-packaged meat during chilled storage contributes to the extension of shelf-life and increased food safety by competition and by the production of antagonistic bacterial compounds (Stiles, 1996). Important antagonistic compounds produced by LAB in meats are organic (lactic and acetic) acids and bacteriocins. Bacteriocins can play a major role in the control of bacterial pathogens in vacuum-packaged chill-stored meats. The current study investigated the efficacy of a colicin V-producing LAB to control *E. coli* O157:H7 in vacuum-packaged chill-stored meat.

Colicin V is a small proteinaceous bacterial toxin that is produced by many strains of *E. coli* and can kill closely related strains of *E. coli* (Waters and Crosa, 1991). In theory a colicin V-producing *E. coli* strain could be added to raw meat to

control pathogenic *E. coli*. However, it would probably not be acceptable to public health authorities to have *E. coli* intentionally added to raw meat. If *E. coli* or any other organism was to be added to meat, public health regulators would require evidence that the organism and the products that it produces are safe to consume in food and safe to release into the environment. In the case of *E. coli*, even if the specific strain was shown to be nonpathogenic it would probably still be unacceptable to most consumers. Furthermore, bacteriocin production by *E. coli* would not occur at typical chill storage temperatures (0 to 4°C) because the minimum growth temperature for *E. coli* is claimed to be 7.4°C (Palumbo *et al.*, 1995). Because of the many limitations of using *E. coli*, a plasmid containing the colicin V structural gene from *E. coli* was constructed and transformed into a suitable LAB host organism.

C. piscicola UAL26 was chosen as the host organism because it has many desirable characteristics. It grows well in vacuum-packaged chill-stored meat, it does not impart undesirable flavors or odors in meat products even after prolonged storage (Burns, 1987) and it would probably be considered Generally Recognized As Safe (GRAS) by government regulators. Leisner *et al.* (1995) reported that *C. piscicola* may not be a suitable antagonistic strain for extending the storage life of meat because the growth was unpredictable at 2°C and that off-odors were detected after 8 weeks of storage. In the current study, growth of *C. piscicola* UAL26 was predictable at 4 and 10°C. *C. piscicola* UAL26 was initially transformed with the plasmid pJKM37 (McCormick *et al.*, 1999), which contained the colicin V structural gene. In this study it was shown that the plasmid pJKM37 was unstable in *C. piscicola* UAL26 in the absence of erythromycin. In a study by Bohaychuk

(unpublished data), it was shown that pJKM67, which is based on the same vector as pJKM37, was unstable in *C. piscicola* UAL26. Because of the instability of pJKM37 in *C. piscicola* UAL26, this transformant could not be used and another plasmid, pCV22, was constructed and electrotransformed into *C. piscicola* UAL26 (Jiang, unpublished data). This study showed that the plasmid pCV22 was stable in the host organism *C. piscicola* UAL26 even after 100 generations at chill and abuse temperatures of 4, 10, 25°C; however, the plasmid was not stable at 37°C. The plasmid's instability at 37°C was not a concern, because the intended application of this colicin V-producing organism was at chill storage temperatures and not at high temperatures. Plasmid stability was determined by screening colonies for antibiotic resistance and bacteriocin activity. Both tests were done because it was not always possible to distinguish between plasmid-carrying and plasmidless organisms on the antibiotic-containing medium therefore colonies were also screened for bacteriocin activity using deferred inhibition assay. The presence of zones of inhibition indicated that colicin V was being produced therefore the plasmid must be present. As expected, all of the colonies that were antibiotic resistant also had inhibitory activity.

The pCV22 plasmid contains the *L. lactis* P32 promoter, the *C. divergens* divergicin A signal peptide, *E. coli* colicin V structural gene and the *L. plantarum* pCaT vector (see Figure 3.1, page 28) The divergicin A signal peptide allows for the export of colicin V via the general secretion pathway therefore dedicated transport protein genes are not required (McCormick *et al.*, 1999). The vector also contains chloramphenicol and streptomycin resistance genes. These genes are useful as genetic markers for isolation and plasmid stability experiments; however, the markers

would have to be removed if the organism was to be used in food products. It is interesting to note that an immunity gene for colicin V was not required because the host organism *C. piscicola* UAL26 is naturally resistant to colicin V. Most bacteriocins including colicin V are only active against closely related organisms (Tagg *et al.*, 1976), therefore it is not surprising that *C. piscicola* UAL26 was not affected by a bacteriocin produced by *E. coli*.

There have been a number of studies investigating the genetic determinants responsible for bacteriocin production, immunity and export (Allison *et al.*, 1995; Worobo *et al.*, 1995; van Belkum *et al.*, 1997; McCormick *et al.*, 1999). However, there have not been applied studies using a genetically modified LAB designed to produce a bacteriocin that specifically inhibits the growth of pathogenic *E. coli* in vacuum-packaged chill-stored meat.

C. piscicola UAL26 naturally produces a chromosomally-mediated bacteriocin that inhibits a broad spectrum of gram-positive organisms, but it is not active against gram-negative organisms. Inhibitory activity includes the important psychrotrophic pathogen *L. monocytogenes*. As expected, the incorporation of the pCV22 plasmid into *C. piscicola* UAL26 broadened the parent strain's inhibitory spectrum to include gram-negative *E. coli*. As a result, *C. piscicola* UAL26 pCV22 possesses the unique ability to control both *L. monocytogenes* and *E. coli*. Previous studies with nisin demonstrated activity against both *L. monocytogenes* and *E. coli* but only with the addition of a chelator (e.g., EDTA), which makes the gram-negative cell wall susceptible to nisin's bactericidal action (Cutter and Siragusa, 1995).

The bacteriocin produced by *C piscicola* UAL26 pCV22 was first detected in late log phase, which indicates that the cell population must reach a large enough population to produce detectable amounts of bacteriocin. In most cases bacteriocin production is greatest at the optimal growth temperature of the producing organism (Tagg *et al.*, 1976). *C. piscicola* UAL26 pCV22 produced colicin V at sub-optimal temperatures such as 4°C, provided that sufficient incubation time was given to allow for growth of *C. piscicola.* In this study it was demonstrated that the antagonistic activity of *C. piscicola* UAL26 pCV22 against *E. coli* was a proteinaceous bacteriocin because activity was lost with protease treatment. Numerous unsuccessful attempts have been made to characterize the bacteriocin produced by *C. piscicola* UAL26 (Rosario, 2001).

The indicator organism *E. coli* DH5α was much more sensitive (32 x) to the colicin V than all of the *E. coli* O157:H7 strains tested. Murinda *et al.* (1996) evaluated colicin V inhibitory activity against diarrheagenic *E. coli* and found only 2 of 11 (18%) O157:H7 strains were sensitive. In contrast, Bradley and Howard (1990) found 18 of 20 (90%) O157:H7 strains were sensitive to colicin V. Prior to conducting large meat experiments, preliminary experiments were done in broth. For preliminary experiments DH5α strain was used because it is very sensitive to colicin V and it is nonpathogenic therefore safer to use in the laboratory. For final studies, pathogenic strains of *E. coli* O157:H7 strains were used. The deferred inhibition assay was used to demonstrate that the 14 strains of *E. coli* O157:H7 used in this study were equally sensitive to colicin V.

Various methods to enumerate *C. piscicola* and *E. coli* in mixed culture were investigated. The method developed for this study was a rapid and simple method that was based on the different temperature and nutrient requirements of the organisms. *C. piscicola* is a psychrotroph, whereas *E. coli* is a mesophile. BHI broth was chosen as the medium to be used for co-incubation experiments because both *C. piscicola* and *E. coli* grew well in this medium. Of the media tested BHI and CMM broth most closely resemble meat extracts. BHI and CMM both have a very low glucose level that is comparable to the carbohydrate content of ground beef. Good growth was obtained in CMM; however, inconsistent enumeration data was obtained from this medium.

The addition of partially purified colicin V initially reduced the population of *E. coli* in broth incubated at 25°C. However, colicin V-resistant cells emerged and grew. Murinda *et al.* (1996) also reported the development of colicin V-resistant strains. Numerous studies have reported the development of resistance in *L. monocytogenes* to the bacteriocins: sakacin A (Schillinger *et al.*, 1991), nisin (Davies and Adams, 1994); and mesenterocin 52, plantaricin C19 and curvacin 13 (Rekhif *et al.*, 1994). A concern with bacteriocin resistance is that cross-protection may develop by subjecting *E. coli* to colicin V at non-lethal (stress) concentrations. Rowe and Kirk (1999) reported that a strain of *E. coli* showed a significantly greater resistance to salt (20% w/v) when prestressed at pH 4.0 using lactic acid as the acidulant.

It appeared that the *E. coli* DH5α did not need to be growing (actively dividing) to be affected by colicin V because cell death was observed in PBS, which does not contain nutrients for growth. There was no growth of *E. coli* DH5α when

incubated in PBS alone. In contrast, the strains of *E. coli* O157:H7 were not affected by colicin V in PBS.

In broth, the growth of colicinogenic *C. piscicola* resulted in a reduction in the number of viable *E. coli* compared with the samples inoculated with noncolicinogenic *C. piscicola.* The reduction observed was due to the production of bacteriocin and not due to competition for nutrients or lactic acid production and subsequent pH change. Once *C. piscicola* UAL26 reach maximum population the *E. coli* growth was inhibited but the number of viable cells did not decrease. The emergence of resistant strains is one of the concerns with the addition of colicin V; however, resistant cells were not observed when colicin V (200AU/ml) was added with either *C. piscicola* UAL26 pCV22 or UAL26. There was no added benefit using the colicinogenic strain in regard to preventing the emergence of resistant cells. The inhibition of the resistant cells is probably due to competitive exclusion and lactic acid production.

During storage at 10°C with the addition of either strain of *C. piscicola* the growth of *E. coli* was inhibited for up to 15 days in the ground beef compared with the control in which there was a 2 log CFU increase of *E. coli* per g of ground beef. Cell numbers did not decrease, as observed in the broth system with the colicinogenic strain. To apply this form of biopreservation it is important that the bacteriocin should reduce the number of pathogenic *E. coli* and not just inhibit their growth because normal refrigeration successfully inhibits growth. However, at abusive retail temperatures of >7°C, either inhibition or reduction of *E. coli* by the bacteriocin would be valuable. Under abusive retail temperatures (10°C), without added carnobacteria, *E. coli* grew slowly and reached a maximum population of 6.5 log

CFU/g during 15 days of storage. A possible explanation for the low final *E. coli* count could be the delayed inhibition caused by the growth of indigenous LAB in the meat. The aseptically-prepared ground beef initially contained a very low number of indigenous LAB, which would have little effect on the *E. coli* until they reached maximum population after 7 days at 10°C. The inhibition of growth was likely the effect of acid production or competitive exclusion from the indigenous microflora. In contrast to the indigenous LAB population, indigenous *E. coli* was not detected during the entire storage period indicating that *E. coli* was either below the detection level or that the indigenous LAB inhibited the growth of *E. coli*. Results from this study showed that addition of bacteriocin directly to meat containing *E. coli* did not affect the viability of the *E. coli* cells. Even with the addition of high bacteriocin concentrations (1600 AU/ml) the *E. coli* cells remained unaffected in the ground beef.

Colicin V production could not be detected in ground beef inoculated with the colicinogenic *C. piscicola* at any of the temperatures tested. Even after 10 days of storage at 10°C, bacteriocin production was not observed in the meat. It is likely that the bacteriocin is being produced but that it is being inactivated by meat components or by proteolytic activity in the meat. The ineffectiveness of nisin in meat products was attributed to poor solubility, food proteases, binding or reaction with proteins or phospholipids, high bacterial loads and poor distribution throughout the meat product (Bell and De Lacy, 1986; Henning *et al.*, 1986; Cutter and Siragusa, 1994; Stringer *et al.*, 1995). However, Rose *et al.* (1999b) determined that the inactivation of nisin was due to an enzymatic reaction with glutathione in fresh meat.

To investigate the inactivation of colicin V, different concentrations of partially purified colicin V were added to meat. At high concentrations, the colicin V could be detected in the fresh meat; however, over time the activity level decreased.

Rose *et al.* (1999a) reported that the use of MALDI-TOF MS is an effective method for detecting bacteriocins in culture supernatant of producer organisms and suggested that this method may have the potential to determine the fate of bacteriocins in food. In this study we successfully used MALDI-TOF MS to detect colicin V produced by colicin V-producing *C. piscicola* in broth media; however, we were not able to detect colicin V produced by colicin V-producing *C. piscicola* in meat extracts.

The pH of meat did not decrease as much as would be expected with other LAB. Carnobacteria do not reduce the pH of media to the same extent as other LAB. The initial pH of untreated ground beef was 5.6 and the pH decreased to 4.9 by day 15 of storage at 10°C due to acid production by the indigenous LAB. Samples inoculated with *C. piscicola* had a slightly higher final pH 5.1 to 5.2. Similar results for pH decline were observed by day 32 of storage at 4°C. Brashears and Durre (1999) reported that *L. lactis* was effective at reducing the population of *E. coli* in a broth system and that pH was primarily responsible for the inhibition that was reported (final pH 4.3).

In spite of excellent sanitation/hygiene practices, spoilage and pathogenic microorganisms will be introduced into ground meat products during processing. Therefore it is necessary to identify and develop innovative methods or procedures for eliminating these contaminating organisms especially a pathogen with such a low

infective dose as *E. coli* O157:H7. *C. piscicola* UAL26 pCV22 possessed characteristics that made it interesting to study as a prototype for use in meat systems.

Although we failed to kill *E. coli* in a meat system with *C. piscicola* UAL26 pCV22, the effective inhibition and killing of *E. coli* in a broth system would justify further study of inhibition in a ground beef system. Colicin V was not detected in the meat incubated with the colicinogenic *C. piscicola* strain and this could be a factor of concentration or inactivation of colicin V in raw meat. Furthermore, in the broth system, resistance of *E. coli* to colicin V was observed at sublethal bacteriocin concentrations, and further studies are needed to investigate the effect of colicin V concentration on development of resistant strains.

The pCV22 construct with its P32 promoter, divergicin A signal peptide and pCaT vector were successful genetic components for the stable production of colicin V in a LAB; however, the colicin V structural gene and the resultant bacteriocin was not as effective as had been expected. It is suggested that the colicin V structural gene be replaced with another bacteriocin gene that is more effective against *E. coli* O157:H7 in a ground beef system. In addition, a broad-spectrum bacteriocin gene or a multiple gene cassette could be introduced that would be effective against a number of different pathogens including other gram-negative foodborne pathogens such as *Salmonella* and *Shigella*.

The use of a genetically modified organism of this type in meat could raise many questions about consumer acceptability of the product and its safety. Future studies must consider the safety aspect if the product is to be approved and marketable. The use of a genetically modified bacteria would require a biosafety

assessment with regard to the safety of the host organism, potential to colonize the gut, horizontal gene transfer in the gut and food, and safety and origin of vectors, markers and foreign genes (Lindgren, 1999; Klijn *et al.*, 1995). The strategy of using a genetically modified LAB such as *C. piscicola* UAL26 pCV22 to control the growth of gram-negative pathogens such as *E. coli* O157:H7 has the potential for improving food safety in vacuum-packaged chill-stored meats.

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