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# INHIBITION OF SPOILAGE AND PATHOGENIC BACTERIA BY A BACTERIOCIN PRODUCING STRAIN OF *LEUCONOSTOC GELIDUM*

BY

BEVERLY J. UIBEL

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND RESEARCH IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN

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INHIBITION OF SPOILAGE AND PATHOGENIC BACTERIA BY A BACTERIOCIN PRODUCING STRAIN OF *LEUCONOSTOC GELIDUM*

SUBMITTED BY BEVERLY J. UIBEL IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF MASTER OF SCIENCE

IN FOOD MICROBIOLOGY

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## ABSTRACT

A lactic acid bacterium, identified as *Leuconostoc gelidum*, was isolated from processed meat packaged in a modified atmosphere. This bacterium can grow at refrigerator temperatures, and in BM broth with 5% NaCl and 200 ppm nitrite. It produces a bacteriocin-like substance which inhibits the growth of many other lactic acid bacteria and strains of *Listeria monocytogenes*.

*Leuc. gelidum* grown in APT broth at 4 and 10°C, dominated in a mixed culture fermentation with strains of *Listeria monocytogenes* which were bacteriocin-sensitive. A mutant of *Leuc. gelidum* which did not produce bacteriocin was used as a control to determine the inhibitory role of the bacteriocin. Decrease in pH of the growth medium due to lactic acid production by *Leuc. gelidum* assisted inhibition. When grown in association with bacteriocin-resistant cultures of *Salmonella typhimurium* at 4 and 10°C and *Pseudomonas fluorescens* at 10°C, *Leuc. gelidum* dominated due to the inhibitory effect of lactic acid rather than the action of bacteriocin. *S. typhimurium* was inhibited to a greater extent than psychrotrophic *P. fluorescens* probably due to the slow growth of salmonella at the low incubation temperatures. Growth of *Leuc. gelidum* was not affected by growth of the competing bacteria.

Addition of protease to a culture of *Leuconostoc gelidum* and *Listeria monocytogenes* at 4 or 10°C did not decrease the inhibitory effect of bacteriocin against the sensitive bacteria. This occurred even though the protease was active at these temperatures and when it was added to supernatant fluids of *Leuc. gelidum* growth of the bacteriocin sensitive organism was not inhibited. Growth of the sensitive bacterium was inhibited when grown in bacteriocin containing supernatant fluids of *Leuc. gelidum*, but subsequent growth occurred with exhaustion of bacteriocin supply.

A bacteriocin-producing organism which can predominate over spoilage flora and inhibit pathogenic flora at cold temperatures has potential for commercial use in modified atmosphere packaged meats.

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## GLOSSARY OF ABBREVIATIONS OF GROWTH MEDIA

| Abbreviation | Medium | Characteristics |
|---|---|---|
| APT | All Purpose Tween medium | Supportive growth medium |
| MRS 5.6 | Lactobacilli deMan Rogosa Sharpe medium pH adjusted to 5.6 | Selective medium for lactic acid bacteria |
| Oxford agar | Oxford agar | Selective medium for *Listeria* spp. |
| TSA | Tryptic Soy Agar | Supportive growth medium |
| TSB | Tryptic Soy Broth | Supportive growth medium |
| BGA | Brilliant Green Agar | Selective medium for *Salmonella* spp. |
| CFC agar | Cephaloridine, Sodium fusidic acid, Centrimide agar | Selective medium for *Pseudomonas* spp.(Mead and Adams, 1977) |

# 1. INTRODUCTION

Carbon dioxide can be used to slow the growth of bacteria (Coyne, 1933). This principle is used in modified atmosphere packaging (MAP) to prolong the storage life of foods. Modified atmosphere storage of meats was used in the 1930s for transoceanic shipment of meat (Wolfe, 1980). Development of new plastic materials with defined gas transmission rates has allowed the use of smaller packages for modified atmosphere storage of foods.

There are two ways in which packaging atmospheres can be modified: vacuum packaging, in which meat is packaged in oxygen impermeable bags with the air drawn out (Seideman and Durland, 1984); and gas flushing, in which the initial gas content of the package is adjusted with or without drawing an initial vacuum. Another process used in transportation and storage of foods is controlled atmosphere, in which specific concentrations of selected gases are maintained throughout the storage period (Wolfe, 1980). The bacterial flora of a food can be affected by changes in the gas atmosphere.

Meat is a food rich in the nutrients which readily support the growth of microorganisms (Bacus and Brown, 1981). During slaughter and handling processes, meat becomes contaminated with spoilage microorganisms and may also be contaminated with pathogenic organisms. Control of these bacteria is essential to extend shelf-life and to ensure safety of meat. Refrigeration of food is important in slowing the grow of bacteria, but addition of antibacterial compounds such as salt and nitrite to processed meats, is also needed to inhibit some bacteria (Bacus and Brown, 1981). Modified atmosphere packaging of meat is another method which can be used to inhibit the growth of bacteria, yeasts and molds (Wolfe, 1980).

Psychrotrophic Gram-negative bacteria predominate and rapidly spoil meat stored in air at 7°C (Reddy *et al.*, 1970). Growth of these bacteria is inhibited by increased carbon dioxide ($CO_2$) and decreased oxygen ($O_2$) atmospheres (King and Nagel, 1967), while lactic acid bacteria are able to grow and predominate the microflora of fresh and

processed meat (Pierson *et al.*, 1970; Blickstad and Molin, 1983). Lactic acid bacteria do not discolor the surface of meat and are slower growing than pseudomads, therefore meat spoils more slowly (Seideman and Durland, 1984). Growth of lactic acid bacteria on fresh meat produces souring that is less noticeable to consumers than the proteolytic degradation products of aerobic spoilage (Reddy *et al.*, 1970). Processed meat stored for 35 days in modified atmospheres was judged by sensory evaluation as acceptable (McMullen and Stiles, 1989).

Lactic acid bacteria are Gram-positive, non-motile and non-spore forming bacteria which produce lactic acid as the main end product of sugar fermentation (Orla-Jensen, 1919). The lactic acid bacteria include the genera *Lactobacillus, Leuconostoc, Pediococcus, Lactococcus* and *Carnobacterium* (Hurst and Collins-Thompson, 1979; Collins *et al.*, 1987).

An increasing demand for extended shelf-life food has increased the need to control bacterial spoilage of meats. Addition of lactic starter cultures to meat products packaged in modified atmospheres would control the fermentation of a product provided they have the ability to predominate the microflora. Inhibitory metabolic end-products produced by lactic acid bacteria can repress the growth of competing microflora (Hurst and Collins-Thompson, 1979), but some of these compounds may cause undesirable changes in meat. Addition of a fermentation liquor containing bacteriocin-like substances produced by *Lactococcus diacetilactis* did not affect the sensory quality of meat (Branen *et al.*, 1975) and enabled a bacterium to predominate over organisms sensitive to that bacteriocin (Reddy *et al.*, 1971). Workers with dairy bacteria observed dominance of bacteriocin-producing bacteria in a mixed population (Baribo and Foster, 1951). Meat lactics were reported to inhibit closely related bacteria on solid media (Ahn and Stiles, in press), but whether bacteriocin production by meat lactics is advantageous and can ensure dominance of the producing organism in mixed fermentation remains uncertain.

The object of this study was to determine the ability of the bacteriocinogenic lactic acid bacterium, *Leuconostoc gelidum* UAL 187, to control or inhibit the growth of potential spoilage and pathogenic bacteria in mixed culture in a broth medium. Pathogenic and spoilage bacteria, sensitive and resistant to the bacteriocin, were used as competitors with *Leuconostoc gelidum* UAL 187. The influence of the bacteriocin was determined using a bacteriocin-free mutant as a control.

# 2. LITERATURE REVIEW

## 2.1. Microflora of meat

The initial microflora of fresh meat includes Gram-positive mesophilic bacteria, mainly *Micrococcus*, *Staphylococcus*, and *Bacillus*, with small concentrations of Gram-negative psychrotrophic organisms (Gill, 1985). Refrigeration temperatures favor growth of psychrotrophic bacteria, allowing rapidly growing pseudomonads to outgrow other psychrotrophic organisms and dominate the microflora of meat (Gill, 1985). Bacteria typically present on aerobically packaged refrigerated meats include *Pseudomonas*, *Acinetobacter*, *Moraxella*, and *Alcaligenes* (Kraft, 1986). Proteolytic breakdown by these psychrotrophic bacteria causes spoilage of the meat, production of off-odors and slime (Kraft, 1986). Smaller concentrations of Gram-positive bacteria, *Corynebacterium*, *Microbacterium*, *Arthrobacter*, and members of the family Enterobacteriaceæ may also be present in the flora of refrigerated meat (Kraft, 1986).

Lactic acid bacteria are also found on meat. Morishita and Shiromizu (1986) studied these lactic acid bacteria and identified 690 isolates of lactobacilli from unpackaged meats and meat products. The predominant lactic acid bacteria were: *Lactobacillus curvatus*, *Lact. sake*, *Lact. crispatus*, *Lact. salivarius*, *Lact. fermentum* and *Lact. viridescens*. Small numbers of *Lact. coryniformis* subsp. *torquens*, *Lact. casei*, *Lact. plantarum*, *Lact. brevis* and *Lact. confusus* were also found. Only 2% of the isolates in this study were identified as Leuconostoc.

## 2.2. Aerobic and anaerobic packaging of meat

Meat can be packaged aerobically or anaerobically in modified atmospheres. Aerobic packaging uses materials which are oxygen permeable so the internal atmosphere is identical to the ambient (Pierson *et al.*, 1970). With modified atmosphere packaging the gas concentration in the package is different from ambient; the atmosphere within the

package is usually higher in $CO_2$ (Christopher *et al.*, 1979). Advantages of modified atmosphere packaging of meats are: extended shelf-life; maintenance of high quality; inhibition of bacteria and fungi; and reduced economic loss (Wolfe, 1980). Disadvantages include: 'added cost, undesirable color changes in red meats, equipment and training requirements, atmosphere maintenance and requirement of temperature regulation' (Wolfe, 1980).

## 2.3. Lactic acid bacteria in packaged meat

Development of vacuum or modified atmosphere (anaerobic) packaging of meats, in which lactic acid bacteria become the predominant bacteria, has increased interest in the lactic acid bacteria in meats (meat lactics). Hitchener *et al.* (1982) and Shaw and Harding (1984) reported that lactobacilli predominate the lactic acid bacteria isolated from vacuum-packaged meat and meat products. Two groupings within the category lactobacilli are obligate heterofermentative bacteria or betabacteria, and facultative homofermentative bacteria or streptobacteria (Snead *et al.*, 1986). Another bacterium often present in low numbers on fresh anaerobically stored meats is leuconostoc, a heterofermentative coccus-shaped lactic acid bacterium (Hitchener *et al.*, 1982).

Hitchener *et al.* (1982) noted the presence of *Leuconostoc mesenteroides* and many atypical lactobacilli on vacuum-packaged fresh meat which could not be identified as any known species. The atypical bacteria were described as atypical betabacteria and atypical streptobacteria. Atypical betabacteria were heterofermentative lactobacilli similar to *Lactobacillus brevis* or *Lact. viridescens* and were identified by their ability to produce only L(+) lactic acid (Hitchener *et al.*, 1982). Atypical streptobacteria were homofermentative lactobacilli similar to *Lact. plantarum* that produced both isomers of lactic acid and did not ferment lactose or maltose (Hitchener *et al.*, 1982). The atypical betabacteria were the main component of the microflora comprising 65%, the streptobacteria were second in predominance comprising 25%. *Leuconostoc*

*mesenteroides* was a small component comprising only 10% of the microflora. In contrast, Mol *et al.* (1971) found atypical streptobacteria as the main bacteria on vacuum-packaged cooked meat. *Leuconostoc mesenteroides* comprised only 1% of all bacteria (Mol *et al.*, 1971). The low percentage of *Leuconostoc mesenteroides* and betabacteria on cooked vacuum-packaged meats may have resulted from higher sensitivity of these bacteria to cooking temperatures.

Further study of the taxonomy of lactic acid bacteria from vacuum-packaged fresh meats and bacon by Shaw and Harding (1984) reported atypical lactic acid bacteria which could not be identified as any previously known species. These bacteria were placed into three clusters or groupings: two groups of non-gas producing streptobacteria (clusters I and II) and *Leuc. paramesenteroides* and unidentifiable *Leuconostoc* strains (cluster III). The streptobacteria were separated by differences in guanine plus cytosine (G+C) content and acid tolerance. Cluster I organisms were unidentifiable with any known species. They were acid sensitive and had a G+C content of 33.2 - 36.9 moles%. Cluster II organisms were identified as acid tolerant *Lactobacillus sake*-like or *Lact. bavaricus*-like organisms with a G+C content of 40.7 - 43.7 moles%. The dominant proportion of the lactic flora was composed of atypical streptobacteria; leuconostocs were found in low concentrations (8-13%) on all fresh meat products but none was isolated from the processed bacon (Shaw and Harding, 1984).

Schillinger and Lücke (1987a) identified lactobacilli in fresh and processed meat. The predominant bacteria were streptobacteria (83%); the majority were identified as *Lactobacillus sake* and *Lact. curvatus*. Bacteria were isolated from a variety of sources, therefore, the flora is not a representative sample of a meat product. Schillinger and Lücke (1987b) characterized the lactic acid bacteria present on vacuum-packaged fresh beef and pork. The majority of the isolates were streptobacteria (47%) and leuconostoc (29%). The remainder were betabacteria (10%) and lactococci (14%). Schillinger and Lücke (1987b) stated that the variation of lactic acid bacteria isolated from vacuum-packaged meats and

meat products was more representative of the variation of abbatoir microflora than any other factor. Therefore, the lack of consensus in the literature regarding predominant bacteria in vacuum-packaged fresh or processed meats was attributed to this factor.

New species of *Lactobacillus* and *Leuconostoc* have been characterized from the atypical bacteria isolated from meat and meat products. Holzapel and Gerber (1983) named a new species of heterofermentative betabacteria, *Lactobacillus divergens*. This bacterium was isolated frequently from vacuum-packaged fresh meat (Schillinger and Lücke, 1987a). *Lactobacillus divergens* was classified with *Lactobacillus piscicola* into a new genus, *Carnobacterium* (Collins *et al.*, 1987). These bacteria differ from *Lactobacillus* sp. on the basis of DNA-DNA hybridization. Shaw and Harding (1985; 1989) characterized and named several new species of lactic acid bacteria: *Lactobacillus carnis*, *Leuconostoc gelidum* and *Leuc. carnosum*. *Lact. carnis* is frequently isolated from vacuum packaged meat (Schillinger and Lücke, 1987a). Using the criteria of Shaw and Harding (1984) this bacterium would be classified within cluster I non-aciduric streptobacteria. It produces small amounts of gas from glucose and has low molar ratios of acetate plus ethanol to lactate suggesting a metabolism that differs from typical heterofermentative lactobacilli (Shaw and Harding, 1985). *Lactobacillus carnis* was named before the genus *Carnobacterium* was proposed. *Lact. carnis* has been reclassified as *Carnobacterium piscicola* based on DNA homology and relatedness of biochemical and chemical data (Collins *et al.*, 1987). *Leuconostoc carnosum* and *Leuc. gelidum*, newly described species isolated from fresh meat (Shaw and Harding, 1989), were differentiated from other *Leuconostoc* sp. by DNA homology, acid fermentation profiles and fatty acid analysis.

## 2.4. Inhibitory substances of lactic acid bacteria

Lactic acid bacteria produce inhibitory substances which may hinder growth of other bacteria, enabling the lactics to predominate (Hurst and Collins-Thompson, 1979).

This phenomenon was first demonstrated by Rogers (1928), who noted the inhibition of *Lactobacillus bulgaricus* by *Lactococcus lactis*. Inhibitory substances which may be produced by lactic acid bacteria include: organic acids, hydrogen peroxide ($H_2O_2$), ammonia, free fatty acids, bacteriolytic enzymes and bacteriocins (Wannamaker, 1980).

The definition of bacteriocins of Gram-positive bacteria has changed over time. In the 1960s bacteriocins were not differentiated from antibiotics and both were investigated and suggested for food use (Marth, 1966). Harold (1970) described bacteriocins as 'antibiotics which interact with highly specific membrane receptors'. In the mid 1970s, bacteriocins were defined as biologically active protein moieties with narrow spectra of bactericidal activity (Tagg *et al.*, 1976). Specific cell receptors for bacteriocin attachment and plasmid-borne location of the genetic determinants of bacteriocins were not yet proven for Gram-positive bacteria, but they were assumed from reports of these characteristics of bacteriocins produced by Gram-negative bacteria (Tagg *et al.*, 1976). Subsequently, Klaenhammer (1988) described bacteriocins of lactic acid bacteria as "proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium".

Interest in bacteriocin-producing bacteria initially involved Gram-negative bacteria. Bacteriocins produced by *Escherichia coli*, named colicins, inhibit closely related bacteria, usually other *E. coli*. Colicin can kill sensitive cells without inhibiting the producer bacterium. However, production of colicin has not been linked to domination *in vivo* (Wannamaker, 1980; Govan, 1986). The mode of action of colicins has been established (Konisky, 1979; 1982). Colicins enter the bacterial cell through established receptors, for example, the receptor used by colicin E is the same receptor used for uptake of vitamin $B_{12}$ (Konisky, 1979). The mode of action of bacteriocins of lactic acid bacteria is not well understood. Davey (1981) reported that absorption of diplococcin produced by *Lactococcus cremoris*, to the cell wall, is non-specific, and a two stage killing mechanism was hypothesized. The initial absorption to the cell is followed by transfer of a lethal

message to specific biochemical targets. The stage of growth of the target organism is important. Cells in the log phase of growth were more sensitive to diplococcin than cells in stationary phase of growth.

Bacteriocins of Gram-positive bacteria have been recognized for many years but, until recently, their characterization and study of their mode of action received little interest (Tagg *et al.*, 1976; Davey, 1981). Oxford (1944) investigated inhibition by lactic acid bacteria and concluded that repression of growth of a sensitive bacterium was due to a low molecular weight protein produced by the inhibiting bacterium. An inhibitory substance produced by *Lactococcus lactis*, nisin, was concentrated and examined for its inhibitory spectrum (Mattick and Hirsch, 1944, 1947; Hirsch *et al.*, 1951). Nisin inhibits clostridia and prevents germination of clostridial spores (Hirsch *et al.*, 1951; Eastoe and Long, 1959). Hurst (1981) proposed addition of nisin to processed to meats lower the amount of nitrite necessary to prevent clostridial spore germination. Nisin has been utilized as a food preservative but only for dairy products and canned foods (Hurst, 1981).

Only dairy lactic acid bacteria were recognised as bacteriocin producers (Marth, 1966). Many researchers investigated the inhibitory action of dairy starter cultures to explain the slow ripening of cheese or inhibition of bacterial spoilage, and hypothesized that domination was due to inhibition by antibiotic substances (Baribo and Foster, 1951; Lightbody and Meanwell, 1955; Mather and Babel, 1959). Hurst (1973) reviewed the literature on the production of bacteriocins by bacteria commonly found in foods. He concluded that the production of bacteriocin by meat lactics needed more investigation. Bacteriocin production was hypothesized because the level of inhibition could not be attributed to decrease in pH or $H_2O_2$ present in the growth medium. Tagg *et al.* (1976) published a comprehensive review of Gram-positive bacteriocin-producing bacteria in which they outlined properties, methods of detection, and genetic determinants of bacteriocins. They concluded that more research was necessary to determine the genetic

control, mode of action and the biological significance of bacteriocins of Gram-positive bacteria.

Hurst (1981) reviewed the literature on nisin emphasizing its biology, chemistry, synthesis by the cell and potential use in food to control microbial cultures. Nisin was not effective in controlling spoilage in meat products, but he considered that its ability to inhibit the outgrowth of *Clostridium botulinum* spores and lactic spoilage organisms in these products warrants further investigation. Hurst (1983) reviewed the literature on inhibitory spectra of lactic acid bacteria and the competitive ability of bacteriocin-producing dairy bacteria in mixed fermentations. *Leuconostoc citrovorum* produced inhibitory substances but no bacteriocin. The only lactic acid bacteria that had been reported to produce bacteriocins were lactobacilli and streptococci (Hurst, 1983). Klaenhammer (1988) reviewed the literature on mode of action, production, expression and genetics of bacteriocins of lactic acid bacteria and proposed the use of bacteriocin-producing bacteria in foods to 'promote competition of the desired microorganism and provide natural agents for food preservation'. The study of genetic control of bacteriocins of lactic acid bacteria to create broad spectrum bacteriocin-producing bacteria which are more competitive and have improved survival characteristics in food was proposed. The genetic control of bacteriocin production may be plasmid mediated (Davey, 1984; Graham and McKay, 1985; Daeschel and Klaenhammer, 1985; Harmon and McKay, 1987; Hoover *et al.*, 1988) or chromosomally determined (Joerger and Klaenhammer, 1986).

Antibacterial activity of lactic acid bacteria from meat was reported by Ahn and Stiles (in press). The influence of growth medium on bacteriocin production and ability of some organisms to produce bacteriocin early in the growth cycle and at refrigerator temperatures was noted. Early production of bacteriocin at refrigeration temperatures could enhance dominance of specific lactics in meat packaged under modified atmospheres. Ahn and Stiles (in press) proposed that lactic acid bacteria isolated from meat are more likely to predominate in meat environments. Spriggs (1986) questioned the use of

producer and sensitive bacteria isolated from different environments in studies of bacteriocins and dominance. He suggested that factors other than bacteriocin production might influence dominance.

Hastings and Stiles (in press) studied a bacteriocin-like inhibitory substance produced by a lactic organism isolated from processed meat packaged in a modified atmosphere. The organism was classified as *Leuconostoc gelidum* according to the description by Shaw and Harding (1989). Most inhibition by leuconostoc bacteria has been attributed to acetate or diacetyl production (Klaenhammer, 1988), but Orberg and Sandine (1984) and Hastings and Stiles (in press) independently isolated leuconostocs that produce bacteriocin-like substances. An inhibitory spectrum which includes lactic acid bacteria as well as *Enterococcus fæcalis* and *Listeria monocytogenes* was reported for the bacteriocin from *Leuconostoc gelidum* UAL 187 (Hasting and Stiles, in press).

An evolutionary role for development of bacteriocin production has been suggested (Hirsch, 1952). Bacteriocins are generally active against closely related organisms and their evolutionary role may be due to competition for the same environmental niche (Govan, 1986; Klaenhammer, 1988). Wannamaker (1980) examined bacterial competition in the human body, emphasizing the role of bacteriocins in predominance of streptococci in the throat. Many studies have examined the antagonistic or bacteriocin-like inhibition of lactic acid bacteria isolated from different sources: the human body (Mårdh and Soltész, 1983; Skarin and Sylwan, 1986; Silva *et al.*, 1987), dairy products (Davey, 1984; Joerger and Klaenhammer, 1986; Harmon and McKay, 1987; Andersson *et al.*, 1988), vegetables (Graham and McKay, 1985; Daeschel and Klaenhammer, 1985) and meat (Hoover *et al.*, 1988; Schillinger and Lücke, 1989; Ahn and Stiles, in press; Hastings and Stiles, in press).

## 2.5. Lactic acid bacteria in mixed culture

Growth of two different bacteria in a mixed culture may result in inhibition or stimulation of growth of one of the bacteria or no effect on either of the bacteria. Production of inhibitory compounds and decrease in pH of the medium occur with growth of lactic acid bacteria. These conditions can inhibit many microorganisms present in foods (Karunaratne *et al.*, 1990; Ashenafi and Busse, 1989; Schillinger and Lücke, 1989).

### 2.5.1. Inhibition of *Pseudomonas* spp. by lactic acid bacteria

Growth of pseudomonads may be inhibited by organic acids (Pinheiro *et al.*, 1968) and $H_2O_2$ (Price and Lee, 1970; Raccach and Baker, 1978b; Collins and Aramaki, 1980) produced by lactic acid bacteria. Other workers reported no effect on growth of pseudomonads on meat when grown with lactic acid bacteria (Reddy and Chen, 1975). Raccach and Baker (1978b) reported that the inhibition of pseudomonads could not be accounted for by $H_2O_2$ alone. Other workers suggested that inhibitory or bacteriocin-like substances produced by lactic acid bacteria contributed to inhibition of pseudomonads (Branen *et al.*, 1975; Dubois *et al.*, 1979). The lactics that were reported to produce antimicrobial substances were *Lactococcus lactis*, *Lactococcus diacetylactis* and *Leuconostoc citrovorum*. The bacteriocin produced by *Lactococcus lactis* is nisin. *Lactococcus diacetylactis* has been reported to produce a protein containing inhibitory substance but the inhibitory effect of *Leuconostoc citrovorum* is probably caused by acid (Klaenhammer, 1988).

Pseudomonads can affect the growth and metabolic function of other bacteria. Collins-Thompson *et al.* (1973) reported a decrease in growth rate, salt tolerance and enterotoxin production of *Staphylococcus aureus* when grown in the presence of *Pseudomonas aeruginosa*. Freedman *et al.* (1989) found that lower iron concentration in the growth medium could increase antagonism by pseudomonads. Inhibition of growth was affected by a bacteriocin-like substance and competition for iron.

### 2.5.2. Inhibition of *Salmonella* spp. by lactic acid bacteria

*Salmonella* spp. is a common pathogen causing foodborne illness associated with meat consumption in the United States (Bryan, 1980). This organism is frequently isolated from raw meat; therefore, inhibition of its growth in meat products could decrease the incidence of foodborne illness associated with consumption of meat. *Salmonella* can be inhibited by addition of $H_2O_2$ or acid directly to meat (Mulder *et al.*, 1987). Differences in inhibition can be observed with addition of different acids used to adjust pH (Speck, 1972; Rubin *et al.*, 1982). Acetic acid was most inhibitory, followed by lactic acid and hydrochloric acid. Lactic acid is one of the main fermentation products of lactic acid bacteria (Hurst and Collins-Thompson, 1979).

Inhibition of salmonella occurs when they are grown together with lactic acid bacteria (Daly *et al.*, 1972; Ashenafi and Busse, 1989; and Nout *et al.*, 1989) or in supernatant fluids from lactic acid bacterial cultures (Sorrells and Speck, 1970). The decrease in pH of the medium which occurs with the growth of *Lactococcus diacetylactis*, *Lactobacillus plantarum*, *Leuconostoc citrovorum* and other non-specified lactic acid bacteria causes the inhibition (Sorrells and Speck, 1970; Daly *et al.*, 1972; Ashenafi and Busse, 1989; Nout *et al.*, 1989). Inhibition of salmonella by lactic acid bacteria is caused [illegible]ly by decrease in pH of the growth medium (Park and Marth, 1972) but, volatile fa[illegible] possibly bacteriocins produced by the lactic acid bacteria may also con[illegible]e inhibition (Gilliland and Speck, 1972, 1977; Raccach and Baker, 1978a; Raccach *et al.*, 1979). The production of bacteriocins by *Pediococcus cerevisiæ*, *Lactobacillus plantarum* (Raccach and Baker, 1978a,b) and *Lactobacillus acidophilus* (Gilliland and Speck, 1972) was hypothesized as the mechanism of inhibition of salmonella as this could not be explained by $H_2O_2$ or acid present in the growth medium. Decrease in temperature and available oxygen may increase inhibition of salmonella by lactics (Chung and Goepfert, 1970). Addition of substances to the growth medium, for

example, 0.3% glucono-delta-lactone and 2.5% nitrate or 5% garlic may cause greater inhibition than lactic acid bacteria alone (El-Khateib and El-Rahman, 1987).

### 2.5.3. Inhibition of *Listeria monocytogenes* by lactic acid bacteria

*Listeria monocytogenes*, a foodborne pathogen often found on meat (Skovgaard and Morgan, 1988) can survive thermal processing to an internal temperature of the meat of 62.8°C in meat (Glass and Doyle, 1989a) and may grow at refrigeration temperatures (Walker *et al.*, 1990). The ability of *List. monocytogenes* to grow on meat and meat products can depend on the meat type. *List. monocytogenes* does not grow on ground beef or liver due to lack of needed nutrients (Shelef, 1989; Johnson *et al.*, 1988; Gouet *et al.*, 1978). Glass and Doyle (1989b) reported growth of *List. monocytogenes* at 4.4°C on ham, bologna, bratwurst, sliced chicken and turkey, and some wiener products but, not on summer sausage, cooked roast beef and other wiener products. Products with pH 6 or greater support growth of *List. monocytogenes*, while products with pH 5 or less do not, therefore growth on meat is limited by pH (Glass and Doyle, 1989b).

Consumption of meat contaminated with *List. monocytogenes* has been associated with listeriosis, a form of meningitis affecting humans (Schwartz *et al.*, 1988; Health and Welfare Canada, 1990). The presence of *List. monocytogenes* on meat may be a concern for food safety. To improve safety of meat products a method to suppress the growth of *List. monocytogenes* at refrigeration temperatures is needed. Inhibition of the growth of *List. monocytogenes* is observed when they are grown in association with lactic acid bacteria. Production of acid by lactic acid bacteria and subsequent decrease in pH inhibits the growth of *List. monocytogenes* in milk (Schaack and Marth, 1988 a,b; Papageorgiou and Marth, 1989 a,b). In blue cheese, the growth of *Penicillium roqueforti* increases the pH of the cheese which results in improved survival of *List. monocytogenes* (Papageorgiou and Marth, 1990b). pH values below which growth of *List. monocytogenes* does not occur range from values pH 4.75 (Schaack and Marth, 1988a) to

pH 5.0 (Glass and Doyle, 1988; Papageorgiou and Marth, 1989b). Gouet *et al.* (1978) reported that *List. monocytogenes* is inhibited by *Lactobacillus plantarum* without a decrease in pH which suggests inhibition by bacteriocin.

Wilkinson and Jones (1977) studied the taxonomy of *Listeria* and related bacteria. These authors suggested that *Lactobacillus* and *Listeria* are similar and that *Listeria* should be included with *Lactobacillus* in the family Lactobacillaceae. As bacteriocins have a narrow spectrum of activity affecting similar organisms (Klaenhammer, 1988) the similarity between *Listeria monocytogenes* and lactic acid bacteria makes susceptibility of listeria to bacteriocins produced by lactics a possibility.

Various lactic acid bacteria produce bacteriocins antagonistic to *List. monocytogenes* when tested on solid media (Hoover *et al.*, 1988; Harris *et al.*, 1989; Carminati *et al.*, 1989; Raccach *et al.*, 1989). Inhibition by a bacteriocin-producing strain of *Lactobacillus acidophilus* was observed in milk (Raccach *et al.*, 1989). Pucci *et al.* (1988) reported inhibition of *List. monocytogenes* in APT broth and cheese foods with addition of dried supernatant fluids from a bacteriocin-producing culture of *Pediococcus acidilactici*. Berry *et al.* (1990) reported that *List. monocytogenes* was inhibited in sausage by associative growth with a bacteriocin-producing strain of *Pediococcus* sp. Attachment of *List. monocytogenes* to the surfaces of fresh meat is inhibited by treatment of the meat with a bacteriocin produced by *P. acidilactici* (Nielsen *et al.*, 1990).

Harris *et al.* (1989) and Schillinger and Lücke (1989) tested antimicrobial spectra and competitive abilities of lactic acid bacteria. Bacteriocin-producing lactic acid bacteria inhibited growth of some strains of *Listeria monocytogenes* and outgrew bacteriocin-sensitive bacteria to dominate the microflora (Harris *et al.*, 1989; Schillinger and Lücke, 1989). Supernatant fluids of lactic acid bacteria inhibited the growth of some bacteria (Schillinger and Lücke, 1989). Growth of *List. monocytogenes* on solid medium was inhibited by supernatant fluids of bacteriocin-producing *Lactobacillus sake* but not by the

supernatant fluids of the bacteriocin-free mutant (Schillinger and Lücke, 1989). Bacteriocin was considered to be responsible for the inhibition of the listeria.

*Pseudomonas fluorescens* inhibits growth of *List. monocytogenes* Scott A in tryptose broth and chicken loaf (Farrag and Marth, 1989; Ingram *et al.*, 1990) but stimulates growth of *List. monocytogenes* in ground beef (Gouet *et al.*, 1978). *List. monocytogenes* can use the degradation products of proteolytic bacteria for growth (Shelef, 1989).

### 2.5.4. Effect of other bacteria on growth of lactic acid bacteria

Stimulation of growth of lactic acid bacteria by *List. monocytogenes* was reported by Gouet *et al.* (1978), who found that high concentrations of *List. monocytogenes* inoculated in ground beef stimulated the growth of *Lactobacillus plantarum.* Litopoulou-Tzanetaki (1987) observed stimulation of the growth of *Pediococcus pentosaceus* when grown together with *Salmonella typhimurium. Bacillus* sp. was reported to inhibit lactic acid production by dairy lactic starter cultures (Martin *et al.*, 1962), but inhibition of growth of the lactic organisms was not reported.

The ability of lactic acid bacteria to inhibit similar bacteria and to dominate a population was observed as early as the 1920s. Researchers have investigated effects of inhibitory compounds produced by lactic acid bacteria on growth of sensitive bacteria. However, the inhibitory activity of a bacteriocin-producing lactic acid bacterium versus the ability of the bacterium to inhibit other bacteria without the bacteriocin has only recently been compared. Few studies have examined the effect of a bacteriocin producer on pathogens which are not sensitive to the bacteriocin. This study examines the ability of a bacteriocin-producing leuconostoc and a bacteriocin-free mutant to inhibit pathogenic and spoilage bacteria sensitive and insensitive to the bacteriocin.

# 3. MATERIALS AND METHODS

## 3.1. Bacterial cultures

The *Leuconostoc gelidum* strain UAL 187 used in this study was isolated from processed meat by the food microbiology laboratory at the University of Alberta The bacteriocin-free mutant strain UAL 187-13 was cured of the plasmid that mediates bacteriocin production (Hastings and Stiles, in press). The non-lactic strains used for competitive growth studies were: (i) *Pseudomonas fluorescens* ATCC 13525; (ii) *Salmonella typhimurium* ATCC 13311; and (iii) *Listeria monocytogenes* ATCC 15313 and strain Scott A.

The lactic acid bacteria used as indicators of bacteriocinogenic activity were: *Carnobacterium piscicola* UAL 8, *Carnobacterium divergens* UAL 9, *Lactobacillus sake*-like organism UAL 12, *Leuconostoc* sp. UAL 13 (all donated by Dr. B.G. Shaw, AFRC Institute of Food Research, Langford, Bristol, U.K.), *Lactobacillus sake*-like organism UAL 3 isolated at the University of Alberta from raw meat, *Leuconostoc mesenteroides* ATCC 23386 (UAL 15), *Lactobacillus plantarum* ATCC 4008 (UAL 16), *Lactobacillus viridescens* ATCC 12706 (UAL 17), *Pediococcus acidilactici* ATCC 8042 (UAL 18), and *Pediococcus parvulus* ATCC 19371 (UAL 19).

The non-lactic indicator strains were: *Enterococcus fæcalis* ATCC 19433, *Enterococcus fæcium* ATCC 19434, *Enterococcus fæcium (durans)* ATCC 11576, *Staphylococcus aureus* ATCC 25923, *Bacillus cereus* NCDO 572, *Pseudomonas fluorescens* ATCC 13525, *Escherichia coli* ATCC 11775, *Klebsiella pneumoniae* ATCC 13883, *Salmonella typhimurium* ATCC 13311, *Serratia liquifaciens* ATCC 27592, *Listeria monocytogenes* ATCC 15313 and Scott A, *Listeria innocua* ATCC 33090, *Brochothrix thermosphacta* ATCC 11509, *Yersinia enterocolitica* ATCC 23715, *Escherichia coli* meat isolate 1840 and *Klebsiella pneumoniæ* meat isolate 2 isolated at the University of Alberta from raw meat (Stiles and Ng, 1980), and *Brochothrix thermosphacta* meat isolates BT 1

and BT 2 (Dr. G.G. Greer, Agriculture Canada, Lacombe Research Station, Lacombe, Alberta).

## 3.2. Maintenance and growth of bacterial strains

The lactic acid bacteria were stored in cooked meat medium (Difco Laboratories Inc., Detroit, MI) held at 4°C and subcultured every 3 months. Prior to use, the cultures were grown in APT broth (Difco) for 18-24 h at 25°C with one additional subculture in the same broth. The non-lactic strains were stored on tryptic soy agar slants (Difco) at 4°C and subcultured every 3 months. Prior to use, the cultures were subcultured first in tryptic soy broth and then in APT broth and incubated for 18-24 h at 25°C in both media.

Competitive growth tests were done in APT broth adjusted to pH 5.4 or 6.5. Lactic acid bacteria were enumerated on lactobacilli MRS agar (Difco) adjusted to pH 5.6 with 85% lactic acid. *Salmonella typhimurium* was enumerated on brilliant green agar (Difco); *Pseudomonas fluorescens* on cephaloridine, sodium fusidic acid, centrimide (CFC) agar (Mead and Adams, 1977); *Listeria monocytogenes* on Oxford agar (Oxoid Canada Inc., Nepean, Ont.). All experiments were also enumerated on tryptic soy agar (Difco).

## 3.3. Methods for detection of inhibitory substances produced by lactic acid bacteria

Testing for bacterial inhibition was done according to methods reviewed by Tagg *et al*. (1976).

### 3.3.1. Direct and deferred antagonism

Test strains were grown in basal medium (BM) broth (Wilkinson and Jones, 1977) for the initial experiments and APT broth for the remainder of the experiments and incubated at 25°C for 20-24 h. These broth cultures were used to spot "producer strains" onto the surface of APT agar with a Cathra replicating inoculator (KVL Laboratory,

Cambridge, Ontario, Canada). The inoculum was dried in a laminar flow hood, after which, the plates were either (a) overlaid with 6 mL of soft APT agar (0.75%) inoculated with 60 µL of an indicator organism (direct antagonism); or (b) incubated at 25°C for 20 - 24 h before being overlaid as described above (deferred antagonism). The overlayer had a bacterial concentration of approximately $10^6$ CFU/mL. The inoculated plates were incubated anaerobically in an atmosphere containing 10% $CO_2$ and 90% $N_2$ for 24 h. After 24 h incubation, the plates were examined for zones of inhibition in the indicator lawn surrounding each test strain. Deferred antagonism was also tested at 4 and 10°C and incubated for 1 week. Plates were periodically checked for zones of inhibition.

### 3.3.2. Test for inhibitory substances in supernatant fluids

Preparation of the supernatant fluids was as follows: bacteriocin producing strains grown in APT broth at 25°C for 24 h were centrifuged (12,100 x g for 10 min at 4°C). The supernatant fluids were decanted then adjusted to pH 6.5 with 5 N NaOH. These supernatant fluids were sterilized by one of two methods, either heated to 62°C for 30 min or mixed 1 part chloroform with 4 parts supernatant fluids, allowed to stand 5 min and centrifuged to separate the chloroform. A 50 µL sample of sterile supernatant fluids was placed in a 9 mm well in an APT agar plate and allowed to diffuse into the medium. This well plate was overlaid with 17 mL of soft APT agar, seeded with 170 µL of a 24 h culture of indicator bacterium, and incubated at 25°C for 24 h under anaerobic conditions. After 24 h, the indicator lawn was examined for zones of inhibition surrounding the wells. Supernatant fluids of *Leuconostoc gelidum* UAL 187, 187-13 and 187 with protease added, grown at 10°C for one week, were prepared at 0°C and overlaid with *Listeria monocytogenes* ATCC 15313. These plates were incubated at 10 C for five days and checked for zones of clearing.

### 3.3.3. Detection of bacteriophage in the supernatant fluids

With one exception, this procedure was identical to the test for inhibitory substances in supernatant fluids. Before overlayering, the well plate was inverted into another Petri dish, exposing the reverse side of the medium. The reverse side was then overlaid with soft APT agar containing an indicator bacterium ($10^6$ CFU/mL). The plates were incubated anaerobically at 25°C for 24 h, after which they were examined for zones of inhibition in the indicator lawn surrounding the wells.

### 3.3.4. Characterization of the bacteriocin

Catalase (Sigma Diagnostics, Mississauga, Ont., Canada) and protease from *Streptomyces griseus* (Sigma) were added to different samples of pH-adjusted, chloroform-sterilized supernatant fluids to give final concentrations of 100 U/mL and 1 μg/mL, respectively. These supernatant fluids were incubated at 35°C for 1 h and spotted onto APT plates which were subsequently overlaid with soft APT agar containing the indicator organism at $10^6$ CFU/mL. The plates were incubated anaerobically at 25°C for 24 h and examined for zones of inhibition.

### 3.3.5. Test for time of bacteriocin production

Producer bacteria were inoculated into APT broth at approximately $10^7$ CFU/mL and incubated at 25°C. Samples were removed at specific time intervals. Supernatant fluids were prepared as described above, heat sterilized, and tested against an overlayer of indicator cells to determine bacteriocin production.

## 3.4. Tests to characterize *Leuconostoc gelidum* UAL 187

The organism was characterized using methods described by Shaw and Harding (1984, 1985, 1989).

All UAL 187 inocula used for the following tests were grown in BM broth at 25°C for 24 h. All of the media were prepared from Difco ingredients.

(i) Gram stain. A 24 h culture of UAL 187 incubated in BM broth at 25°C was used for this test.

(ii) Gas production from glucose in MRS broth without ammonium citrate. This medium was prepared in test tubes containing inverted Durham vials. UAL 187 was inoculated into the broth and held at 25°C for 7 d. Periodically the Durham vials were examined for gas production.

(iii) Growth on acetate agar adjusted to pH 5.6. UAL 187 was streaked onto the surface of the acetate agar. The plates were incubated anaerobically at 25°C and examined for growth after 24 and 48 h.

(iv) Reduction of TTC (2,3,5-triphenyltetrazolium chloride) in BM agar. UAL 187 was streaked onto a BM agar plates containing 0.01% TTC. The plates were incubated at 25°C, and examined after 24 and 48 h for reduction of the TTC, indicated by a red coloration surrounding the bacterial growth.

(v) Production of ammonia from arginine. UAL 187 was grown in BM broth with 0.05% glucose for 48 h at 25°C. After 48 h, an aliquot of this broth was mixed with Nessler's reagent (BDH Chemicals, Toronto, Ont., Canada) and checked for production of ammonia by the immediate development of an orange pigment.

(vi) Growth at pH 3.9. Ability to grow in APT broth, with phosphate omitted, containing 2% glucose and adjusted to pH 3.9 with 85% lactic acid. UAL 187 was inoculated into this broth and incubated at 25°C for 7 d.

(vii) Terminal pH after 7 d growth in MRS broth without phosphate and with 0.3% sodium citrate adjusted to an initial pH 6.8 (La broth). After 7 d growth in La broth at 25°C the pH of the medium was determined using an Extech pH meter, model 671 (Boston, MA., USA).

(viii) Carbohydrate fermentation. Carbohydrate fermentation patterns were determined on BM agar prepared according to Shaw and Harding (1985), containing filter-sterilized 0.004% chlorophenol red and a final concentration of 0.5% carbohydrate

(carbohydrate fermentation agar). The carbohydrates chosen were amygdalin, arabinose, cellobiose, galactose[1], glucose, glycerol, inositol, lactose, maltose, mannitol, mannose, melezitose, melibiose, raffinose, rhamnose, ribose, salicin, sorbitol, sucrose, trehalose, and xylose. The test organisms were inoculated onto carbohydrate fermentation agar plates with a replicating inoculator (Cathra). The plates were dried in a laminar flow hood and incubated anaerobically at 25°C. The plates were checked for carbohydrate fermentation after 24 and 48 h. A positive result was recorded when color change of the medium surrounding the colony was greater than the color change seen on the control plate which contained no carbohydrate.

(ix) Production of D (-) lactate. This test was performed on UAL 187 by Hasting and Stiles ( in press).

(x) Maximal temperature of growth in BM broth. The test organisms were incubated at 1, 4, 15, 25, 30, 35, 40 and 45°C. Increased turbidity were checked visually for 3 d.

(xi) Dextran production from sucrose. The test organisms were grown on MRS Agar without glucose with 5% sucrose sucrose. (Schillinger and Lücke, 1987a).

(xii) Assay for bacteriocin activity. pH neutralized, chloroform-sterilized supernatant fluids of *Leuc. gelidum* UAL 187 were grown at 25°C for 36 h. The activity of the supernatant fluids was determined by 1:1 serial dilutions of the supernatant fluids spotted onto APT agar. The highest dilution that formed an inhibitory zone in overlays of *Carnobacterium divergens* UAL 9 was recorded. The reciprocal of this dilution was reported as the arbitrary activity units (AU/mL) of UAL 187 bacteriocin activity.

(xiii) Production of inhibitory compounds at low pH. APT broth adjusted to pH 5.5 and 5.0 with 85% lactic acid was inoculated with $10^4$ CFU of *Leuc. gelidum* UAL 187/mL of broth and incubated at 25°C for 48 h. At specific time intervals samples were

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[1] The testing for this characteristic was preformed by John Hastings at the University of Alberta.

removed and assessed for pH and bacteriocin production. Assessment of pH was with an Extech pH meter. Bacteriocin assessment was by the previously described well plate technique overlaid with UAL 9.

## 3.5. Ability of *Leuconostoc gelidum* UAL 187 to survive conditions simulating those found in processed meats

The test media used for this experiment included the following: (i) BM broth containing 100, 150 and 200 ppm of nitrite ($NaNO_2$), (ii) BM broth prepared with 5, 7.5, 10 and 12.5% NaCl, (iii) BM broth containing 5% NaCl and 100, 150 or 200 ppm of nitrite, (vi) growth at 4 °C and (v) growth in APT broth adjusted pH to 5.0 and 5.5. All tests were incubated at 25°C for 48 h and inspected for visible signs of growth.

## 3.6. Competitive growth studies

### 3.6.1. Bacterial inocula

*P. fluorescens, S. typhimurium* or *List. monocytogenes* was inoculated independently into medium containing *Leuc. gelidum* at $10^3$ CFU/mL. The bacteria were inoculated at concentrations of $10^3$ CFU/mL into the test broth, with the exception of higher inoculum trials with *List. monocytogenes*, in which the inoculum was $10^6$ CFU/mL, while the *Leuc. gelidum* inoculum remained at $10^3$ CFU/mL. For each bacterium, controls were done in which test organisms were grown alone. The growth medium was tempered to the experimental temperature for 24 h prior to inoculation. Differentiation of the bacteria in competitive growth experiments was done with differential growth media.

### 3.6.2. Temperatures of propagation

The competitive growth experiments were done at 10°C with *P. fluorescens*, at 4 and 10°C with *S. typhimurium* and at 4, 10 and 25°C with *List. monocytogenes*.

### 3.6.3. Addition of protease to the growth medium

Protease from *Streptomyces griseus* (Sigma) was added to tubes of APT broth to give a final concentration of 1 mg/mL. The addition of protease was done prior to the addition of bacteria to the medium. The bacteria grown independently or in competition with *Leuc. gelidum* were grown in APT broth with and without added protease.

### 3.6.4. Experimental design

The experiments were run in duplicate test tubes concurrently for each bacterium at each experimental temperature. Two samples were enumerated from each test tube at each sampling time.

## 3.7. Testing breakdown of protein by protease at different temperatures

Catalase (Sigma) and protease (Sigma) were suspended in sterile distilled water to a concentration of 1 mg/mL, adjusted to 4, 10 and 37°C, mixed and held for one hour before adding a drop of the enzyme mixture to a drop of $H_2O_2$ on TSA agar to check for release of gas.

A further experiment was done in which protease was added to the supernatant fluids of *Leuc. gelidum* UAL 187. Supernatant fluids were prepared from 36 h cultures of UAL 187 and UAL 187-13 incubated at 25°C. The fluids and protease from *Streptomyces griseus* (Sigma) were adjusted to 4, 10 and 25°C. Protease was added to give a final concentration of 1 mg/mL in the reaction mixture, except for the controls, and held at the reaction temperatures for one hour. The tubes were placed in a 65°C water bath for 30 min to inactivate the protease, and 50 µL of each sample was transferred into a well on an APT plate, overlaid with soft agar containing the indicator strains *C. divergens* UAL 9 or *List. monocytogenes* ATCC 15313 and incubated at 25°C for 24 h. After 24 h incubation the plates were checked for zones of inhibition surrounding the wells.

### 3.8. Determination of protein concentration

The protein concentration of the supernatant fluids and APT broth was determined spectrophotometrically using a 8451A Diode Array Spectrophotometer (Hewlett Packard) at $OD_{595}$ according to the Bradford protein standard assay procedure (Bradford, 1976). Bovine serum albumin (BSA) (Bio-Rad Laboratories (Canada) Inc., Mississauga, Ont.) containing 1.43 mg BSA/mL was used to prepare a standard curve to determine protein concentration. Supernatant fluids of *Leuc. gelidum* UAL 187 were prepared from a culture grown at 25°C for 36 h. The supernatant fluids were adjusted to pH 6.5 and chloroform sterilized before determining the protein content. APT broth (Difco) was prepared using the standard method. A standard curve was plotted and the protein concentrations of the fluids were read from the standard curve.

### 3.9. Competitive growth of *Listeria monocytogenes* ATCC 15313 and *Leuconostoc gelidum* UAL 187 in low protein broth

Due to the difference in protein concentration between supernatant fluids and APT broth, a broth based on APT with a lower protein content was formulated (Table 1). This broth was inoculated with *List. monocytogenes* ($10^6$ CFU/mL) and *Leuc. gelidum* ($10^4$ CFU/mL) and incubated at 25°C. Samples were removed over a 24 h period for enumeration of viable bacteria on Oxford, MRS 5.6 and TSA agars. The procedure was the same as that described for competitive growth experiments (section 3.6).

### 3.10. Growth of *Listeria monocytogenes* ATCC 15313 in supernatant fluids from *Leuconostoc gelidum* UAL 187

The supernatant fluids from *Leuc. gelidum* grown in APT broth at 25°C for 36 h, adjusted to pH 6.5 and chloroform sterilized were used as the growth medium for *List. monocytogenes* ATCC 15313. *List. monocytogenes* ATCC 15313 was inoculated at $10^6$ CFU/mL into 100% prepared supernatant fluids or a 1:4 or 1:10 dilutions of supernatant

**Table 1.** Ingredients in low protein and APT broths.

| Ingredients | APT broth (Difco) (g) | Low Protein broth (g) |
|---|---|---|
| Yeast extract | 7.5 | 3.75 |
| Tryptone | 12.5 | 6.25 |
| Dextrose | 10.0 | - |
| Saccharose | - | 10.0 |
| Sodium citrate | 5.0 | 5.0 |
| Thiamine hydrochloride | 0.001 | 0.001 |
| Dipotassium phosphate | 5.0 | 5.0 |
| Manganese chloride | 0.14 | 0.14 |
| Magnesium sulfate | 0.8 | 0.8 |
| Ferrous sulfate | 0.04 | 0.04 |
| Tween 80 | 0.2 | 0.2 |
| Sodium chloride | 5.0 | 5.0 |
| Water | 1000 mL | 1000 mL |

fluids with APT broth. The viable count was determined on samples removed at 0, 1, 3, 7, 9 and 24 h, plated onto Oxford and TSA agars and incubated at 30°C.

### 3.11. Serotyping of Listeria

*Listeria* strains used in this study were serotyped with antisera (Difco) according to the manufacturer's instructions using the rapid slide technique. Cultures were subcultured twice in tryptose broth (Difco) at 35°C for 24 h, streaked in duplicate onto tryptose agar slants and incubated at 35°C for 24 h. These slants were washed with 3 mL of Difco FA buffer, the fluid was collected, heated to 80°C for 1 h, centrifuged (1600 x g for 30 min) and 2 mL of the supernatant fluids removed. The pellet was resuspended in the remaining buffer. This suspension was mixed with Bacto-Listeria O Antisera types 1, 4 and polyvalent on a glass slide for 1-2 min and observed for agglutination.

### 3.12. Statistical analysis

Biological count data from competitive growth experiments were analyzed using analysis of variance (ANOVA) to compare the effect of bacteriocinogenic or nonbacteriocinogenic strains of *Leuconostoc gelidum* on pathogenic or spoilage microorganisms (Steele and Torrie, 1980). Where appropriate Duncan's Multiple range test was used to rank means (Steele and Torrie, 1980).

# 4. RESULTS

## 4.1. Selection of a lactic acid bacterium for competitive growth experiments

Lactic acid bacteria from processed meats were evaluated for production of inhibitory compounds. Initially, lactic acid bacteria were screened for production of inhibitory compounds by overlayering with other lactic strains and looking for clear zones around the colony. Later, non-lactic meat bacteria were used to screen for inhibition. The bacterium *Leuconostoc gelidum* UAL 187 was chosen for further study, based on characteristics described in the next section. The antimicrobial spectrum for this bacterium against Gram-positive bacteria is illustrated in Table 2. UAL 187 was also tested against Gram-negative bacteria, but it was not found to inhibit any of the strains that were tested.

## 4.2. Characteristics of *Leuconostoc gelidum* UAL 187

The bacterium was characterized for taxonomic purposes (Table 3) and growth characteristics (Table 4). Carbohydrate fermentation profiles of the bacterium were also determined (Table 5). The inhibitory substance produced by UAL 187 was considered a bacteriocin because inhibition of sensitive bacteria was not inactivated when the supernatant fluids were adjusted to pH 6.5, when catalase was added to the supernatant fluids or when APT agar plates that had been spotted with supernatant fluids were inverted and overlaid to test for bacteriophage. Addition of protease to the medium stopped inhibition of sensitive bacteria indicating that the inhibitory compound contains protein. Bacteriocin production at 25°C was demonstrated by well plate overlayer technique after 4 hours incubation. At that point the bacterial population had reached $9x10^6$ CFU/mL from an initial population of $7x10^6$ CFU/mL.

**Table 2.** Spectrum of activity of *Leuconostoc gelidum* UAL 187 against other Gram-positive bacteria by deferred antagonism.

| Indicator bacteria | Lab code | Inhibition by *Leuconostoc gelidum* UAL 187 |
|---|---|---|
| *Lactobacillus sake*-like organism | UAL 3 | + |
| *Carnobacterium piscicola* Shaw isolate | UAL 8 | + |
| *Carnobacterium divergens* Shaw isolate | UAL 9 | + |
| *Lactobacillus sake*-like organism Shaw isolate | UAL 12 | + |
| *Leuconostoc* sp. Shaw isolate | UAL 13 | + |
| *Leuconostoc mesenteroides* ATCC 23368 | UAL 14 | + |
| *Lactobacillus plantarum* ATCC 4008 | UAL 15 | + |
| *Lactobacillus viridescens* ATCC 12706 | UAL 16 | + |
| *Pediococcus acidilactici* ATCC 8042 | UAL 17 | - |
| *Pediococcus parvulus* ATCC 19327 | UAL 19 | + |
| *Enterococcus fæcalis* ATCC 19433 | | + |
| *Enterococcus fæcium* ATCC 19434 | | +/- |
| *Enterococcus fæcium (durans)* ATCC 11576 | | + |
| *Staphylococcus aureus* ATCC 25923 | | - |
| *Listeria monocytogenes* ATCC 15313 | | + |
| *Listeria innocua* ATCC 33090 | | + |
| *Brochothrix thermosphacta* ATCC 11509 | | - |

+/- weak inhibition

**Table 3.** Characteristics of *Leuconostoc gelidum* UAL 187 and SML 9 based on the criteria of Shaw and Harding (1984, 1989).

| Characteristics | UAL 187 | SML 9† |
|---|---|---|
| Gram-positive | + | + |
| Growth at 1°C | + | + |
| Growth at 35°C | - | - |
| Gas production from glucose in MRS broth with ammonium citrate | + | + |
| Growth on acetate agar at pH 5.6 | +/- | |
| Production of dextran from sucrose | + | + |
| Reduction of 0.01% TTC | - | - |
| Production of ammonia from arginine | - | - |
| Ability to initiate growth at pH 3.9 growth in APT without phosphate and with 2% glucose | +/- | |
| pH after 7 days growth in La broth at 25°C | 4.5 | |
| Production of D (-) lactate†† | + | + |

† *Leuconostoc gelidum* strain SML 9 characteristics according to Shaw and Harding (1989)

†† Test performed by John Hastings at the University of Alberta.

+/- Poor growth

**Table 4.** Ability of *Leuconostoc gelidum* UAL 187 to growth in conditions found in processed meats.

| Conditions | |
|---|---|
| Ability to grow at | |
| 1°C | +/- |
| 4°C | + |
| 15°C | + |
| 25°C | + |
| 35°C | - |
| 40°C | - |
| 45°C | - |
| Ability to grow in NaCl | |
| 5% | +/- |
| 7.5% | - |
| 10% | - |
| 12.5% | - |
| Ability to grow in nitrite | |
| 100 ppm | + |
| 150 ppm | + |
| 200 ppm | + |
| Ability to grow in NaCl and nitrite | |
| 5% and 100 ppm | +/- |
| 5% and 150 ppm | +/- |
| 5% and 200 ppm | +/- |
| Ability to produce bacteriocin at pH | |
| 5.5 | + |
| 5.0 | + |
| Ability to produce bacteriocin at | |
| 4°C | + |

+/- weak growth

**Table 5.** Carbohydrate fermentation profile of *Leuconostoc gelidum* UAL 187 and SML 9.

| Carbohydrate | Fermentation (48 h @ 25°C) | |
|---|---|---|
| | UAL 187 | SML 9† |
| Amygdalin | +/- | + |
| Arabinose | + | - |
| Cellobiose | + | + |
| Galactose | -†† | - |
| Glucose | + | + |
| Glycerol | - | - |
| Inositol | - | - |
| Lactose | +/- | - |
| Maltose | + | - |
| Mannitol | +/- | - |
| Mannose | - | + |
| Melezitose | - | - |
| Melibiose | + | + |
| Raffinose | + | + |
| Rhamnose | - | - |
| Ribose | + | + |
| Salicin | + | + |
| Sorbitol | +/- | - |
| Sucrose | + | + |
| Trehalose | + | + |
| Xylose | + | + |

† *Leuconostoc gelidum* strain SML 9 characteristics according to Shaw and Harding (1989)
†† Test performed by John Hastings at the University of Alberta
+/- Slight fermentation

## 4.3. Differentiation of bacteria in competitive growth experiments

Differentiation of the bacteria in competitive growth experiments was done with differential, selective agars. These media inhibited growth of the competing organisms but not that of the bacteria to be enumerated. Table 6 illustrates the inhibition and growth of test organisms on the enumeration media. *P. fluorescens* was able to grow on brilliant green agar which was used to enumerate *S. typhimurium*. However, colonies of *P. fluorescens* were small, not pink and did not grow in the first 24 h, making them easy to differentiate from colonies of *S. typhimurium*.

## 4.4. pH changes in the growth medium of the competitive growth experiments

Changes in pH of the growth medium in the competitive growth experiments was similar to the changes in pH in the medium with *Leuc. gelidum* grown alone. Temperature of growth affected the time taken to reach pH 4.5 (Figure 1). The change in pH that occurred with growth of *Leuc. gelidum* UAL 187 was similar to that of *Leuc. gelidum* UAL 187-13.

## 4.5. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Pseudomonas fluorescens* ATCC 13525

*Pseudomonas fluorescens* was grown alone and in competition with $Bac^+$ (UAL 187) and $Bac^-$ (UAL 187-13) strains of *Leuc. gelidum* at 10°C in APT broth. When *P. fluorescens* was grown alone there was no change in the pH throughout the 25 days of the experiment. However, in trials in which *Leuc. gelidum* and *P. fluorescens* were grown together, *Leuc. gelidum* caused a drop in pH to 4.7 within 12 days. The growth of *P. fluorescens* at 10°C is illustrated in Figure 2. After day four there is a significant difference ($P \leq 0.05$) between the growth of *P. fluorescens* in the control and competitive growth samples, but the difference is of limited importance. Inhibition of *P. fluorescens* was slightly greater when grown in competition with UAL 187-13 than with UAL 187,

**Table 6.** Growth of test organisms on differentiating selective media in 24 h.

| Organism | UAL 187 | UAL 187-13 | *Listeria monocytogenes* | *Salmonella typhimurium* | *Pseudomonas fluorescens* |
|---|---|---|---|---|---|
| | log CFU | | | | |
| Incubation temperature | 25°C | 25°C | 30°C | 35°C | 25°C |
| Brilliant Green Agar | NG[1] | NG | NG | 8.73 | NG* |
| Oxford Agar | NG | NG | 9.11 | NG | NG |
| CFC Agar[2] | NG | NG | NG | NG | 7.48 |
| Lactobacilli MRS 5.6 Agar[3] | 8.66 | 8.73 | NG | NG | NG |
| Tryptic Soy Agar | 8.77 | 8.96 | 9.20 | 8.80 | 8.04 |

* No growth in 24 h, small colonies after extended incubation

1 NG - No growth

2 CFC - Cephaloridine, Sodium fusidic acid, Cetrimide agar (Mead and Adams, 1977)

3 Lactobacilli MRS 5.6 - de Man Rogosa Sharpe lactobacilli selective agar adjusted with 85% lactic acid to pH 5.6

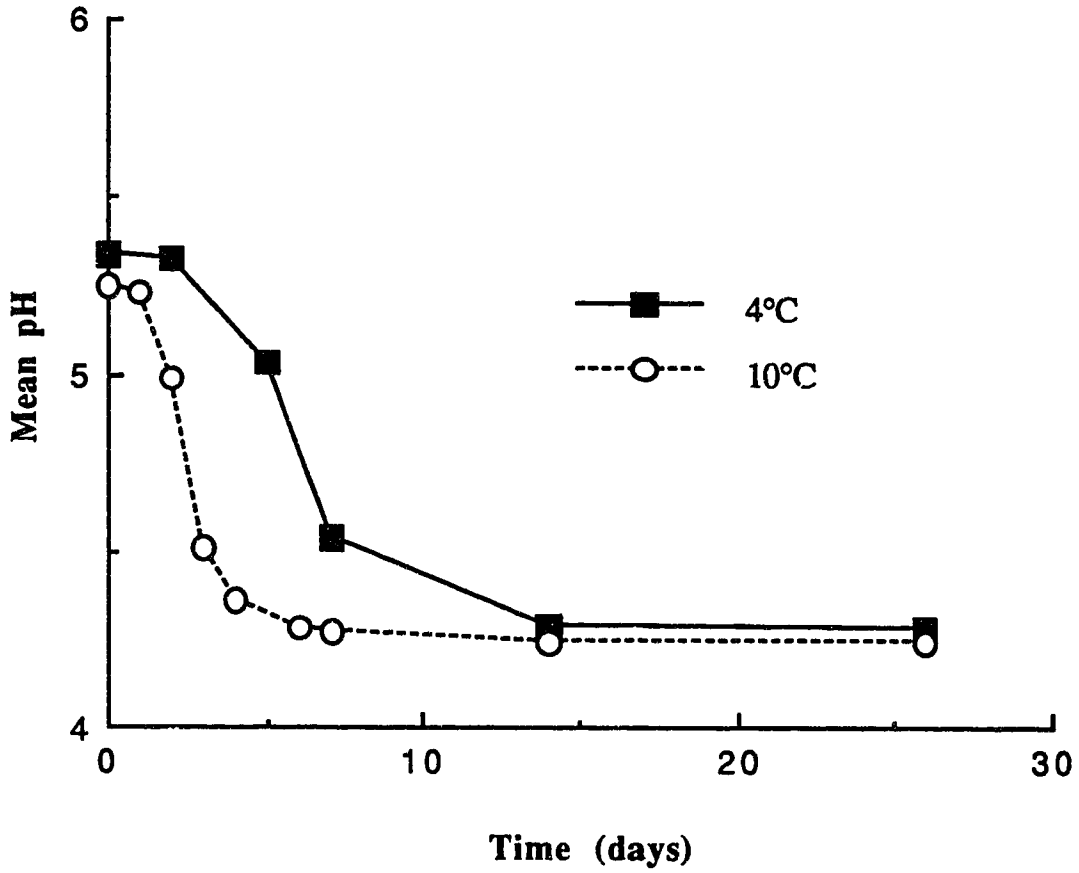


**Figure 1.** pH changes in growth medium of competitive growth experiments at 4 and 10°C in APT broth with *Leuconostoc gelidum* present.

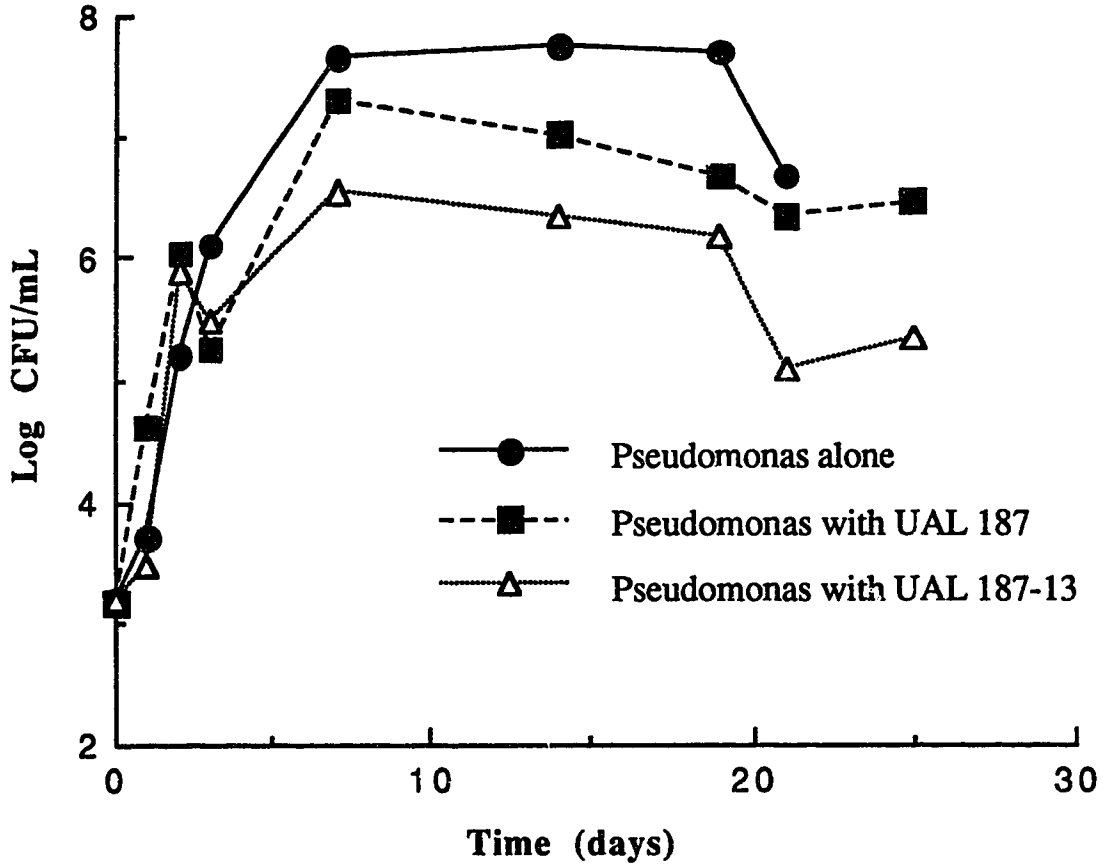


**Figure 2.** Competitive growth of *Pseudomonas fluorescens* strain ATCC 13525 with *Leuconostoc gelidum* strains UAL 187 and UAL 187-13 in APT broth at 10°C.

indicating that bacteriocin production does not affect *P. fluorescens*. The highest populations of *P. fluorescens* occurred when no other bacterium was present indicating that *Leuc. gelidum* has an inhibitory effect on the growth of *P. fluorescens*. There was no difference in the growth of *Leuc. gelidum* whether grown alone or in the presence of *P. fluorescens*.

### 4.6. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Salmonella typhimurium* ATCC 13311

*Leuc. gelidum* UAL 187 and *Salmonella typhimurium* were grown in APT broth at 4 and 10°C. Within 12 days at 4°C and 8 days at 10°C, the pH of the APT broth decreased to 4.4 - 4.8 as a result of the growth of *Leuc. gelidum* UAL 187. There was no change in pH of the medium inoculated with salmonella alone during 28 days incubation at 4°C. In contrast, salmonella alone at 10°C caused a slow drop to pH 4.5 over 28 days. At 4°C, *S. typhimurium* survived well over the 28-day duration of the experiment, when grown alone and in the presence of either the $Bac^+$ or $Bac^-$ strains of UAL 187 (Figure 3). The decrease in pH to 4.8 with UAL 187 and 187-13 did not affect survival of *S. typhimurium* at 4°C. At 10°C, *S. typhimurium* grew to $10^7$ CFU/mL within 8 days (Table 7). In the presence of UAL 187 or UAL 187-13 there was an initial increase in cell numbers of *S. typhimurium* followed by a die-off of the cells, coinciding with the decrease in the pH of the medium to 4.7. pH had an inhibitory effect on *S. typhimurium* under conditions where salmonella can grow (10°C) in contrast to no effect under conditions where salmonella can not grow (4°C).

Further testing was done to determine the effect of pH on *S. typhimurium*. The pH of APT broth was adjusted to pH 4.0, 4.5, 5.0, and 5.5 using lactic and hydrochloric acids. The killing effect was greater when lactic acid was used to adjust the pH (Figure 4). *S. typhimurium* was unable to grow in broths adjusted with lactic acid. However, when the medium was adjusted to pH 5.5 with HCl the salmonella was able to grow. When the

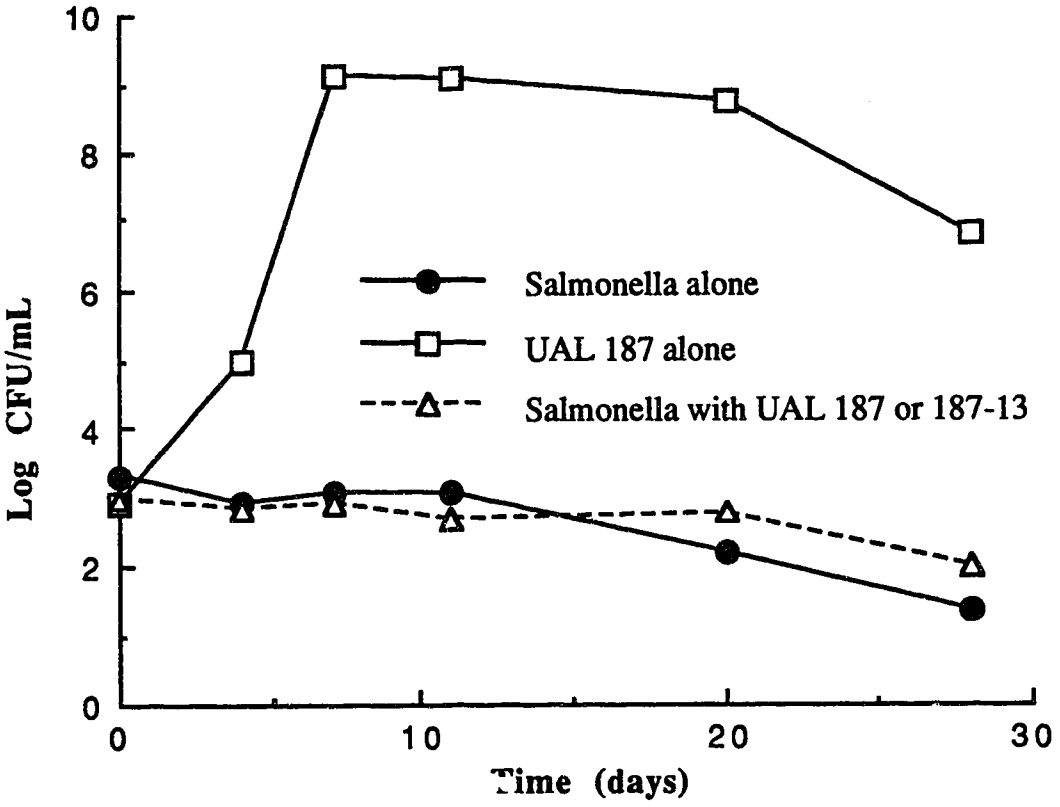


**Figure 3.** Competitive growth of *Salmonella typhimurium* ATCC 13311 with *Leuconostoc gelidum* UAL 187 in APT broth at 4°C.

**Table 7.** Competitive growth of *Salmonella typhimurium* ATCC 13311 with *Leuconostoc gelidum* UAL 187 in APT broth at 10°C.

| Time (days) | Growth of *Salmonella typhimurium* (Log CFU/mL) | | |
|---|---|---|---|
| | *Salmonella* alone | *Salmonella* grown with UAL 187 | *Salmonella* grown with UAL 187-13 |
| 0 | 3.03 | 3.01 | 2.93 |
| 5 | 5.00 | 4.03 | 3.75 |
| 8 | 7.11 | 3.70 | <3.00 |
| 12 | 7.14 | <3.00 | <3.00 |
| 21 | 7.22 | <3.00 | <3.00 |
| 28 | 6.25 | 1.00 | 1.00 |

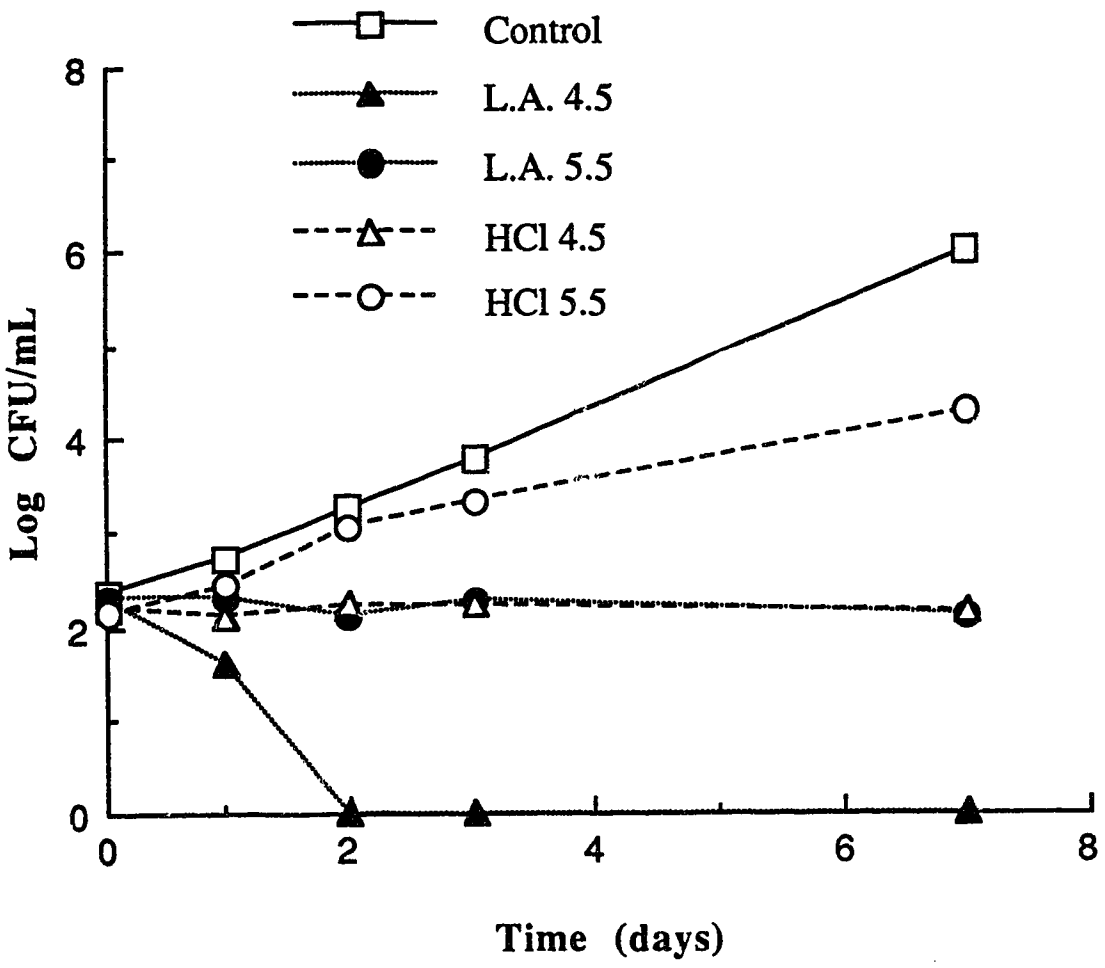


**Figure 4.** Growth of *Salmonella typhimurium* ATCC 13311 at 10°C in APT broth with added lactic (L.A.) or hydrochloric (HCl) acids.

pH of the broth was adjusted to 4.5 with HCl there was no killing effect on the salmonella for 7 d. Whereas, when the pH was adjusted to 4.5 with lactic acid, there was a rapid die-off of salmonella to undetectable levels within two days.

## 4.7. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Listeria monocytogenes* at 4°C

*Listeria monocytogenes* strains Scott A and ATCC 15313 were grown separately in APT broth with the bacteriocinogenic ($Bac^+$) *Leuconostoc gelidum* strain UAL 187 or the $Bac^-$ mutant UAL 187-13. The experiments were done with inoculum levels of $10^4$ or $10^6$ CFU of listeria per mL of APT broth. The change in pH of the medium during growth of *List. monocytogenes* Scott A and UAL 187 at 4°C is representative of the pH changes during competitive growth of these organisms. The final pH of the medium with listeria inoculated alone was 5.0, but with leuconostoc present the pH of the medium dropped to 4.4 during the first 10 days of incubation and remained at this pH to the termination of the experiment at 28 days. Initial pH of the broth in the experiment with lower inoculum was adjusted to pH 5.4, whereas the pH of the broth for the experiment with the higher inoculum was 6.5. Starting pH of the medium did not affect the final pH reached.

Figures 5 and 6 illustrate growth and survival of listeria when incubated with the leuconostoc strains at 4°C. Marked differences were observed between the two listeria strains. For the initial 8 days, differences in *Listeria monocytogenes* Scott A (Figure 5) counts with or without UAL 187-13 present was not significant ($P \geq 0.05$). However, in the presence of *Leuc. gelidum* UAL 187 the viable count of listeria decreased by one log cycle at day eight. Thereafter the listeria counts decreased dramatically in the presence of UAL 187 and UAL 187-13, coinciding with the decrease in pH of the medium to 4.4. In contrast, *List. monocytogenes* ATCC 15313 is resistant to low pH (Figure 6). The decrease in viable count of this strain in the presence of UAL 187 is attributed to production of bacteriocin. After a reduction from $2 \times 10^6$ to $7 \times 10^3$ CFU/mL, the viable

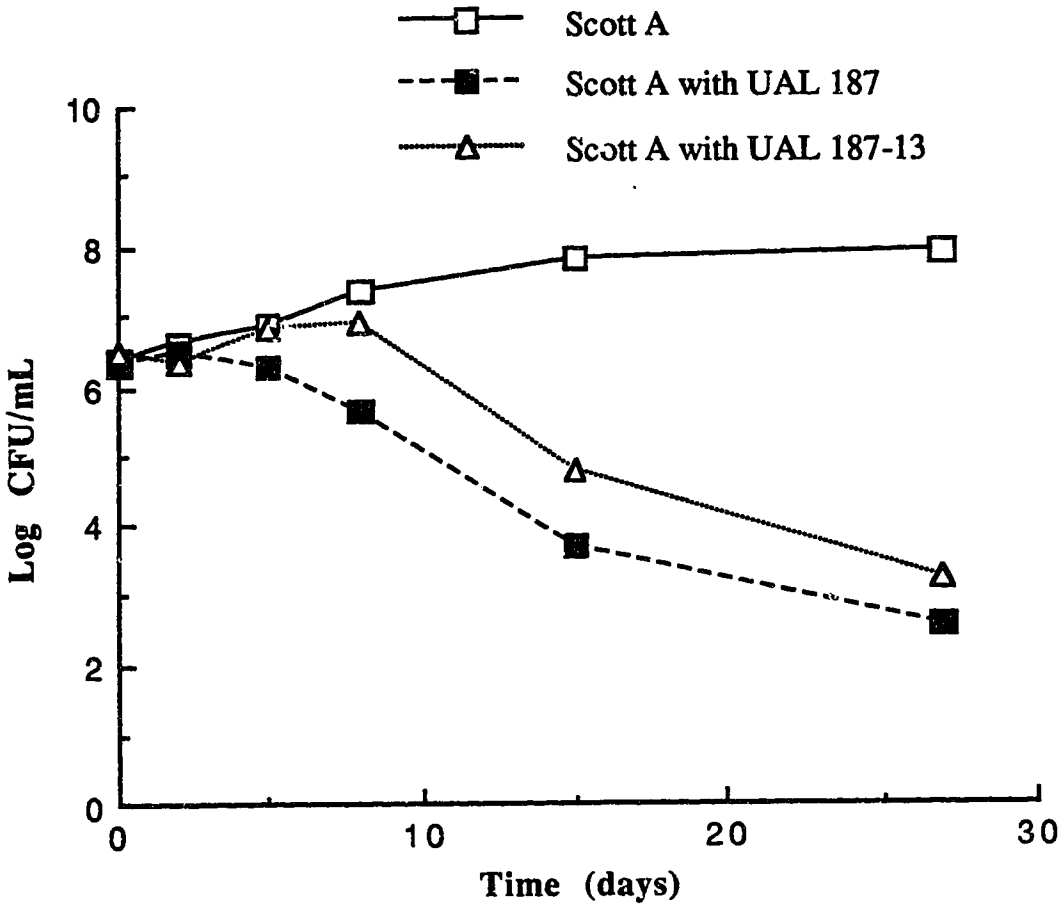


**Figure 5.** Competitive growth of *Listeria monocytogenes* strain Scott A at 4°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum.*

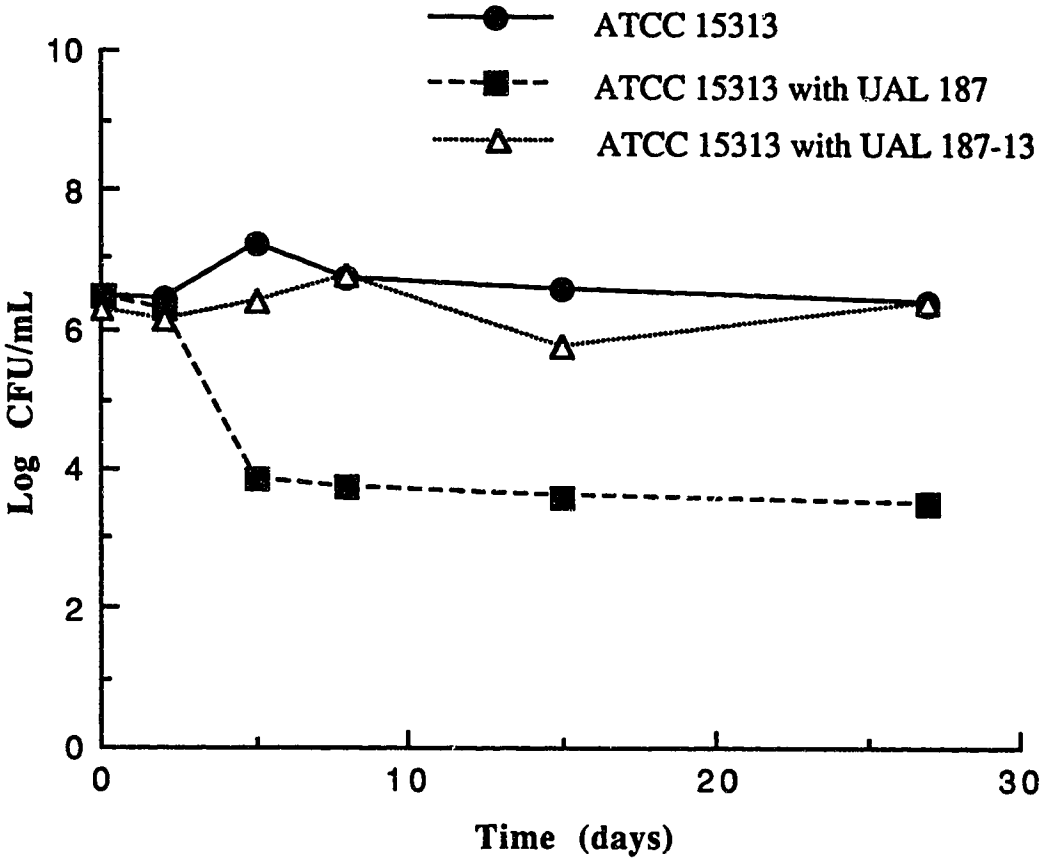


**Figure 6.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 at 4°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum*.

count of listeria cells remained constant to the end of the experiment. The change in viable count of *List. monocytogenes* ATCC 15313 with initial inoculum levels of $10^4$ CFU/mL is shown in Figure 7. At the lower inoculum level the listeria in pure culture grew. In the presence of UAL 187-13 there was no change in listeria count during the 28-day experiment, whereas in the presence of UAL 187 a two log reduction in count was observed. Decreases in viable count of *List. monocytogenes* were consistently greater when grown with UAL 187 than when they were grown with UAL 187-13. The differences in population decrease were not always found to be statistically significant ($P \geq 0.05$) at the lower inoculum levels. In contrast, in Figures 5 and 6 the differences in growth of listeria with UAL 187 and UAL 187-13 were found to be significant ($P \leq 0.05$) after day two of the experiment.

### 4.8. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Listeria monocytogenes* at 10°C

The inoculum levels of the experiment at 10°C were the same as at 4°C. The initial pH of all trials done at 10°C was 5.4 to 5.5. The minimum pH reached during these experiments was 4.3 in 5 days of incubation, except with *List. monocytogenes* ATCC 15313 grown alone in which the pH of the medium decreased to 5.0. By day seven of the experiment, the population of *Leuc. gelidum* UAL 187 reached the maximum of 6.34 x $10^8$ CFU/mL and the pH dropped to 4.3. This pH resulted in a marked die-off of *List. monocytogenes* Scott A in all competitive growth experiments (Figure 8). When grown with UAL 187 the combined action of the bacteriocin and lowered pH caused strain Scott A to decrease to undetectable levels, two log units lower than the effect of pH alone. Under the same conditions, *List. monocytogenes* ATCC 15313 initially increased in count, followed by slight inhibition of growth in the presence of $Bac^-$ *Leuc. gelidum* UAL 187-13 and a two log decrease in the presence of $Bac^+$ *Leuc. gelidum* UAL 187 (Figure 9).

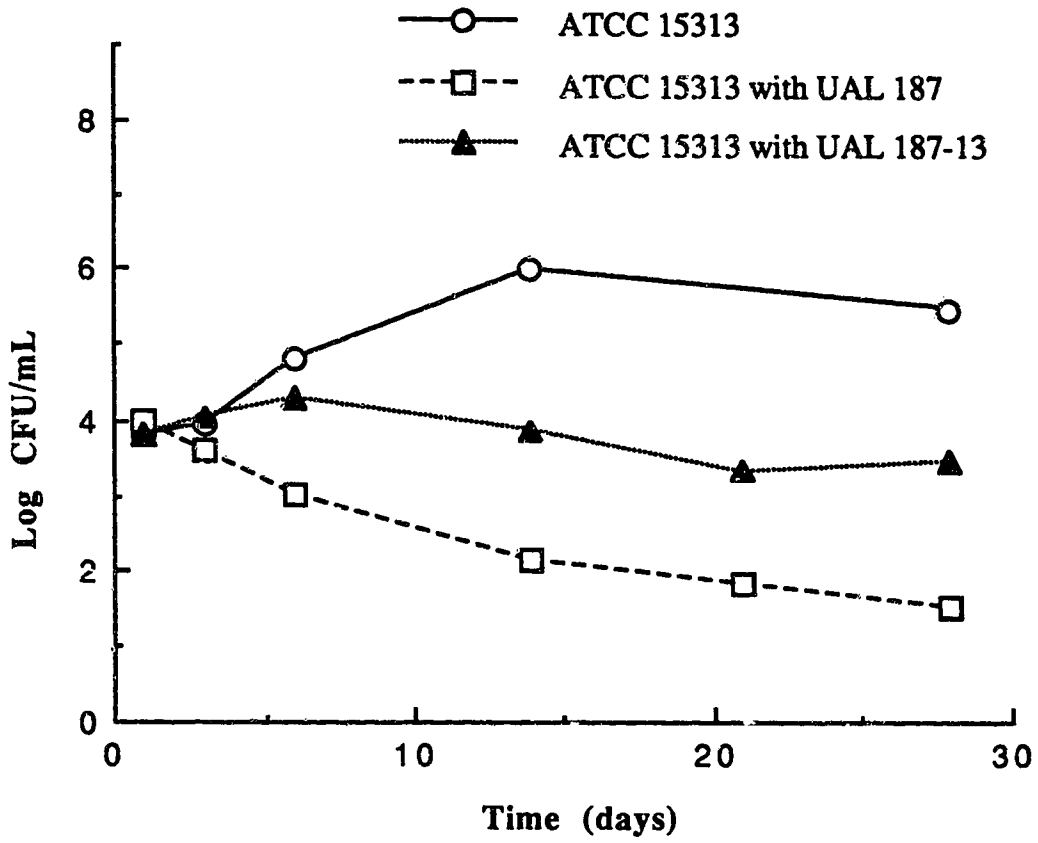


**Figure 7.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 at 4°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum* with cultures inoculated at $10^4$ CFU/mL.

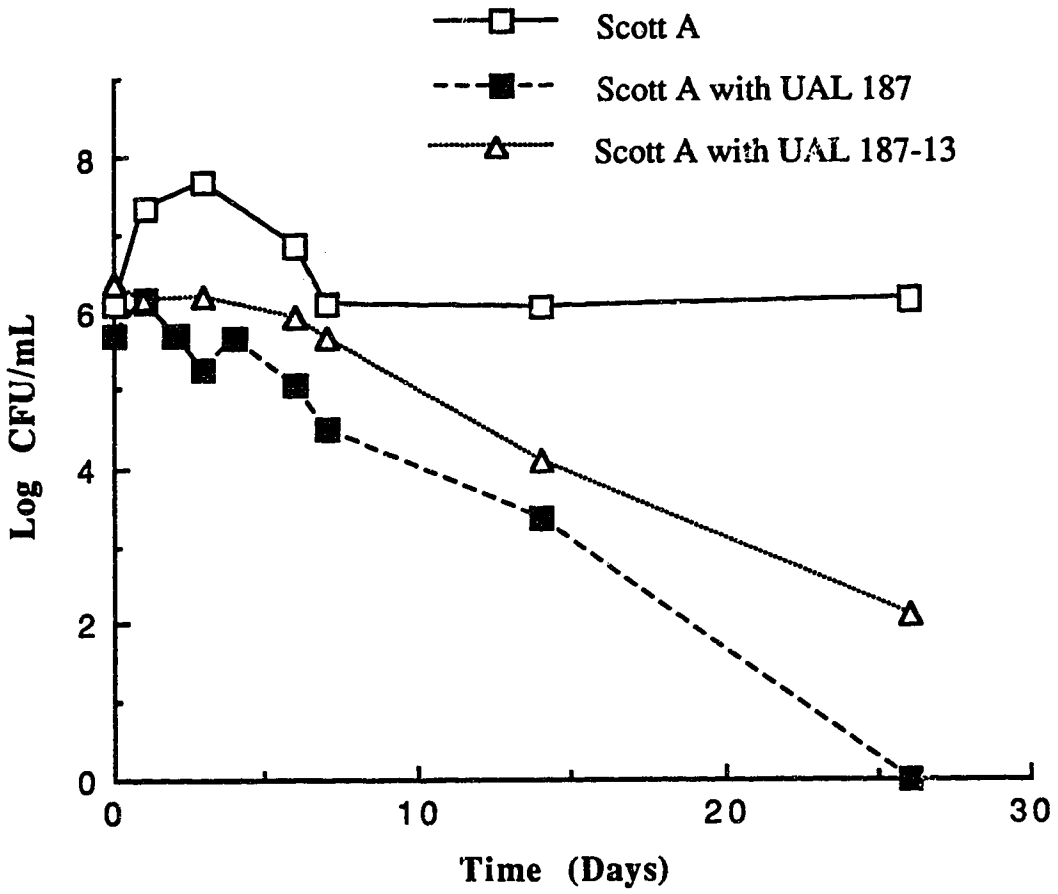


**Figure 8.** Competitive growth of *Listeria monocytogenes* strain Scott A at 10°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum.*

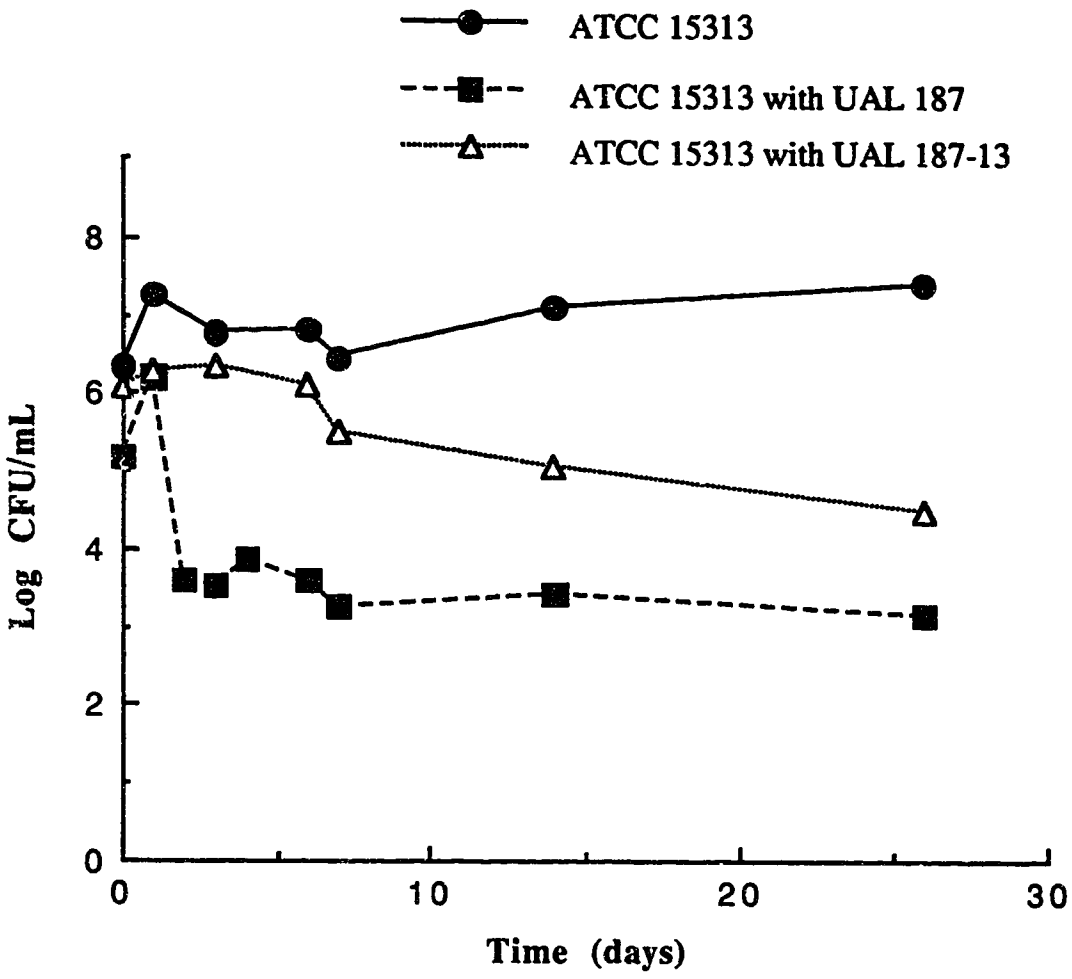


**Figure 9.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 at 10°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum*.

Changes in viable count of *List. monocytogenes* ATCC 15313 inoculated at $10^4$ CFU/mL are shown in Figure 10. At lower inoculum levels, listeria alone increased to $10^6$ CFU/mL, whereas in the presence of UAL 187-13 they only increased to $10^5$ CFU/mL. The difference in counts between counts of *List. monocytogenes* ATCC 15313 alone and grown in the presence of *Leuc. gelidum* UAL 187-13, from day five to day 20 of the experiment was significant ($P \leq 0.05$). In the presence of UAL 187 there was an initial two log decrease in the viable count of listeria, followed by a slower die-off of the cells. Growth of *List. monocytogenes* ATCC 15313 with UAL 187 was significantly different ($P \leq 0.05$) than when grown alone or with UAL 187-13. The initial decrease in population was attributed to the action of a bacteriocin produced by UAL 187.

## 4.9. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Listeria monocytogenes* at 25°C

Experiments were done at 25°C with inoculum levels of $10^4$ CFU/mL of both listeria and lactic organisms. APT broth used as the suspending medium was adjusted to an initial pH of 5.2 to 5.4 with 85% lactic acid. Medium which contained either strain of *List. monocytogenes* in pure culture showed no change in pH over the 24 h of the experiment. In the trials with UAL 187 the pH decreased to 4.7 in 24 h. However, in one trial with listeria strain Scott A there was little decrease in pH over 48 h but inhibition of the listeria still occurred.

The data presented in Figures 11 and 12 illustrate the changes in viable counts of the test strains of *List. monocytogenes* incubated at 25°C in the presence of *Leuc. gelidum* There was no difference in the growth of *List. monocytogenes* Scott A during the first 12 h of incubation at 25°C, irrespective of whether it was grown alone or with UAL 187, 187-13, or 187 with protease (Figure 11). Between 12 and 24 h there was a 3-log decrease in population of Scott A cells in all of the samples with associative growth of listeria and lactic organisms, while Scott A grown alone increased to $2.0 \times 10^8$ CFU/mL.

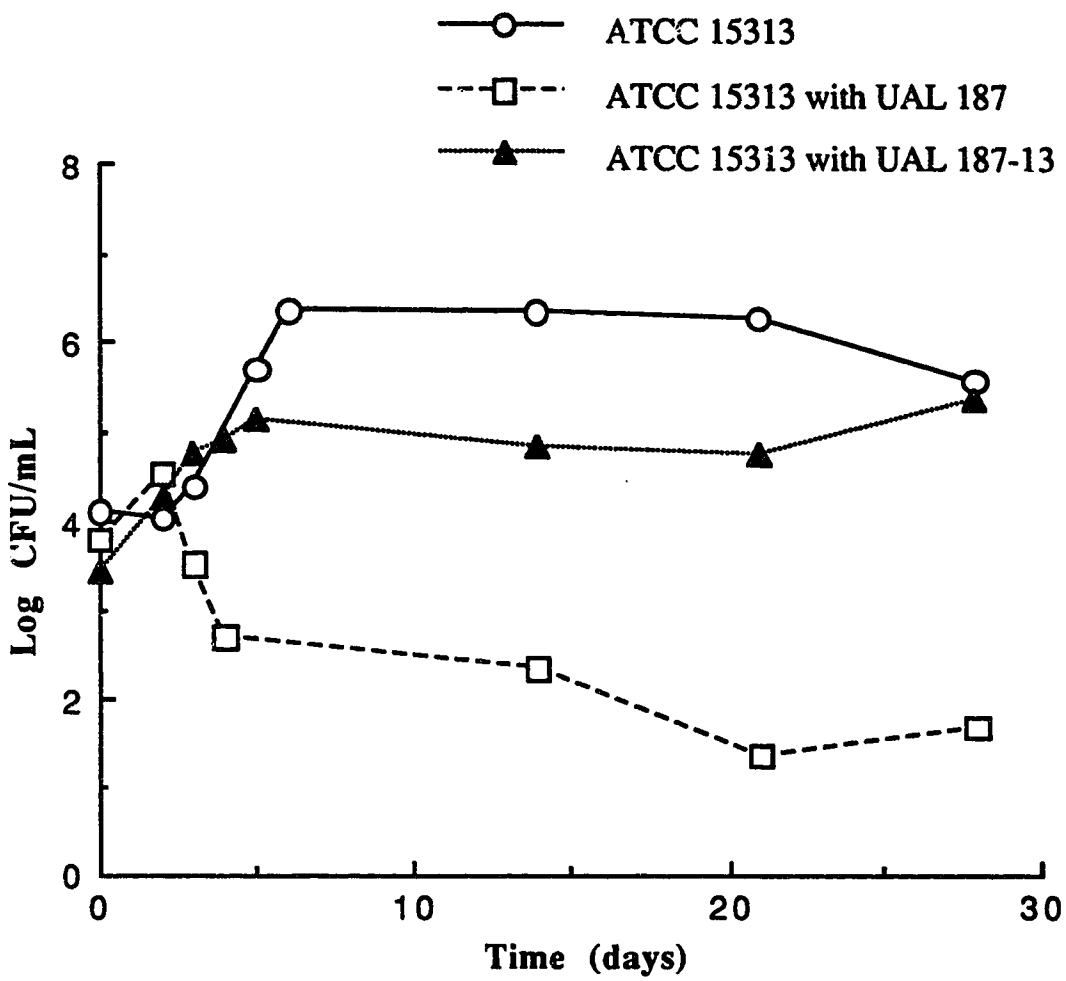


**Figure 10.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 at 10°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum* with inoculum of $10^4$ CFU/mL.

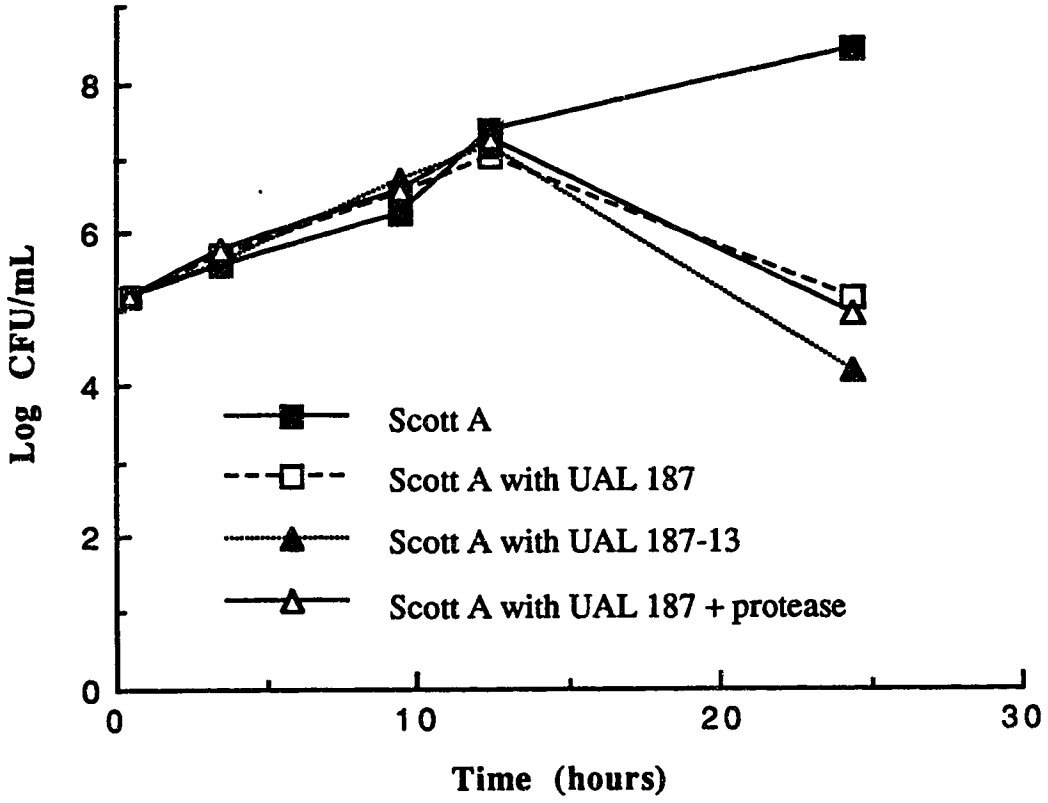


**Figure 11.** Competitive growth of *Listeria monocytogenes* strain Scott A at 25°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum*.

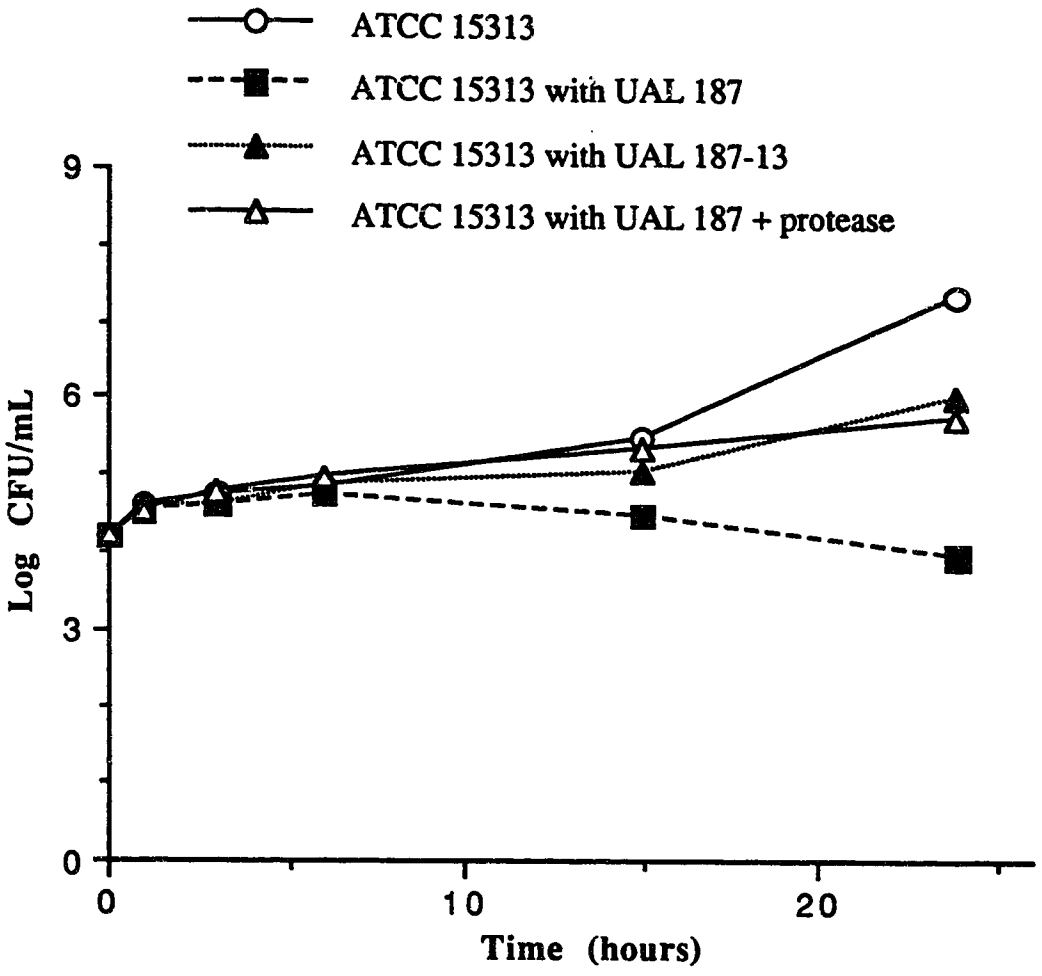


**Figure 12.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 at 25°C with bacteriocinogenic (UAL 187) and nonbacteriocinogenic (UAL 187-13) strains of *Leuconostoc gelidum.*

In contrast, *List. monocytogenes* ATCC 15313 was affected by the bacteriocin produced by UAL 187 (Figure 12). The viable count of *List. monocytogenes* ATCC 15313 in the presence of UAL 187-13 or UAL 187 with added protease was the same as growth of the listeria alone up to 6 hours (Figure 12); from 8 to 24 h listeria grown in association with lactic bacteria did not increase as quickly as listeria grown alone due to acid development by the lactic strain. Listeria grown with Bac$^+$ UAL 187 decreased slowly over 24 h due to combined inhibition of lactic acid and bacteriocin.

## 4.10. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Listeria monocytogenes* grown in APT broth with added protease

Protease from *Streptomyces griseus* was added to APT broth prior to inoculation with bacteria. Addition of protease to APT broth should stop the bacteriocinogenic effect produced by UAL 187 and should affect *List. monocytogenes* in the same way as growth in competition with the Bac$^-$ UAL 187-13 (see Figure 12). At 4 and 10°C, both strains of *List. monocytogenes* exhibited similar death curves when grown with *Leuc. gelidum* UAL 187 in the presence or absence of protease. Figure 13 illustrates the survival of *List. monocytogenes* Scott A and ATCC 15313 in competitive growth with *Leuc. gelidum*. This graph is representative of the changes that occurred with either of the listeria strains with and without added protease at all incubation temperatures. Strain ATCC 15313 exhibited immediate decreases in population when grown with UAL 187 whether protease was present or not. Strain Scott A showed a more prolonged die-off than ATCC 15313, but the survival rate was similar whether protease was present or not. The death rates and final populations of the listeria strains grown with protease present were similar to growth in association with the Bac$^+$ UAL 187.

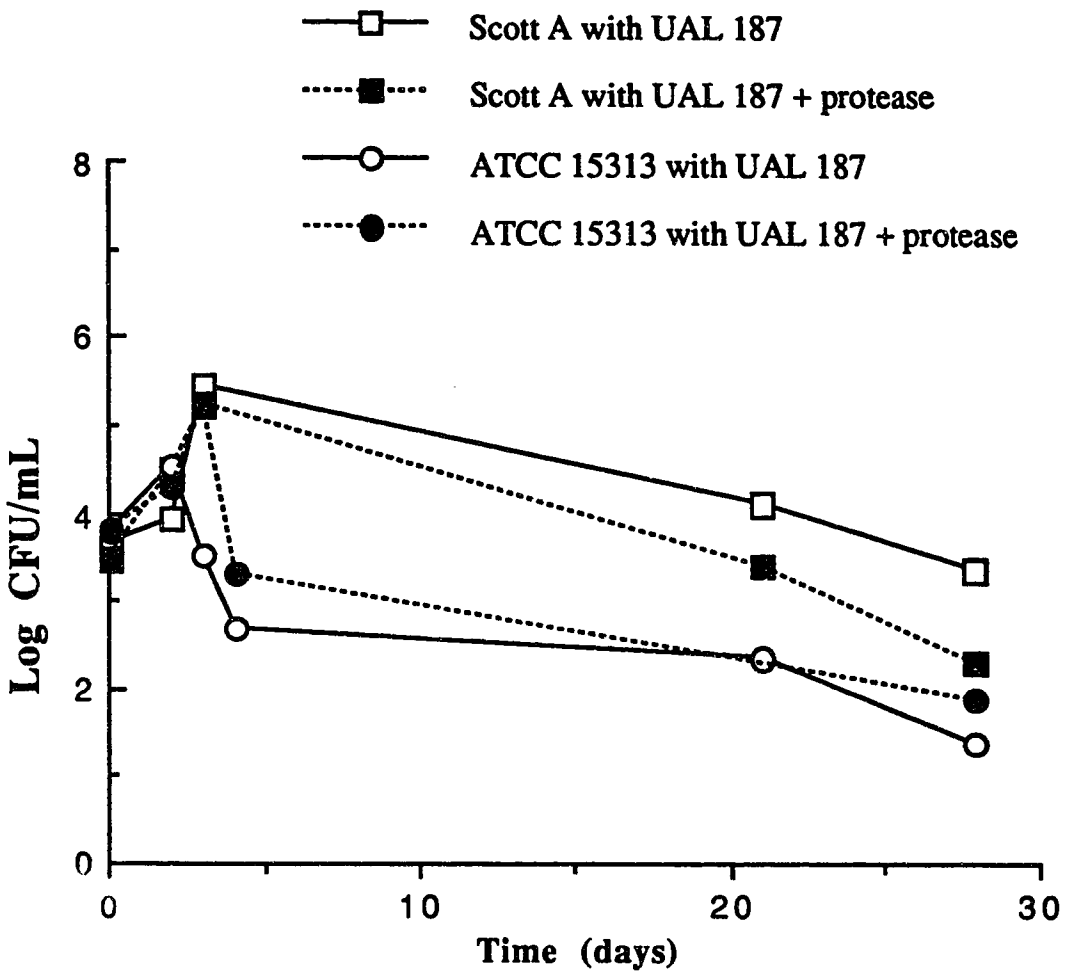


**Figure 13.** Survival of *Listeria monocytogenes* at 10°C inoculated with the bacteriocinogenic strain of *Leuconostoc gelidum* UAL 187 in APT broth with and without added protease.

## 4.11. Activity of protease in the test broth

### 4.11.1. Presence of bacteriocin in the APT test broth incubated at 10°C with protease and *Leuconostoc gelidum* UAL 187 present

Supernatant fluids from competitive growth experiments with *List. monocytogenes* were assayed for the presence of bacteriocin on days 7 and 26 of incubation at 10°C. Activity was tested by the overlayer technique using *List. monocytogenes* ATCC 15313 as the indicator bacterium. No zones of inhibition were produced by supernatant fluids from UAL 187 with added protease, or from UAL 187-13. Supernatant fluids from test broths containing UAL 187 produced zones of inhibition on day 7 but not on day 26.

### 4.11.2. Effect of temperature on protease activity

Protease from *Streptomyces griseus* did not inactivate the inhibitory activity of UAL 187 in APT broth inoculated with *Leuc. gelidum* and *List. monocytogenes*, therefore, the activity of protease at 4 and 10°C was tested using catalase. After 1 h at 37°C the catalase was inactivated by protease but after 1 hour at 4 and 10°C no inactivation occurred, indicating that the protease was not active at these temperatures. Inactivation of the bacteriocin of UAL 187 by protease was determined at 4, 10 and 25°C. Inactivation of the bacteriocin by protease was noted after 1 h at all of these temperatures.

### 4.11.3. Cold harvesting of protease-containing supernatant fluids

Harvesting the supernatant fluids at room temperature (25°C) exposes them to temperatures which allow protease present in the medium to break down the bacteriocin. Therefore, supernatant fluids from cultures of *Leuc. gelidum* UAL 187 grown at 10°C with protease present were prepared at 0°C. No inhibition of the *List. monocytogenes* strain ATCC 15313 was observed by well plate overlayer of supernatant fluids from *Leuc. gelidum* UAL 187 with protease or UAL 187-13, but inhibition occurred in the presence of supernatant fluids from UAL 187.

### 4.12. Production of bacteriocin early in the growth cycle of *Leuconostoc gelidum* grown at 10°C

*Leuc. gelidum* UAL 187 was grown in APT broth at 10°C for 126 h to determine when bacteriocin was produced. *Leuc. gelidum* UAL 187 bacteriocin was first detected in supernatant fluids after 98 h of growth. No bacteriocin was detected in supernatant fluids of *Leuc. gelidum* UAL 187 grown with added protease at any time during the experiment.

### 4.13. Growth of *Listeria monocytogenes* ATCC 15313 in supernatant fluids of *Leuconostoc gelidum* UAL 187

*List. monocytogenes* ATCC 15313 was inoculated into supernatant fluids from 36 h cultures of *Leuc. gelidum* UAL 187 grown at 25°C and into 1:10 and 1:4 dilutions of supernatant fluids in APT broth. The supernatant fluids were treated in three ways: no addition of protease; addition of protease prior to inoculation of *List. monocytogenes*; and addition of protease to the enumeration medium after plating (UAL 187; UAL 187P; and UAL 187+P, respectively). In supernatant fluids of *Leuc. gelidum* UAL 187, *List. monocytogenes* was inhibited and the inhibition was not reversed by the addition of protease to the plating medium (UAL 187+P), but it was reversed by addition of protease to the supernatant fluids (UAL 187P) (Figure 14). The most effective inhibition occurred when listeria was grown in supernatant fluids of UAL 187. The data for the growth of listeria in 10% supernatant fluids (Figure 14) is representative of what also occurs at 25% concentration of supernatant fluids.

### 4.14. Effect of protein concentration on activity of *Leuconostoc gelidum* supernatant fluids and APT broth

Protein concentration in APT broth might be a factor influencing protease activity against bacteriocin. Protein concentration of fresh APT broth and supernatant fluids was determined. Fresh APT broth contained 57 µg of protein/ mL compared with 27.5 µg of protein/ mL in supernatant fluids.

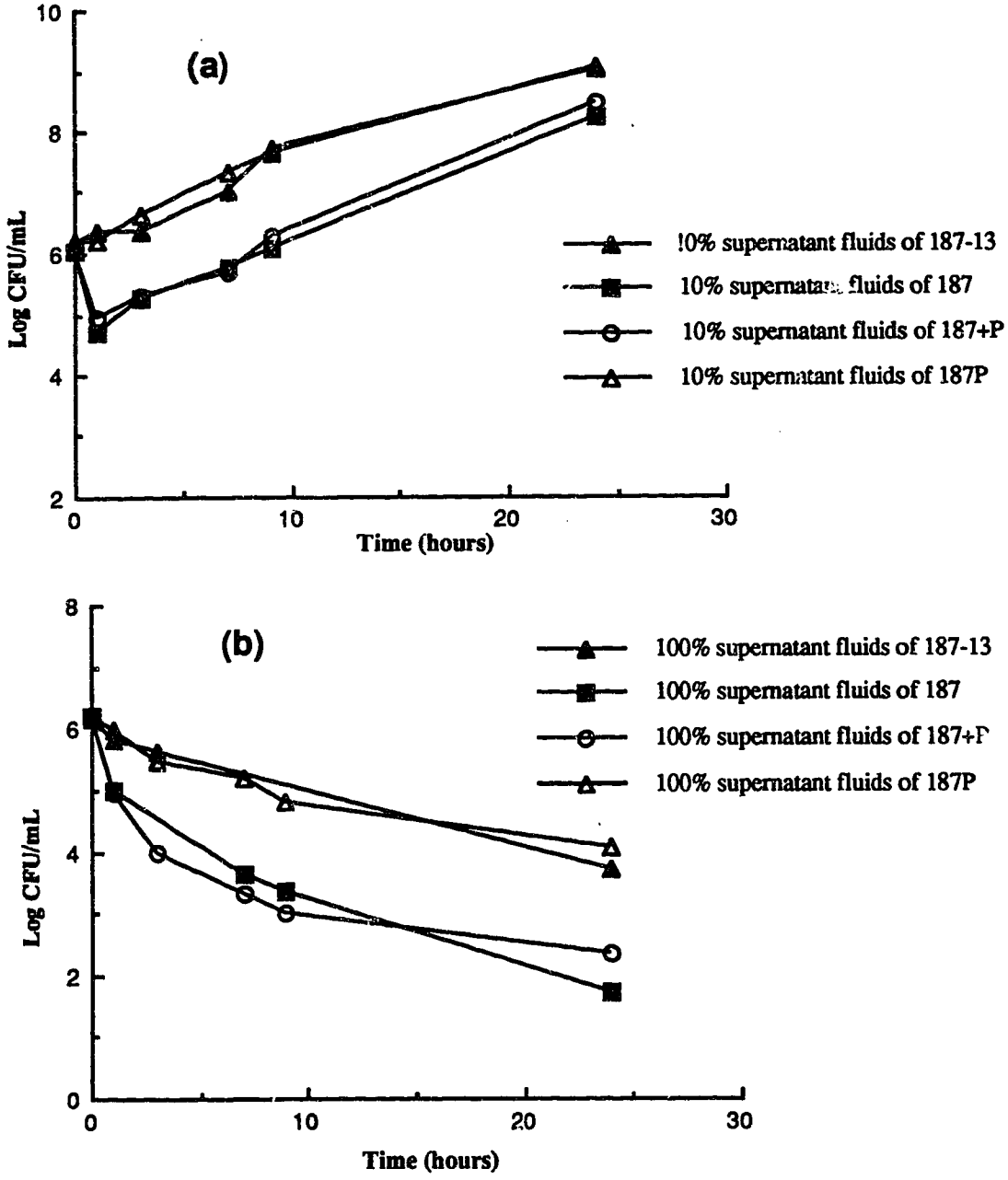


**Figure 14** Growth of *Listeria monocytogenes* ATCC 15313 at 25°C in (a) 10% and (b) 100% concentrations of supernatant fluids of *Leuconostoc gelidum* strain UAL 187, a bacteriocin producer: UAL 187-13, a mutant of UAL 187 which does not produce bacteriocin; UAL 187+P, UAL 187 with protease added to the enumeration medium at the time of plating; and UAL 187P, UAL 187 with protease added to the growth medium prior to inoculation of the listeria.

## 4.15. Competitive growth of *Leuconostoc gelidum* UAL 187 and *Listeria monocytogenes* in low protein broth at 25°C

Because supernatant fluids were lower in protein than fresh APT broth and protease added to supernatant fluids of UAL 187 inactivated the bacteriocin, a low protein broth was formulated with decreased protein concentration. The data in Figure 15 illustrate the growth curves of *List. monocytogenes* ATCC 15313 with *Leuc. gelidum* UAL 187 in low protein broth at 25°C. The growth curve for strain UAL 187 alone in the low protein broth represents the growth of all UAL strains grown separately or in associative growth with *List. monocytogenes*. From an initial inoculum of $10^2$ CFU/mL the UAL 187 strain reached a population of $10^8$ to $10^9$ CFU/mL within 60 h. There was initial growth of *List. monocytogenes* ATCC 15313 in all experiments, but by the time that Bac$^+$ UAL 187 reached $10^6$ CFU/mL there was a noticeable inhibitory effect on the growth of *List. monocytogenes* ATCC 15313. *Listeria monocytogenes* ATCC 15313 growth was similar when grown with UAL 187 whether protease was present or absent. After 48 hours, the viable count of *List. monocytogenes* had decreased to $10^6$ CFU/mL and remained at that level to the termination of the experiment. The growth of *List. monocytogenes* with UAL 187-13 was similar to growth of *List. monocytogenes* alone.

## 4.16. Ability of supernatant fluids of *Leuconostoc gelidum* UAL 187 to inhibit *Listeria monocytogenes* ATCC 15313 or Scott A on solid medium

Supernatant fluids from cultures of *Leuc. gelidum* grown in APT broth were used to test the inhibition of *List. monocytogenes* on solid medium. Testing for activity of bacteriocin at 4 and 10°C revealed differences in susceptibility between the two *List. monocytogenes* strains. Irrespective of temperature, *List. monocytogenes* ATCC 15313 was inhibited to the same degree by the supernatant fluids of *Leuc. gelidum* UAL 187. However, *List. monocytogenes* Scott A was less affected by the bacteriocin at lower temperatures. At 10°C there was a smaller zone of inhibition than at 25°C and at 4°C there

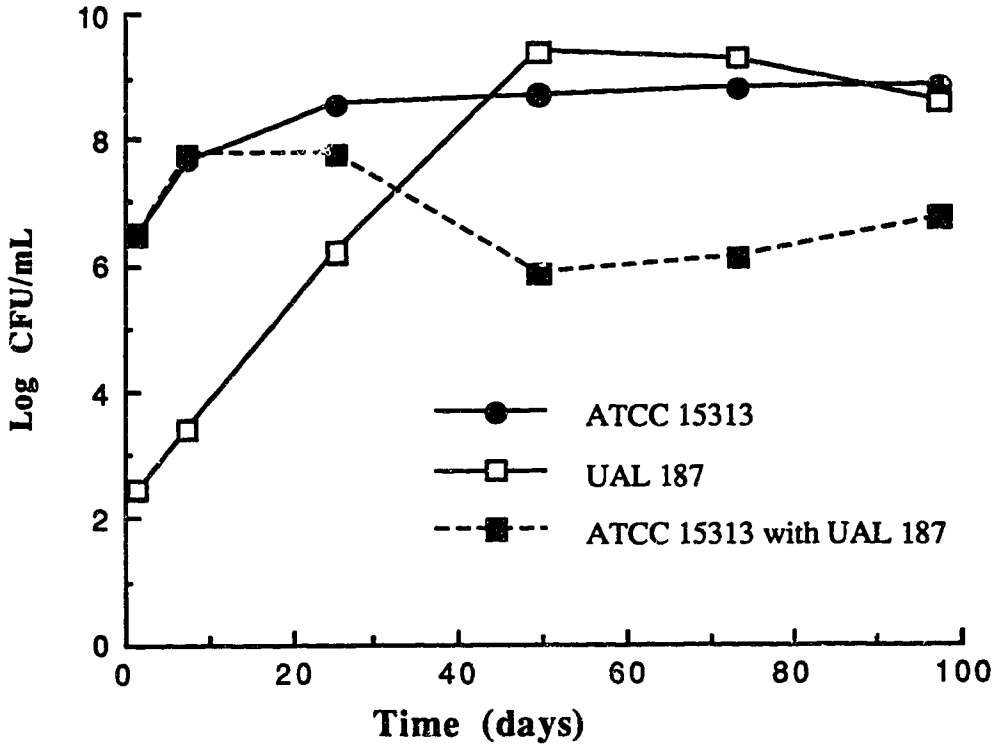


**Figure 15.** Competitive growth of *Listeria monocytogenes* strain ATCC 15313 with a bacteriocinogenic strain of *Leuconostoc gelidum* UAL 187 at 25°C in modified APT broth with lowered protein concentration.

was no inhibition. Supernatant fluids from *Leuc. gelidum* UAL 187-13 and UAL 187 grown in APT broth with protease added did not inhibit growth of either strain of *List. monocytogenes* whether grown at 4, 10 or 25°C.

### 4.17. Serotyping of wild type *Listeria monocytogenes* and their inhibition by *Leuconostoc gelidum* UAL 187

*List. monocytogenes* Scott A and *List. innocua* of dairy origin were serotyped with Bacto antisera Poly 4b. *List. monocytogenes* ATCC 15313, two dairy isolates, and thirteen of the fourteen raw hamburger isolates were serotyped with Poly 1b. One raw hamburger *List. monocytogenes* isolate could not be serotyped with the antisera available.

Fourteen strains of *List. monocytogenes* isolated from raw hamburger and 2 strains isolated from dairy farms were tested by deferred and direct antagonism techniques for inhibition by *Leuc. gelidum* UAL 187 and 187-13. One strain of *List. innocua* of dairy origin was also tested. All strains were inhibited by UAL 187 and none was found to be inhibited by UAL 187-13. Strain Scott A had a smaller sized zone of inhibition than all other listeria tested.

# 5. DISCUSSION AND CONCLUSIONS

The purpose of this study was to investigate the effect of bacteriocin produced by a lactic acid bacterium on competition with other bacteria in a mixed culture. Inhibitory metabolic end-products produced by lactic acid bacteria can repress the growth of competing microflora (Hurst and Collins-Thompson, 1979) and enabled the lactic acid bacteria to predominate (Hurst, 1983). Inhibition of pathogens by bacteriocin may occur through direct or deferred inhibition (Graham and McKay, 1985; Hoover *et al.*, 1988; Ahn and Stiles, in press; Hastings and Stiles, in press), addition of bacteriocin to the medium (Pucci *et al.*, 1988; Nielsen *et al.*, 1990) or supernatant fluids spotted onto a lawn of bacteria or the agar well technique (Schillinger and Lücke, 1989; Ahn and Stiles, in press; Hastings and Stiles, in press). Examination of inhibition of pathogenic bacteria through addition of the bacteriocin-producing bacteria to the medium has not been previously studied.

## 5.1. Identification of a bacteriocin-producing lactic acid bacterium isolated from meat

A bacterium which produced a relatively broad spectrum bacteriocin (bacteriocinogenic bacterium) early in its growth cycle was isolated from processed meat at the University of Alberta. The bacterium was identified as *Leuconostoc gelidum* through taxonomic testing as described by Shaw and Harding (1989). The classification was based upon the following characteristics: inability to grow at 35°C; ability to produce acid from amygdalin, arabinose, cellobiose, glucose, melibiose, raffinose, ribose, salicin, sucrose, trehalose or xylose; inability to produce acid from galactose, glycerol, inositol, melezitose or rhamnose; gas production from glucose; inability to hydrolyze arginine; dextran production and predominance of D(-) lactate. The carbohydrate fermentation profiles of *Leuc. gelidum* UAL 187 were similar to those described for the newly designated species by Shaw and Harding (1989).

## 5.2. Growth of *Leuconostoc gelidum* UAL 187 vs UAL 187-13

*Leuconostoc gelidum* UAL 187 was cured of the bacteriocin-producing plasmid creating a non-bacteriocinogenic mutant strain (Hastings and Stiles, in press). Growth and acid production of the Bac$^+$ and Bac$^-$ strains of UAL 187 were similar. Schillinger and Lücke (1989) also reported no difference in growth rate between Bac$^+$ and Bac$^-$ strains of *Lactobacillus sake.*

## 5.3. Competitive growth of *Leuconostoc gelidum* in APT broth

Growth of *Leuc. gelidum* UAL 187 in the competitive growth experiments was not affected by the presence of pathogenic or spoilage bacteria. Farrag and Marth (1989) and Shelef (1989) also observed no effect of the growth of *Listeria monocytogenes* on aerobic meat spoilage flora. Stimulation of growth of lactic acid bacteria was observed by Gouet *et al.* (1978) between *Lactobacillus plantarum* and *List. monocytogenes* and by Litopoulou-Tzanetaki (1987) between *Pediococcus pentosaceus* and *Salmonella typhimurium.*

*Leuc. gelidum* UAL 187 grown competitively with other bacteria inhibited their growth and predominated. Other workers have also reported inhibition of competing pathogenic microflora by lactic acid bacteria (Karunaratne *et al*, 1990; Ashenafi and Busse, 1989; Schillinger and Lücke, 1989).

## 5.4. Inhibition of *Pseudomonas fluorescens* by *Leuconostoc gelidum*

Competitive growth of *Leuc. gelidum* with *Pseudomonas fluorescens* ATCC 13525 decreased the maximum population achieved by *Pseudomonas fluorescens* in APT broth at 10°C. Other leuconostoc species such as: *Leuconostoc mesenteroides* (Dubois *et al.*, 1979) and *Leuconostoc citrovorum* (Branen *et al.*, 1975) were found to inhibit pseudomonads by decreasing the pH of the growth medium. The population of

*P. fluorescens* ATCC 13525 did not increase after the pH of the competitive growth test broth had decreased to 5.0, which supports the findings of Shelef (1977) and Pinheiro *et al.* (1968). The decreased maximum population of *P. fluorescens* in competitive growth experiments could be explained by the decrease in pH below the growth range of *P. fluorescens*. Champagne *et al.* (1990) reported no significant difference between growth of *Pseudomonas putida* grown with or without lactic acid bacteria cultures, but this experiment was conducted in milk which may buffer and support the growth of pseudomonads. *P. putida* may also be more acid tolerant than *P. fluorescens*.

Greater inhibition was exerted on the growth of *P. fluorescens* in association with UAL 187-13 than UAL 187. However, no difference in pH or in population was noted between UAL 187 and UAL 187-13. No explanation could be found to account for the difference in inhibition. *P. fluorescens* population did not reach $10^7$ CFU/mL when grown in association with UAL 187-13 during the 28 days of the experiment.

### 5.5. Inhibition of *Salmonella typhimurium* by *Leuconostoc gelidum*

*S. typhimurium* ATCC 13311 was inhibited when grown in competition with *Leuc. gelidum* at 4 and 10°C in APT broth. At 4°C *S. typhimurium* does not grow and at 10°C it grows slowly. Fedio (1986) also reported no die off or growth of *S. typhimurium* ATCC 13311 at 4°C. The inhibitory substances produced by *Leuc. gelidum* did not affect *S. typhimurium* ATCC 13311 unless it was actively growing. Therefore, *Leuc. gelidum* UAL 187 would not be a dependable inhibitor of salmonella in foods stored at 4°C. *Leuc. gelidum* inhibited growth of *S. typhimurium* at 10°C. Raccach *et al.* (1979) observed repression of growth of salmonella by *Lactobacillus plantarum* at 15°C.

Growth of *S. typhimurium* was similar in the presence of bacteriocinogenic and non-bacteriocinogenic strains of *Leuc. gelidum* UAL 187 on solid medium. Inhibition of the growth of salmonella coincided with decrease in pH of the growth medium. Inhibition of salmonella was therefore due to decrease in pH and not bacteriocin production.

Inhibition of *S. typhimurium* occurred at pH 4.7. Chung and Goepfert (1970) reported that salmonella is unable to initiate growth below pH 4.4, but pH adjustment in their study was by addition of lactic acid not by lactic acid bacteria growing directly in the medium. Ashenafi and Busse (1989) correlated inhibition of *S. infantis* with decrease in pH of the medium, but the data reported in this experiment does not support this statement. pH 5.0 did not inhibit the growth of *S. infantis* when grown alone, but when grown competitively with *Lactobacillus plantarum* there was a one log decrease in salmonella with no decrease in pH. Therefore, inhibition must be caused by a factor other than decrease in pH. Other workers observed inhibition of salmonella by antibiotic-like substances produced by lactic acid bacteria (Frank and Marth, 1977; Gilliland and Speck, 1977).

Adjustment of pH of the medium with different acids revealed that lactic acid is more inhibitory to growth of salmonella than other acids. Less inhibition was noted in medium adjusted to pH 4.5 with hydrochloric acid than adjustment to pH 5.5 with lactic acid. This concurs with the findings of Chung and Goepfert (1970), who reported growth of salmonella at pH 4.05 when adjusted with hydrochloric acid but only above pH 4.4 when adjusted with lactic acid. Decreased oxygen concentrations and temperature can increase minimum pH for growth of salmonella (Chung and Goepfert, 1970). Rubin *et al.* (1982) reported a bactericidal effect of pH 5.5 on salmonella when the medium was adjusted with lactic acid.

### 5.6. Inhibition of *Listeria monocytogenes* by *Leuconostoc gelidum*

*Leuc. gelidum* inhibited the growth of *List. monocytogenes* at 4, 10 and 25°C, but inhibition was greater at lower temperatures. This agrees with findings of Shelef (1989) who noted that *List. monocytogenes* is a poor competitor in meat held at lower temperatures. Decrease in competitive ability may be due to slower growth rate of *List. monocytogenes* by increasing lag and generation times at lower temperatures (Walker *et al.*, 1990). Growth of *List. monocytogenes* in APT broth was inhibited by the

bacteriocin produced by UAL 187. *List. monocytogenes* reacted differently to competitive growth with the bacteriocinogenic ($Bac^+$) strain UAL 187 compared with the non-bacteriocinogenic ($Bac^-$) strain UAL 187-13. This observation was also made for inhibition of a bacteriocin-sensitive strain of *Lactobacillus sake* by a bacteriocin-producing strain of *Lactobacillus sake*, but not by its bacteriocin-free mutant (Schillinger and Lücke, 1989).

The two strains of *List. monocytogenes*, Scott A and ATCC 15313, utilized in associative growth experiments reacted differently to the bacteriocin products of *Leuc. gelidum* UAL 187. Growth of *List. monocytogenes* ATCC 15313 was inhibited and *List. monocytogenes* Scott A was affected by both bacteriocin and decrease in pH of the medium. Schillinger and Lücke (1989) reported inhibition of *List. monocytogenes* by a bacteriocin-producing strain of *Lactobacillus sake*. Schaack and Marth (1988a) reported inhibition of *List. monocytogenes* by acid development when grown in association with lactic acid bacteria. Inhibition of *List. monocytogenes* by *Leuc. gelidum* UAL 187-13 was caused by decrease of the medium pH to 4.5. The minimum pH for growth of *List. monocytogenes* was reported to be 4.75 by Schaack and Marth (1988a) and 5.0 by Glass and Doyle (1989b), using strain Scott A. Differences in growth medium such as meat (Glass and Doyle, 1989b), broth or skim milk (Schaack and Marth, 1988a), and the strain of bacteria used could explain the differences in effect of pH. Survival of *List. monocytogenes* Scott A was reported at pH 4.3 by Papageorgiou and Marth (1989a). Schaack and Marth (1988b) reported death of *List. monocytogenes* at pH 4.0. The resistance to lower pH reported in these two experiments may result from a more supportive medium (milk products) in which experiments were conducted.

Decrease in incubation temperature resulted in decreased inhibition of strain Scott A by *Leuc. gelidum* UAL 187 on solid medium. This supports the findings of Sorrells *et al.* (1989) that increased survival and decreased antimicrobial activity occurred at lower incubation temperature, and those of Ahamad and Marth (1990) that a nine-fold increase in

length of survival period occurred for listeria cells exposed to acid when incubated at lower temperatures. In contrast, Parish and Higgins (1989) reported no difference in rate of death in cultures of *List. monocytogenes* Scott A with similar reductions in pH of the medium when incubated at 4 or 30°C. However, in this experiment the acid was not added directly to the medium but produced by the growth of lactic acid bacteria, which may have a different effect on the listeria.

Serotyping distinguishes differences within a species by the somatic antigens present. Bacteriocins adsorb to specific cell-envelop receptors (Tagg *et al.*, 1976). Because *List. monocytogenes* Scott A (serotype 4b) reacted differently from *List. monocytogenes* ATCC 15313 (serotype 1b) during growth in competition with *Leuc. gelidum* UAL 187 or as a result of exposure to UAL 187 bacteriocin, it was hypothesized that differences in cell wall make-up might explain differences in effect of bacteriocin against these strains. Deferred bacteriocin testing of UAL 187 against other listeria serotype 4b indicated differences in inhibition between these listeria and Scott A, therefore, no further testing of these strains for increased survival at 4°C was done.

Although a large decrease in numbers of *List. monocytogenes* occurred when grown with *Leuc. gelidum*, a resistant group of listeria cells remained in all experiments. Growth of *Leuc. gelidum* exhausted the medium which did not contain the necessary nutrients to allow the resistant listeria to grow. This was shown by the inability of *List. monocytogenes* to initiate growth in supernatant fluids of *Leuc. gelidum*, even if UAL 187 bacteriocin was not present and when the pH of the medium was neutralized. The population of *List. monocytogenes* in competitive growth experiments decreased to a steady-state from which it could not increase.

Lactic acid bacteria at concentrations greater than $10^8$ CFU/mL were reported to inhibit pathogens (Schillinger and Lücke, 1989; Raccach *et al.*, 1989). These bacterial concentrations would lead to early spoilage in meat. *Leuc. gelidum* UAL 187 at a concentration of $10^6$ CFU/mL inhibited sensitive pathogenic bacteria. UAL 187 could

inhibit and dominate when grown with a sensitive bacterium even when the latter was inoculated at higher concentrations. The ability to inhibit sensitive bacteria inoculated at higher concentrations was also observed by a bacteriocin-producing *Klebsiella pneumoniae* (De Lorenzo *et al.*, 1984). Inhibition of pathogens by bacteriocin-producing lactic acid bacteria inoculated at low concentrations could protect meat by preventing growth of pathogenic bacteria without sacrificing the shelf-life of the meat.

Inhibition of *List. monocytogenes* by *Leuc. gelidum* UAL 187 also occurred on solid media. All strains of *List. monocytogenes* tested by deferred and supernatant well plate techniques were inhibited by the bacteriocin produced by *Leuc. gelidum* UAL 187. The inhibition of all strains of *List. monocytogenes* tested by the same bacteriocin was also observed by Harris *et al.* (1989). In contrast, Hoover *et al.* (1988) tested a bacteriocin from *Pediococcus* sp. and found it effective against only three out of five strains of *List. monocytogenes* tested. *Pediococcus* sp. were tested by Harris *et al.* (1989) and they produced bacteriocins against all *List. monocytogenes* tested, therefore, the differences in inhibition should not be due to differences between species and must be due to differences in the bacteriocin produced by the bacteria tested.

Supernatant fluids of *Leuc. gelidum* UAL 187 inhibited the growth of *List. monocytogenes* ATCC 15313. In undiluted supernatant fluids no growth was observed in the $Bac^+$ or $Bac^-$ supernatant fluids. In the $Bac^+$ supernatant fluids inhibition was due to high concentrations of bacteriocin, and in $Bac^-$ supernatant fluids inhibition was probably due to depletion of nutrients in the medium. At lower concentrations of bacteriocin the listeria was inhibited initially and was able to grow later, probably after the bacteriocin in the medium was depleted.

A bactericidal mode of action of *Leuc. gelidum* UAL 187 against *List. monocytogenes* ATCC 15313 was indicated by addition of protease after exposure to the bacteriocin and the inhibitory effect was not reversed. Hasting and Stiles (in press) reported a bacteriostatic mode of action with this bacteriocin against *Carnobacterium*

*divergens* UAL 9 using the same experimental procedure. Therefore, this bacteriocin has a different effect dependent upon the type of bacterium.

Protease was ineffective in stopping bacteriocin inhibition of *List. monocytogenes* produced by an actively growing culture of *Leuc. gelidum* UAL 187 at 4 and 10°C. Decrease in temperature did not inhibit breakdown of bacteriocin by protease, and protein in the medium did not interfere with bacteriocin breakdown. The rate of reaction between UAL 187 bacteriocin and *List. monocytogenes* may be faster than between UAL 187 bacteriocin and protease, therefore, the bacteriocin reacted with the sensitive bacteria before the reaction with protease could take place.

This study provides evidence that bacteriocin production by *Leuc. gelidum* aids domination when the organism is grown in association with a sensitive bacterium. Growth of resistant bacteria was inhibited by lactic acid produced by *Leuc. gelidum.* This bacterium is not ideal for use in food because its spectrum of activity does not include any Gram-negative bacteria or all Gram-positive pathogenic bacteria commonly found on meat.

A further search for bacteriocin-producing lactic acid bacteria which inhibit these pathogenic and spoilage bacteria commonly found on meat is warranted. The inability of protease to breakdown bacteriocins requires further investigation to decide whether attachment of bacteriocin to *List. monocytogenes* occurs at a faster rate than the breakdown of bacteriocin by protease. Examination of domination among other bacteriocin-producing lactic acid bacteria from meat would aid in determining whether all bacteriocins aid domination of the producer strains. Also the ability of bacteriocin-producing lactic acid bacteria to dominate in meats must be examined before this procedure can be considered for use in foods.

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