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CHARACTERIZATION OF BROAD SPECTRUM BACTERIOCINS PRODUCED BY LACTIC ACID BACTERIA ISOLATED FROM VACUUM PACKAGED RAW BEEF

By

© **Charumati V. Mishra**

A THESIS

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research, for acceptance, a thesis entitled

Characterization of broad spectrum bacteriocins produced by lactic acid bacteria isolated from vacuum packaged raw beef

submitted by **Charumati V. Mishra**

in partial fulfillment of the requirements for the degree of **MASTER OF SCIENCE**

in **FOOD MICROBIOLOGY.**

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Date June 5/92

DEDICATED TO

MY PARENTS

ABSTRACT

Lactic acid bacteria isolated from vacuum packaged and chill stored beef were screened for broad spectrum antibacterial activity. Two strains (176 and 185) were detected that showed a broad spectrum of activity against Gram-positive organisms including *Listeria*, *Staphylococcus*, *Enterococcus* and *Brochothrix* spp. and heat activated spores of *Bacillus* and *Clostridium* spp., The meat strains 176 and 185 were biochemically similar to the reference strain *Lactococcus lactis* subsp. *lactis* ATCC 11454. However, growth on acetate agar, proportion of lactate isomers, fermentation of lactose, susceptibility to penicillin and minimum growth temperature indicated that strains 176 and 185 differ from the reference strain. The biochemical data were not sufficient to assign the meat strains to a definite taxonomic position because they do not resemble an established species other than *L. lactis* subsp. *lactis*.

Strains 176 and 185 have identical plasmid profiles consisting of 4 plasmids compared with *L. lactis* subsp. *lactis* ATCC 11454 that contains 6 plasmids. However, restriction analysis of the plasmids showed clear differences between all three strains tested. The low molecular weight RNA profiles also showed distinct patterns for all 3 organisms.

Strains 176 and 185 produce a proteinaceous inhibitory substance (bacteriocin) with a similar spectrum of activity to

that of the nisin-producing reference strain. The inhibitory substances from strains 176 and 185 are produced early in the growth cycle in contrast to nisin production by *L. lactis* subsp. *lactis* ATCC 11454. The inhibitory substances are heat stable and they are produced over a wide range of pH.

The practical application of the producer strains for production of bacteriocins in raw meats and in meat-based synthetic media at 4 and 10°C was investigated. The strains grew in raw meat at 4 and 10°C but bacteriocin was not recovered. However, bacteriocins were produced in cooked meat medium containing fermentable carbohydrate, which suggests that strains 176 and 185 and their bacteriocins have the potential for use as preservative agents in processed meats, possibly replacing or reducing the level of nitrite used in processed meats.

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1. INTRODUCTION

Meat is a rich source of nutrients, which makes it an excellent environment for microbial growth and therefore extremely perishable. Cooked meat has been implicated in more outbreaks of foodborne illness than any other food. Today, the food industry is developing new products with novel or modified formulations and alternative packaging strategies to fulfill the consumer's demand for fresh, high quality, convenient meals. Modified atmosphere, including vacuum packaging, may be used to extend the shelf life of foods by inhibiting the growth of aerobic spoilage bacteria. Unfortunately, this practice may provide an atmosphere conducive to the growth of *Clostridium botulinum* while inhibiting potential competitors which are indicators of incipient spoilage. It is possible that during the extended shelf life of a modified atmosphere packaged (MAP) product, growth of pathogenic organisms may occur or even be stimulated before spoilage is evident. There is little margin of safety in this instance and additional hurdles should be incorporated into the product.

Furthermore, the ability of pathogenic bacteria to grow at refrigeration temperature has generally been underestimated by food microbiologists and public health officials. Temperature abuse of a refrigerated product may occur at any stage from production to consumption but quantifying the

degree of abuse is a difficult task (Hutton *et al.*, 1991). The possible occurrence and survival of psychrotrophic, mesophilic as well as injured bacteria create a need for strict handling and processing of food products. Though it is difficult to prevent contamination the rapid build-up of the potential spoilage and pathogenic microflora can be significantly reduced or delayed. Various methods have been reported in the literature such as use of nitrites and antibiotics in meat products. But the use of antibiotics is not permitted because of the potential for the development of resistant strains of pathogens, the potential for human hypersensitivity to antibiotic residues, the costs and difficulties involved in monitoring their use. The use of nitrite in meat has been questioned because it interacts with amines present in meats to produce nitrosamines which are believed to be potent carcinogens.

The challenge to develop safe and acceptable antimicrobial agents as preservatives is obvious. Fermentation with lactic acid bacteria (LAB or lactics) is one of the oldest known methods of food processing and preservation. The antagonistic activity of various LAB starter cultures or their fermentation products is well recognized for the preservation of foods such as dairy products, meats, cereals, fruits and vegetables. Lactic acid production by LAB is the primary mechanism for antagonism; however, other inhibitory activity of lactics has also been

reported in nonfermented food products such as cooked ham, bacon and ground beef. Although there are many instances of spoilage due to LAB, their essential role in preservation and flavour production in fermented foods far outweighs their spoilage potential. Interest in biocontrol is presently experiencing a resurgence in the food industry.

The predominance of LAB in foods is believed to be the result of antimicrobial activity caused by lactic acid, hydrogen peroxide and antimicrobial substances (bacteriocins). Antimicrobial peptides (bacteriocins) from LAB are bactericidal to many Gram-positive bacteria associated with food spoilage and foodborne illnesses, and in some cases have potential to be used as biological preservatives without acidification of the food (Klaenhammer, 1988; Stiles and Hastings, 1991). Bacteriocin production may be one of the factors that promotes the predomination of the producer organism in a mixed population of bacteria. All indications are that they are safe for consumption and can be used to enhance the safety and storage life of many foods (Hurst, 1981; Klaenhammer, 1988). By definition, bacteriocins have a narrow spectrum of activity (Bhunia *et al.*, 1991), except nisin and some pediocins, which have relatively wide spectra of bactericidal activity. Nisin produced by *Lactococcus lactis* subsp. *lactis* is the most highly characterized bacteriocin produced by LAB and is widely used as a food preservative in dairy products (Delves-Broughton, 1990).

However, the value of nisin for use in meat is doubtful. Pediocins produced by *Pediococcus acidilactici* and *Pediococcus pentosaceus* have not been well characterized and are not currently used for food preservation.

Exploitation of the bacteriocin producing ability of lactic acid bacteria of meats is the best choice for improving the microbial safety of the meat products because of their natural adaptation to the meat environment (Schillinger and Lücke, 1989). LAB of meat are not well defined except for some specific starter organisms that are used in fermented meat products. The efficacy and spectrum of antimicrobial activity of bacteriocins needs further investigation. This may lead to further screening for broad spectrum bacteriocins and may involve genetic or protein engineering of such compounds.

The objectives of this study were:

1. To screen lactic acid bacteria isolated from vacuum packaged meat for broad spectrum bacteriocin producers active against both spoilage and foodborne pathogenic organisms, including aerobic and anaerobic sporeforming organisms.
2. To identify and characterize bacteriocinogenic strains.
3. To characterize bacteriocins and confirm their antagonistic activity.
4. To seek a practical approach of using the producer strains, by adding them to meat under different

conditions of storage and to evaluate their survival, growth and production of bacteriocin.

2.1 Microflora of Meat

Many kinds of microorganisms gain access to meat during slaughter and subsequent handling. The organisms found on hides and the surfaces of carcass meats may originate from soil, water, vegetation, air or from the skin of the animal (Egan, 1983). Contaminated lymph nodes are also thought to be a source of bacteria for deep spoilage of post rigor meat. The factors that determine the storage life of meat are the types of microorganisms present, the extent of initial contamination, storage temperature and type of packaging material used.

The predominant types of bacterial genera reported on raw meats are: *Acinetobacter, Aerococcus, Alcaligenes, Brevibacterium, Brochothrix, Corynebacterium, Flavobacterium, Lactobacillus, Micrococcus, Pediococcus, Pseudomonas* and *Staphylococcus*. Quite often *Clostridium perfringens* and Enterobacteriaceae including *Salmonella* are found on red meats. *Campylobacter jejuni* (Palumbo, 1986), *Yersinia enterocolitica* (Stern and Pierson, 1979) and *Listeria monocytogenes* (Farber and Peterkin, 1991) have also been isolated from meats.

The concentration of residual glucose in post-mortem tissue plays an important role in the time of onset of spoilage and the types of organisms found in meat (Lambert *et al.*, 1991). Dark, firm and dry (DFD) raw meat, caused by

exposing the animals to stress before slaughter, has a high pH (> 6.0) and a low residual glucose concentration. The predominant microorganisms of DFD meat are *Shewanella putrefaciens*, *Enterobacter liquefaciens* and *Y. enterocolitica*. These species are not present in significant numbers on meat of normal pH (Gill and Newton, 1979).

2.1.1 Aerobically stored meat and its microflora

In general, the whole carcass presents a surface of fat and connective tissue affording little opportunity for bacterial growth. The surfaces of meat cuts are moist and support the growth of bacteria, and ground meat offers not only ample and desirable surfaces, but a thorough inoculation of the meat during grinding.

During processing, meat carcasses or primal cuts are held near 0°C to reduce the rate of biochemical and microbial changes and this results in the mesophilic surface contaminants being superceded by psychrotrophic organisms at a rate determined by the temperature and relative humidity. Proteolytic, fast growing, aerobic *Pseudomonas* - type bacteria are the main psychrotrophs present in aerobically stored, refrigerated meat. The associated putrid odour is the consequence of protein degradation, but this only occurs in the advanced stage of bacterial growth. *Acinetobacter*, *Moraxella*, *Shewanella* and psychrotrophic Enterobacteriaceae

are often present as minor components of the microflora (Lambert *et al.*, 1991).

Bacteriology and chemistry of meat spoilage are discussed in several reviews (Dainty *et al.*, 1975; Jay and Shelef, 1978; Gill, 1979, 1983). When carcasses are chill stored, surface drying may slow microbial growth, but the extent of drying is difficult to control. On lean tissue, glucose is utilized preferentially for growth until its rate of diffusion from the bulk of the meat becomes a limiting factor for growth at the meat surface. At that point, proteolysis begins, and it is followed by amino acid catabolism. The metabolism of free amino acids and peptides leads to the formation of hydrogen sulphide, ammonia and various amines and diamines. The H_2S reacts with myoglobin at low oxygen levels forming the green colour, sulphmyoglobin. Defects of meat are off-odour, slime formation and discoloration.

2.1.2 Anaerobically stored meat and its microflora

A number of methods of preserving meats is known, including 12 D thermal processing, acidification, freezing, reduction of water activity, the use of curing salts and refrigeration. Refrigeration is probably the least secure method of preservation because of the potential for temperature abuse. The shelf life for aerobically packaged retail meat cuts is limited to about 3 days. The shelf life of fresh meat can be extended by vacuum or modified atmosphere packag-

ing with elevated levels of carbon dioxide (VP or MAP, respectively). Both methods have been used for the preservation of meats since the early 1960s. Under VP or anaerobic MAP, chilled meats undergo adventitious lactic fermentation that extends their storage life from a few days to several weeks. The application of this type of packaging technology expanded during the past decade to include fresh meats and meat products.

Vacuum packaging using gas impermeable film alters the gas phase in which meat is stored. The integrity of the package must be maintained or the preservative effect will be lost. The gas phase in the package is determined by the rate of gas permeation of the film, oxygen consumption by the meat tissue as well as microbial respiration. Storage of VP, chilled meat inhibits the growth of aerobic spoilage bacteria. Lactic acid bacteria dominate the microflora, and *Brochothrix thermosphacta* may be present as a minor component of the microflora (Nielsen, 1983; Lee *et al.*, 1984). The composition of this meat microflora is determined by the pH of the meat and the permeability of the packaging film to oxygen, CO_2 and water vapour. In DFD meat and under anaerobic conditions, *Shewanella putrefaciens* produces H_2S resulting in a green discoloration of meat.

The gas mixture of MAP contains CO_2 to inhibit microbial growth, particularly Gram-negative bacteria; it may also

contain O_2 to maintain the pigment in its oxygenated form (oxymyoglobin); and often contains nitrogen as an inert filler (Daniels *et al.*, 1985). In MAP, the antimicrobial effect of CO_2 is influenced by several interrelated factors including the types and number of microorganisms present on meat, the time of application of MAP, the gas concentration and the storage temperature. Growth of anaerobic organisms, specifically *C. botulinum*, could occur under these conditions. The growth of other pathogens such as *Salmonella* spp., *S. aureus*, *Campylobacter* spp., *Y. enterocolitica* and *L. monocytogenes* may not be influenced by elevated level of CO_2 (Lambert *et al.*, 1991). The greatest antimicrobial effect is obtained at a level of 100% CO_2. However, this concentration affects the colour of red meat. Finne (1982) found that 15-20% CO_2 partially inhibits bacterial growth and maintains the bright colour of fresh meat. The shelf life achieved in MAP may be less than that attained by VP, but the bright red colour of meat which appeals to the consumers is maintained.

2.1.3 Domination of lactic acid bacteria in VP/MAP meat

LAB have little effect on the shelf life of fresh and cured meats stored under aerobic conditions because they constitute only a small proportion of the bacterial population that develops on the meat. However, LAB dominate the microflora of fresh meat stored either under vacuum or in

modified atmospheres with increased CO_2 (Christopher *et al.*, 1980a,b; Hitchener *et al.*, 1982; Shaw and Harding, 1984; Borch and Molin, 1988; Grant and Patterson, 1991). The ability of these organisms to grow at low temperature (as low as 1°C) and the stimulatory effect of CO_2 on their growth, enables them to proliferate in VP and MAP meats. Many strains also produce inhibitory substances against other microorganisms. There are some conflicting results reported in the literature for the faster growth rate of *B. thermosphacta* than LAB on VP or MAP beef and pork (Shaw *et al.*, 1980; Erichsen and Molin, 1981). Differences in pH, proportion of fat in different products and/or the permeability of the packaging material could account for the discrepancies.

2.1.4 Spoilage of MAP/VP meat due to lactic acid bacteria

The predominating LAB on MAP/VP meat eventually cause the spoilage of the product. Storage life varies depending upon the types of LAB that grow and dominate the microflora. Spoilage under these conditions is normally characterized as 'sour, cheesy' and off-odour. Off-flavours can be caused by minor fermentation products, such as diacetyl and acetoin or as a result of lipolytic and proteolytic activities of these organisms which lead to the formation of short-chain fatty acids and accumulation of amines. Other defects attributed to LAB include off-flavours due to formation of methanethiol and hydrogen sulphide and blowing of packaged meats due to

production of CO_2 (Sharpe and Pettipher, 1983). *Lactobacillus plantarum, Lactobacillus casei* and *Leuconostoc* spp. can cause spoilage by producing copious amounts of slime on meat surfaces.

It is important to note that spoilage occurs some weeks after the population of bacteria reaches a maximum level and thus a total count alone is of little value in predicting shelf life (Egan, 1983). The souring type of spoilage that results is less objectionable than aerobic putrefaction. A shelf life of 12-14 weeks can be achieved if vacuum packaged beef is held at -1°C.

2.2 Control of microorganisms in meats

The prevention of contamination of food with human pathogens and control of proliferation of toxin-producing microorganisms is important to ensure food safety.

2.2.1 Safety concerns

In general, as long as chill temperatures are maintained, there is no reason for concern about growth of pathogens in red meats (Genigeorgis, 1985). When a refrigerated food with high water activity, a pH >4.6 and no chemical preservatives is subjected to temperature abuse at any stage from production to consumption, then the presence of competitive microorganisms is the only defense against pathogenic organisms.

If the growth of the competitive microflora has been retarded by MAP or eliminated by pasteurization, then only

refrigeration exists as a barrier to prevent the germination and outgrowth of spores of *C. botulinum*. In many MAP systems, the O_2 concentration is adjusted to inhibit growth of *C. botulinum*. However, changes in gas levels can occur due to the growth of microorganisms and/or permeability of the packaging material. Therefore, over time, an anaerobic environment can develop within a food container. Nonproteolytic strains of *C. botulinum* (particularly in seafoods) may have time to grow if the shelf life is extensive. This organism can produce toxins in food before the development of detectable spoilage (Post *et al.*, 1985). Proteolytic strains of *C. botulinum* may grow if there is temperature abuse (Conner *et al.*, 1989). The possible consequences of prolonged refrigerated storage of meat products contaminated with *L. monocytogenes* and *Y. enterocolitica* and other psychrotrophic pathogens have not been thoroughly investigated. More and more of the traditional food poisoning bacteria as well as emerging pathogens are being recognized as capable of growth within or near the range of 0-5°C (Roberts, 1989). It is essential to build into a refrigerated product an additional line of defense to guard against the growth of these psychrotrophic pathogens.

2.2.2 Control of microorganisms

Food products that are microbiologically stable and safe are recognized to be the consequence of several preservative factors acting in combination, often at levels that singly would not be inhibitory. While the growth limiting values of individual factors are well documented, particularly temperature (Michener and Elliott, 1964) and water activity (Troller, 1980, 1986) determination of growth limiting values of combinations of antimicrobial agents and physical factors poses difficulties both in experimentation and expressing the results (Roberts, 1989).

It has been clearly established that the main factors controlling microbial growth are temperature, pH, gas atmosphere, water activity, addition of preservatives, and the presence of competitive microflora. The United States Food and Drug Administration (FDA) recently recommended that retail store production of vacuum packaged refrigerated foods should not be allowed unless antibotulinal barriers are present (Gombas, 1989). One of the barriers specified was the presence of high levels of nonpathogenic competing organisms. The presence of competitive microorganisms in a food can greatly influence the safety and stability of a refrigerated food. In the following sections, biocontrol that involves the incorporation of lactic acid bacteria and/or bacteriocins for retention of quality and safety of meat is reviewed.

2.3 Lactic acid bacteria in meat

2.3.1 Lactic acid bacteria

These organisms are Gram-positive, asporogenous cocci, coccobacilli or rods that produce lactic acid as a major end product of metabolism. They include the following genera: *Lactococcus*, *Pediococcus*, *Leuconostoc*, *Lactobacillus*, *Carnobacterium*, *Bifidobacterium* and *Propionibacterium*. The homofermentative LAB convert carbohydrates primarily to lactic acid, while the heterofermentative LAB produce lactic and acetic acids, ethanol and CO_2. The homofermenters use the glycolytic (hexose diphosphate) pathway to degrade carbohydrates, while the heterofermenters use the phosphoenolketolase pathway as well as alternative pathways (Kandler, 1983). Studies have shown that some homofermenters become heterofermentative under conditions of environmental stress or glucose limitation (Borch *et al.*, 1991).

2.3.2 Taxonomy of lactic acid bacteria from meat

The LAB are a phylogenetically diverse group of microorganisms. Fast, reliable identification of LAB is hampered by the fact that taxonomic methods relying on physiological or biochemical criteria are ambiguous and time consuming. Furthermore, determination of carbohydrate fermentation is not convenient and may be misleading because fermentation patterns are similar for several species. As stated by Stackebrandt and Teuber (1988), phylogenetic and traditional classifica-

tions of LAB often disagree at the genus level. This probably occurs because some characteristics such as morphology are overemphasized. The phylogenetically redefined taxa must be confirmed, but a broad spectrum of characteristics can be used to define the border of each taxon.

The taxonomy of LAB from meat sources has been a problem mainly because meat lactics do not conform to the species primarily of dairy origin that have been described in the literature. Many atypical strains have been described and these have proven difficult to identify (Egan, 1983). Most lactics isolated from meat have not been, or could not be identified at the species level (Mol *et al.*, 1971; Hitchener *et al.*, 1982; Lee and Simard, 1984; Lewus *et al.*, 1991). Different test systems can produce quite different results with the same strain (Smith *et al.*, 1972), as a result, all characteristics of new isolates should finally be compared with results of conventional methods to determine their most probable identity.

In a numerical classification of LAB isolated from refrigerated VP meats, Shaw and Harding (1984) divided them into 4 clusters, aciduric, nonaciduric, *Leuconostoc* and unclustered strains. The previously ill-defined homofermenters or atypical streptobacteria were split into two groups. Cluster I included the nonaciduric group of lactobacilli. These strains are characterized as being unable to grow on

acetate agar at pH 5.6 and growth ceases at a terminal pH >4.15 in La broth. None is capable of growing at pH 3.9. Cluster II includes the aciduric group of lactics that are able to grow on acetate agar at pH 5.6, the terminal pH in La broth is <4.15 and about 60% are capable of growth at pH 3.9.

Schillinger and Lücke (1987) proposed a rapid and simple identification scheme for lactobacilli from meat. The scheme was based on sugar fermentation and other easily determinable physiological characteristics. Borch and Molin (1988) suggested that identification based on carbohydrate fermentation is not enough. They compared the fermentation patterns for 34 type strains of *Lactobacillus* with the identification key in Bergey's Manual of Systematic Bacteriology (1986). About 47% of the type strains fully agreed with the key or only deviated in a single test; while six of the tested type strains differed in the fermentation of at least 5 carbohydrates. The existence of such variations has been reported by other researchers (Benno and Mitsuoka, 1983; Lee and Simard, 1984).

Hastings and Holzapfel (1987), using a more conventional methodology, reported fermentation patterns for *Lb. sake* DSM 20017T and *Lb. farciminis* DSM 20184T that also deviated from the key of Bergey's Manual. Grant and Patterson (1991) carried out a numerical study of LAB isolated from irradiated pork and chicken. They found that 80% of strains from pork

and 86% of strains from chicken were clustered with *Lb. sake* strains. This compares well with the results of the study by Hastings and Holzapfel (1987).

Recently, many changes have been made in the taxonomy of LAB. The application of nucleic acid hybridization, sequencing techniques and molecular typing has provided new insights into the relationship among various organisms and has led to important changes in their taxonomy and nomenclature. In particular, DNA-23S rRNA hybridization and superoxide dismutase studies have shown that 'lactic streptococci' form a distinct group from the 'pyogenic' streptococci and enterococci and a new genus *Lactococcus* has been proposed (Schleifer *et al.*, 1985). However, the group is still biochemically and physiologically ill-defined and previous typing based on serological methods, phage typing, bacteriocins, plasmid profile and chromosomal DNA restriction endonuclease analysis have met with only limited success. *L. lactis* and *L. garvieae* are phenotypically closely related and there is no simple means of distinguishing between these taxa. *Lactococcus garvieae* was originally isolated from bovine mastitis and also associated with human clinical infection. Recently Rodrigues *et al.* (1991) proposed specific and intraspecific molecular typing of *Lactococcus* spp. based on polymorphism of DNA encoding rRNA. Highly discriminatory restriction patterns were obtained. Collins *et al.* (1989) suggested a new genus,

Vagococcus, for motile *Lactococcus* spp. The 16S ribosomal RNA sequence analysis data show that this group is phylogenetically unrelated to *Lactococcus* spp. but closer to *Enterococcus* spp. (Collins *et al.*, 1989). Based upon DNA-DNA hybridization, Collins *et al.* (1987) also proposed a new genus *Carnobacterium* which was previously described as nonaciduric *Lactobacilli* by Shaw and Harding (1984).

2.4 Lactic acid bacteria for preservation by biocontrol

2.4.1 History

The literature is replete with studies showing that perishable foods can be preserved by fermentation with LAB. Traditionally, the fermentation process was an empirical process in which raw foods were observed to undergo a change which resulted in a different food with enhanced keeping qualities. This has been developed commercially into the selection and use of highly specific starter strains, particularly for dairy products, but also for meat products, fish, silage and sourdough breads. If the starter culture functions properly, the growth of both spoilage and pathogenic microorganisms is retarded during the manufacture and subsequent storage of the fermented product. Gilliland and Speck (1975) proposed use of LAB as GRAS (generally regarded as safe) microorganisms.

It was not until the 1970s that studies investigated the reasons why fermented foods did not support the growth of

pathogenic bacteria. A great deal of research was concentrated on contaminated fermented sausages, particularly for growth of *S. aureus* and *Salmonella* spp. Goepfert and Chung (1970) studied the fate of *Salmonella* in Thuringer sausage fermented with either *Pediococcus* or *Lactobacillus* spp. Growth of *Salmonella* declined in properly fermented products. Similar results were obtained in Lebanon bologna (Smith *et al.*, 1975a), pepperoni (Smith *et al.*, 1975b), and in cultured milk (Park and Marth, 1972).

It was reported by Riemann *et al.* (1972) that there was an interaction between pH and salt concentration on the growth and enterotoxin production of *S. aureus*. Reduction in pH due to lactic acid produced by LAB was the main inhibitory agent noted in the majority of studies. In some cases, additional barriers contributing to inhibition were also noted. Gilliland and Speck (1972) found that growth of *Salmonella* and *S. aureus* were still inhibited in cultured milk even when pH was continually adjusted to 6.6. Raccach *et al.* (1979) also reported other aspects of the growth of LAB, such as production of antimicrobial compounds, that were credited with contributing to the inhibitory action of LAB. In some fermented sausages (Mettwurst), high acidity is not desirable (Schillinger and Lücke, 1990). Gilliland and Speck (1975) demonstrated inhibition of *Pseudomonas fragi* in refrigerated ground beef inoculated with *L. lactis*, *Pediococcus cerevisiae*

and *Lb. bulgaricus*. Reddy *et al.* (1975) also reported the inhibition of Gram-negative spoilage bacteria and an extended period of sensory acceptance of refrigerated ground beef without any appreciable decrease in pH with the addition of *L. lactis* and *Leuconostoc citrovorum*.

2.4.2 Inhibitory compounds produced by lactic acid bacteria

Antimicrobial activity of LAB isolated from food has been the subject of intensive research due to the potential for application of these bacteria as protective cultures in biological preservation (Tagg *et al.*, 1976). The major groups of inhibitory compounds produced by LAB are:

(i) Lactic acid and other volatile acids and the resulting decrease in pH.

(ii) Other inhibitory primary metabolites such as H_2O_2, CO_2, and diacetyl.

(iii) Bacteriocins - special antimicrobial compounds.

Each of these groups of compounds, especially a combination of them, can be used to extend the shelf life and safety of food products. Antibiosis of LAB has been reviewed by Daeschel (1989) and by Lindgren and Dobrogosz (1990).

Lactic acid is the major metabolite produced by LAB and, depending on the substrate and microorganisms, lactic acid has been reported as having good, average or poor antimicrobial properties. Gill and Newton (1982) observed that the inhibitory effect of lactic acid on Gram-negative psychrotrophic

bacteria found on meat appeared to be due to the decrease of pH and not to the undissociated acid. Acetic acid is another organic acid produced by LAB. Both acids and their salts are generally regarded as safe (GRAS) by the United States Food and Drug Administration. Acetic acid and its salts assert their antimicrobial activity up to pH 4.5 and the effect is due to undissociated molecules (Doores, 1983). Acetic acid has a stronger bactericidal effect on spoilage organisms than lactic acid (Subramanian and Marth, 1968).

Hydrogen peroxide produced by LAB is inhibitory to both Gram-negative *Pseudomonas* spp. (Price and Lee, 1970) and Gram-positive *S. aureus* (Dahiya and Speck, 1968). Because LAB do not possess catalase (Kandler and Weiss, 1986), H_2O_2 accumulates in the surrounding medium, resulting in anaerobic conditions. It may also function via the lactoperoxidase-thiocyanate system. The H_2O_2 oxidizes the thiocyanate to release toxic oxidation products that are detrimental to foodborne pathogens (Fernandes *et al.*, 1987). Use of H_2O_2 has been approved by the United States Food and Drug Administration as an antimicrobial agent in raw milk for the manufacture of certain cheese types and in whey processing. According to Baldry (1983), H_2O_2 is more effective as a sporicide than as a bactericide.

Diacetyl (2,3 butanedione) is synthesized by certain species of LAB from pyruvate. It is an important flavour

component of fermented milks, but it has also been shown to inhibit Gram-negative bacteria, Gram-positive bacteria other than LAB and yeasts (Jay, 1982). Diacetyl interferes with arginine utilization by reacting with arginine-binding proteins of Gram-negative organisms (Jay, 1986).

Bacteriocins produced by LAB are the subject of intense research because of their antimicrobial activity against foodborne bacteria e.g. *L. monocytogenes, S. aureus, B. cereus, C. botulinum* and several others (Gilliland and Speck, 1972; Klaenhammer, 1988; Hoover *et al.*, 1989; Spelhaug and Harlander, 1989; Stiles and Hastings, 1991). Bacteriocins have considerable promise for application as natural food preservatives. In the following section, information about bacteriocins produced by LAB is reviewed.

2.5 Bacteriocins

2.5.1 General characteristics of bacteriocins

Although not exactly defined, bacteriocins differ from classical antibiotics. The characteristics used to define bacterocins are generally based on those for colicins (from *E. coli*) and include: a narrow inhibitory spectrum, usually active against closely related species; a biologically active protein moiety; a bactericidal mode of action; attachment to specific cell surface receptors; plasmid-borne genetic determinants; host cell bacteriocin immunity and production by lethal biosynthesis (Tagg *et al.*, 1976). However, a number of

bacteriocins produced by Gram-positive bacteria and especially LAB show discrepancies (Upreti and Hindsdill, 1973; Barefoot and Klaenhammer, 1983). It is difficult to make a specific definition for bacteriocins because many inhibitory compounds have not been sufficiently characterized to fulfill any classification (Tagg *et al.*, 1976).

According to Klaenhammer (1988) bacteriocins are a heterogeneous group of bacterial antagonists that vary considerably in molecular weight, biochemical properties, range of sensitive hosts and mode of action. He redefined them as follows: "Bacteriocins are proteins or protein complexes with bactericidal activity directed against species that are usually closely related to the producer bacterium".

In the past few years many bacteriocins or bacteriocin-like compounds have been reported and characterized (Lewus *et al.*, 1991; Papathanasopoulos *et al.*, 1991; Skyttä *et al.*, 1991; Stiles and Hastings, 1991). Many of these substances do not comply with the definition of bacteriocins proposed by Klaenhammer (1988). Bacteriocins are generally active against closely related species and thus the inhibitory spectrum is very narrow (especially bacteriocins from LAB). However, nisin from *L. lactis* (Hurst, 1981) and pediocin A from *Pediococcus pentosaceus* B61 and L-7230 (Daeschel and Klaenhammer, 1985) are active against a broader range of foodborne pathogens. Recently it was claimed that bacteriocins from

Pediococcus damnosus and *P. pentosaceus* inhibited the growth of Gram-negative organisms such as *Y. enterocolitica*, *Pseudomonas fragi* and *P. fluorescens* (Skyttä *et al.*, 1991).

Lewus *et al.* (1991) reported that the bacteriocins from *P. pentosaceus* 43200 and 43201 are insensitive to proteases and are not protein in nature. However, it was proposed: (i) that these bacteriocins may be glycoproteins; (ii) that only minor components may be proteinaceous; or (iii) that active domains are not affected by the proteases. Most bacteriocins produced by LAB are bactericidal. However, lactocin 27 from *Lb. helveticus* (Upreti and Hindsdill, 1975), bacteriocin from *Lb. sake* 148 (Sobrino *et al.*, 1991), and bacteriocin from *Leuconostoc gelidum* (Hastings and Stiles, 1991) have a bacteriostatic effect.

These examples do not comply with Klaenhammer's definition of bacteriocins, which states that they have a bactericidal mode of action, that they are protein-containing molecules and that they have a narrow spectrum of antibacterial activity. The term 'bacteriocin-like substance' was suggested by Tagg *et al.* (1976) for those antagonistic substances that do not fit the traditional definition of a bacteriocin.

There is no clear cut boundary between antibiotics, bacteriocins or microcins. Like antibiotics, bacteriocins are bacteriostatic or bactericidal with narrow or broad ranges of

activity, that could be included in a family of peptide antibiotics. Antibiotics are synthesized nonribosomally by multi-step enzyme pathways. There are many ribosomally synthesized bacteriocins. While many antibiotics promote the development of resistant strains, development of resistance to bacteriocins is rare. However, the possibility of resistance to bacteriocins may have been overlooked (Jarvis and Farr, 1971; Hurst, 1981; Harris *et al.*, 1989). Many antibiotics can be chemically synthesized. There is no report to date in which bacteriocins are chemically synthesized. However, it is appropriate to expect that with genetic engineering, it will be possible to construct and design analogues of bacteriocins.

Another class of low molecular weight bacteriocins produced by Enterobacteriaceae are the microcins. The production of colicins is regulated by the host cell SOS system (Pugsley and Oudega, 1987). This means that the transcription of the colicin structural gene is normally repressed by the product of the chromosomal *lexA* gene. Induction of colicin production by SOS-system inducing agents are required to activate the product of the chromosomal *recA* gene, which then catalyses cleavage of LexA protein and allows RNA polymerase to engage the colicin operon promoter and start transcription. In contrast to colicins, microcins are not induced by SOS-system inducing agents such as mitomycin C treatment or UV irradiation. However, many unclassified bacteriocin-like

substances produced by Gram-positive organisms such as *Streptococcus, Lactobacillus* or *Listeria* spp. may resemble microcins in that they are also low molecular weight (<10,000) and not inducible by mitomycin C (Baquero and Moreno, 1984). There is considerable overlap in the definition of antimicrobial substances. In general, bacteriocins are a heterogeneous group of proteinaceous compounds that may vary in spectrum of activity, mode of action, molecular weight, genetic origin and biochemical properties. Their ability to inhibit other bacteria and the fact that all are proteinaceous may be the only common features of this mixed group of substances (Stiles and Hastings, 1991).

Lipid and (or) carbohydrate moieties can be associated with the proteinaceous compounds and be part of the bacteriocin complex. Bacteriocins can be either cell bound or released extracellularly. Bacteriocins can be produced early or late in the growth cycle. They are susceptible to proteases and their stablity at different pH and temperature varies.

2.5.2 Bacteriocins and immunity

Cells that produce a particular bacteriocin are immune to the lethal action of that bacteriocin and sometimes might also be immune to other types of bacteriocins. In E-group colicins, an extra immunity gene was found which is specfic for a different E-type colicin (Lau and Condie, 1989). The mechanism of immunity is not well understood. In well characterized

colicins E2-P9 and E3-CA38, which are DNA and RNA nucleases, respectively, the immunity proteins bind strongly to their homologous colicin molecules, thus preventing nuclease activity on producer cells. These immunity proteins are released from the producing cells as a complex with their homologous colicins (Oudega *et al.*, 1984; Luirink *et al.*, 1987). Although host cell death or suicidal activity of producer strains is not a common phenomenon in LAB, they are very common in *E. coli*. The colicin immunity and lysis or release genes seem to be organized in one of two ways. In some colicin operons, the immunity gene is co-transcribed with the colicin structural gene, resulting in survival of the host cell. Whereas some colicin operons do not include the immunity gene, resulting in host cell death (Pugsley and Oudega, 1987). Host cell death is also known to be caused by the lysis gene located distal to the SOS promoter of the colicin operon (De Graaf and Oudega, 1986; Pugsley and Oudega, 1987). Kanon *et al.* (1991) observed that in highly induced colicin E2, the lysis protein causes host cell death.

2.5.3 Bacteriocins of lactic acid bacteria

The first detailed characterization of bacteriocinogenic activity of lactobacilli was reported by De Klerk and Coetzee (1961). Since then, research has been on-going on bacteriocins from various LAB, notably on nisin.

Nisin is produced by *Lactococcus lactis* subsp. *lactis* of dairy origin. Nisin is the only bacteriocin that is accepted as a food preservative. It has a broad spectrum of activity against Gram-positive organisms including sporeforming bacteria. Nisin occurs as several types, A, B, C, D or E, that differ in amino acid composition and biological activity (Hurst, 1981).

Nisin is a pentacyclic cationic polypeptide, referred to as a lantibiotic. It contains 34 amino acids and is synthesized by posttranslational processing of ribosomally synthesized precursors. Processing events include dehydration of serine and threonine to dehydro forms, some of which react with cysteine residues to form thioether cross linkages. In addition, a leader peptide is cleaved and the mature bacteriocin is exported from the cell. The modified peptide bacteriocin is characterized by the occurrence of the sulphur-containing amino acids lanthionine and ß-methyllanthionine. The unusual amino acids of nisin, dehydroalanine(DHA) and dehydrobutyrine (DHB), are thought to inactivate sulphydryl groups in germinated bacterial spores (Liu and Hansen, 1990). The biochemistry, genetics and mode of action of nisin A has been extensively reviewed (Hurst, 1981; Klaenhammer, 1988; Ruhr and Sahl, 1985; Buchman *et al.*, 1988; Delves-Broughton, 1990).

According to Klein *et al.*(1992) lantibiotics can be divided into two subgroups: (1) linear and (2) globular

shaped. Linear lantibiotics include nisin (Mattick and Hirsch, 1944; Ingram, 1970), subtilin (Banerjee and Hansen, 1988), epidermin (Allgaier *et al.*, 1986), gallidermin (Kellner *et al.*, 1988) and Pep 5 (Sahl and Brandis, 1981). Globular lantibiotics include cinnamycin (Benedict *et al.*, 1952), Ro09-0198 (Kessler *et al.*, 1987), lanthiopeptin (Naruse *et al.*, 1989), duramycin (Shotwell *et al.*, 1958) and ancovenin (Wakamiya *et al.*, 1985). All globular lantibiotics are synthesized by *Streptomyces* spp. and have only weak antimicrobial activities. Nisin and subtilin (from *Bacillus subtilis*) possess similar activity spectra and a generally similar peptide structure. Epidermin (from *Staphylococcus epidermidis*) and gallidermin (from *Staph. gallinarum*) differ only at the amino acid residue in position 6. The general structure of lantibiotic genes are the same for all lantibiotics described to date. The conservative organization of all linear lantibiotic structural genes suggests that the enzymes involved in lantibiotic maturation are also homologous for different producers (Klein *et al.*, 1992).

Lactococcal strains that have been reported to produce lantibiotics are listed in Table 2.1. Several other non-lantibiotic bacteriocins from LAB were reviewed by Klaenhammer (1988). Their inhibitory spectra are quite restricted.

Table 2.1 Lantibiotics produced by *Lactococcus lactis* subsp. *lactis*

Strain	Reference
ATCC 11454	Ingram, 1970
6F3	Kaletta and Entian, 1989
FI5876	Dodd *et al.*, 1990
NIZO 22186	Moulders *et al.*, 1991
CNRZ 481	Piard *et al.*, 1992

2.5.4 Mechanisms of action of bacteriocins

Little is known about the mechanism of action of bacteriocins. For nisin it was reported that the cytoplasmic membrane is the main target, because treatment with nisin causes rapid, nonspecific efflux of amino acids and cations, and rupture of the cell membrane resulting in the death of sensitive cells (Ruhr and Sahl, 1985; Gao *et al.*, 1991). Gao *et al.* (1991) showed that the phospholipid composition of the membrane may be influential in the effectiveness of nisin. The combined results obtained in cells, vesicles and liposomes, indicate that the specificity of lactococcin A from *Lactococcus lactis* may be mediated by a receptor protein associated with the cytoplasmic membrane (Van Belkum *et al.*, 1991). Bhunia *et al.* (1991) also observed that treatment of the cell walls to remove lipoteichoic acid prevented the binding of pediocin ACH from *P. acidilactici*. It has also been suggested that lipoteichoic acid molecules, that are present only in Gram-positive organisms may be one of the binding sites for pediocin ACH. This may be the reason why LAB bacteriocins are adsorbed to Gram-positive bacteria and not to Gram-negative bacteria. They suggested that cell lysis is associated with cell death, and that this may depend on the strains of sensitive bacteria, presence of nonspecific receptor sites, such as lipoteichoic acid, and specific receptor(s).

2.5.5 Genetic determinants for bacteriocins

The gene or genes encoding bacteriocin production may be located on the chromosome or on plasmids. The production of pediocin A (Daeschel and Klaenhammer, 1985), leucocin A (Hastings and Stiles, 1991) and carnobacteriocins (Ahn and Stiles, 1990b) are plasmid-mediated; while production of helveticin J (Joerger and Klaenhammer, 1986) and lactacin B from *Lb. acidophilus* N2 (Barefoot and Klaenhammer, 1983) are chromosomally-mediated. In the case of nisin, it is not clear whether the gene is located on a plasmid or on the chromosome, because no physical evidence linking this phenotype to a distinct plasmid has been obtained. The conjugal transfer of plasmid encoded genes could not be detected in lysates of transconjugants of *L. lactis* 11454 (Gonzalez and Kunka, 1985; Steele and McKay, 1986). Little attention has been directed towards the chromosome of these organisms.

Recently, the application of transposable elements in genetic analyses has received attention in the genus *Lactococcus*. A broad distribution of the insertion elements has been observed in many lactococcal plasmids and chromosomal DNA, encoding lactose fermenting ability (Schäfer *et al.*, 1991), proteinase activity (Haandrikman *et al.*, 1990), and bacteriophage resistance (Romero and Klaenhammer, 1990). In the case of nisin biosynthesis many researchers observed the involvement of insertion elements (IS) in the nisin gene.

Dodd *et al.*, (1990) observed multiple copies of IS*904* within the lactococcal chromosome downstream of the nisin gene. Buchman *et al.* (1988) cloned the gene that encodes the nisin prepropeptide. The nucleic acid sequence of the nisin precursor gives rise to a prepropeptide of 57 amino acids, including a 23 amino acid leader region and a 34 amino acid structural region (*spaN*). No promoter or rho-independent terminator was found, leading to the conclusion that the *spaN* gene is translated from polycistronic mRNA.

Steen *et al.* (1991) reported that genes for nisin production in *L. lactis* ATCC 11454 are located on the chromosome. The restriction patterns indicate that the size of *L. lactis* genome is about 2,500 kb. The sizes of the hybridizable restriction fragments range up to 625 kb (*Not*I) and 1,100 kb (*Sfi*I). The fact that the nisin gene lies on a DNA restriction fragment that is one-half the size of the genome rules out the possibility of it being plasmid encoded, unless it is a plasmid that rivals the size of the chromosome, which is unprecedented (Steen *et al.*, 1991). Horn *et al.*, (1991) found a conjugative transposon Tn5301 in *L. lactis* NCFB894 which encodes nisin production and sucrose catabolism.

To date, antibiotic resistance vectors have been used to study the genetics of LAB because of the ease with which the desired clones can be selected, but these markers are unsuit-

able for food-grade bacteria. Ideally, a food-grade cloning vector should be constructed from DNA derived from a micro-organisms that are approved for food use and contain a selectable marker that does not compromise human drug therapy (Froseth and McKay, 1991). A major objective at present is the development of homologous vector systems based only upon LAB DNA with metabolic or otherwise acceptable selection phenotypes for food use, such as genes associated with carbohydrate metabolism, bacteriocin production and resistance. Recently, Froseth and McKay (1991) developed a food-grade cloning vector, pFM011, employing the Nis^r phenotype as a selectable marker, and this may lead to a better understanding of LAB bacteriocin genes.

2.6 Use of lactic acid bacteria and (or) their bacteriocins in meat preservation

There are many reports of the inhibitory activity of LAB or bacteriocins against various organisms in artificial media (Bhunia *et al.*, 1988; Daeschel, 1989; Ahn and Stiles, 1990a; Hastings and Stiles, 1991). However, for practical purposes, it is important to study their effects in food where intrinsic and extrinsic factors may interact and the food itself may provide protection for the spoilage and pathogenic organisms. In addition, inactivation of bacteriocins by food constituents or enzymes may occur (Asperger *et al.*, 1989).

Antibiosis of LAB can be exploited in food systems either by incorporation of LAB or by adding pure bacteriocin. Tanaka *et al.* (1980) introduced a new technique which involves the incorporation of LAB and a fermentable carbohydrate in the food. One application of this technique is the 'Wisconsin process' used in bacon manufacture in the United States. This method was approved by the U.S. Department of Agriculture (USDA) in 1986 for use in nitrite-reduced bacon to assure protection against botulinum toxigenesis (Hutton *et al.*, 1991). The principle is that LAB grow at abusive temperatures resulting in sufficient acid production to preclude toxigenesis of *C. botulinum*. In their experiments, Tanaka *et al.* (1985) selected a strain of *P. acidilactici* (0.7% sucrose and $5x10^7$ cells/g). This strain was chosen for minimal growth at 7°C but rapid growth and acid production at 23-27°C. Greater protection was obtained against the botulinal toxin with only 40 or 80 µg of nitrite/kg of meat than bacon made with 120 µg of nitrite/kg of meat and no sucrose. There was very slow fermentation of sucrose at 7°C and the pH declined from 6.2 to 5.3 after 45 days of storage. The same technique has been applied to chicken salad to prevent botulinum toxigenesis under conditions of temperature abuse (Hutton *et al.*, 1991). Riemann *et al.* (1972) and Christiansen *et al.* (1975) also recommended the combined use of glucose or sucrose with starter culture. Evaluation of these preservative strategies

requires challenge studies on a product by product basis because the efficacy depends on a number of factors such as: initial pH, inoculum level and type, amount and type of carbohydrate, presence of inhibitory compounds and the buffering capacity of the product.

2.6.1 Bacteriocins produced by meat lactics and their potential for use in meat products

The potential for use of bacteriocins of LAB in meat preservation was recently reviewed by Stiles and Hastings (1991).

2.6.1.1 Effectiveness of nisin produced by *Lactococcus* spp. in meat

To date, there is no report of bacteriocin production by *Lactococcus* spp. of meat origin. However, nisin which is produced by lactococci of dairy origin has been approved for use as a food preservative in over 45 countries. In 1988, it was approved by the U.S. Food and Drug Administration for use in pasteurized cheese to inhibit outgrowth of spores of *C. botulinum*. Nisin is of particular interest because of its effectiveness against a broad range of Gram-positive organisms including *L. monocytogenes*, *C. botulinum* and *S. aureus*. Nisin is being exploited as a food preservative mainly in dairy foods. It is nontoxic and digested by intestinal enzymes. It is heat stable and does not contribute to off-flavours. It has no value in medical therapy because it is

practically insoluble in blood at physiological pH (Hurst, 1981). The large size of the molecule precludes absorption if intramuscular injection is used. However, nisin can be used for topical applications.

The use of nisin as a preservative in meat is doubtful (Hauschild, 1989). Reasons proposed for the poor preservative effect of nisin as observed in meat systems are: the binding of nisin to meat particles, uneven distribution of nisin, poor solubility in meat and interaction with phospholipids (Scott and Taylor, 1981a,b; Delves-Broughton, 1990). In contrast, nisin is relatively easy to incorporate into processed cheese spreads and it is stable in cheese emulsions.

Nisin at 550 ppm in combination with 60 ppm nitrite did not prevent the growth of *C. botulinum* spores when added to pork slurries at pH 5.8 (Rayman *et al.*, 1983). The antibotulinal effectiveness of nisin depends on the pH and level of spore inoculum. Nisin is unstable and becomes inactivated at high pH (Hurst, 1981). Notable properties of nisin are that its solubility and stability increase dramatically as the pH is lowered (Liu and Hansen, 1990). The mechanism of inactivation is unknown but it could be a consequence of denaturation, chemical modification or a combination of both. The activity of nisin is reduced by calcium and magnesium ions, L-alanine, phenylalanine, pyruvate and polysaccharides (Holley, 1981). Chung *et al.* (1989) studied the effect of

nisin on growth of bacteria attached to raw beef and stated that recovery of nisin from meat and meat emulsions has been poor and variable. Nisin is more stable during storage at 5°C than at room temperature. Nisin delayed the growth of *L. monocytogenes* for more than 2 weeks at 5°C in raw meat. At room temperature nisin delayed the growth of *L. monocytogenes* and *S. aureus* for at least 1 day. Although nisin can delay the growth of Gram-positive bacteria attached to meat, nisin alone may not be sufficient because many nisin-resistant organisms as well as Gram-negative organisms are not affected.

Slow and incomplete diffusion of nisin occurs in bacon (Taylor and Somers, 1985). A high level of nisin is required (100-150 ppm in combination with 120 ppm nitrite) for bacon inoculated with spores of *C. botulinum* to provide 1 week extension of shelf life at 27°C. In bacon, nisin alone had no antibotulinal effectiveness. Addition of nisin at levels up to 500 ppm allowed only a 1 week extension of the shelf life of chicken frankfurter emulsions challenged with spores of *C. botulinum* and incubated at 27°C (Taylor *et al.*, 1985). There is only one report in which raw meat was inoculated with a selected strain of nisin producing LAB to examine its effect on the shelf life of a processed and vacuum packaged sausage (Wang *et al.*, 1986). About 10^9 cells of *L. lactis* were inoculated per gram of fresh beef containing 0.5% dextrose. After stored for 3, 5 and 7 days at 7°C the beef was used to

prepare frankfurters that were vacuum packaged and stored at 3°C. There was no appreciable reduction in pH; however, the growth of psychrotrophic organisms in meat was slower. Growth patterns indicate that nisin played a role in the reduced growth of bacteria. However, it is unclear whether the inhibition of psychrotrophs was due to the presence of nisin itself or competition from the heavy inoculum of *L. lactis*.

The use of nisin needs to be evaluated for each food type. Results of most studies indicate that high concentrations of nisin may be required. The maximum concentration of nisin recommended for use in food is 400 IU/g (Hurst, 1981). Other problems likely to limit its application in meat include the cost of nisin, development of resistant strains, inactivation of nisin by nisinase which is produced by some organisms, insolubility of nisin at pH 6.0, lack of growth of nisin producer strains at chill temperatures and late production of nisin in the growth cycle of the producer strain.

2.6.1.2. Pediocin from *Pediococcus* spp.

Bacteriocins have been characterized from *P. acidilactici* (Bhunia *et al.*, 1988; Ray *et al.*, 1989; Nielsen *et al.*, 1990), *P. pentosaceus* (Daeschel and Klaenhammer, 1985; Skyttä *et al.*, 1991) and *P. damnosus* (Skyttä *et al.*, 1991). Pediocins from pediococci are active against a wide range of organisms and their genetic determinants are plasmid-borne (Daeschel, 1989). Pediocin ACH produced by *P. acidilactici* that was isolated

from meat has a broad spectrum of activity, including: *S. aureus*, *C. perfringens* and *L. monocytogenes* and the Gram-negative bacterium, *Pseudomonas putida*. However, the effectiveness of *P. acidilactici* in meat has not been reported.

Pediocin PA1 from the commercial starter culture, *P. acidilactici* PAC10, has a narrow spectrum of activity. Addition of a crude extract of pediocin PA1 to beef inoculated with *L. monocytogenes* reduced the number of attached bacteria by 0.5 to 2.2 log cycles depending on the concentration of bacteriocin (Nielsen *et al.*, 1990).

Berry *et al.* (1991) examined the behaviour of *L. monocytogenes* on fully processed frankfurters which were surface-inoculated with the pediocin JD1-23 producing strain of *P. acidilactici*. Varying degrees of inhibition of listeria were observed dependent upon cell concentration, temperature and package atmosphere. They found that at 4°C, a high level (10^7 cfu/g) of *P. acidilactici* inoculum is required to inhibit the growth of *L. monocytogenes* for up to 60 days. With a low level inoculum (10^3 cfu/g) the listeria grow, although their lag time is increased. At 15°C, an inoculum of 10^7-10^8 cfu/g controlled the growth of *L. monocytogenes* for up to 15 days under anaerobic conditions. Skyttä *et al.* (1991) inoculated minced meat with *P. damnosus* and *P. pentosaceus*. These organisms as well as their crude extract of bacteriocin showed strong inhibition of *Y. enterocolitica*, *P. fragi*, *P. fluoresc-*

ens and *L. monocytogenes*. A high inoculum of producer cells was required, however, the inhibitory action was not due to acidity because crude extracts also showed similar results.

2.6.1.3 Bacteriocins produced by *Leuconostoc* spp.

Among *Leuconostoc* spp., leucocin from *L. gelidum* is the most extensively characterized bacteriocin isolated from vacuum packaged meats (Hastings and Stiles, 1991). This bacteriocin is of interest because of its early production in the growth cycle at 1, 5 and 25°C and its stability over a wide pH range (4.0-6.5). This bacteriocin is heat stable (100°C for 1 h) and it is active against *L. monocytogenes*, *Enterococcus faecalis* and a wide range of LAB strains. Leucocin production is plasmid-mediated. The gene for its production has been cloned, however, phenotypic expression of leucocin was not evident from the clone (Hastings *et al.*, 1991). There are no reports of leucocin being used, to date, as a meat preservative.

2.6.1.4 Bacteriocins from *Carnobacterium* spp.

Bacteriocin production by *Carnobacterium divergens* L66 (Schillinger and Holzapfel, 1990) and *C. piscicola* LV17 (Ahn and Stiles, 1990a) and *C. piscicola* UAL26 (Burns, 1987) have been reported. Bacteriocin production by *C. piscicola* UAL26 is chromosomally-mediated. This bacteriocin is active against spores of *Bacillus* and *Clostridium* and other Gram-positive organisms. Bacteriocin production by *C. piscicola* LV17 is

plasmid-mediated, producing one bacteriocin early and two bacteriocins late in the growth cycle in contrast to *C. piscicola* UAL26 which is a late producer. The gene for early bacteriocin production is on a 49 MDa plasmid and it has been cloned into *C. piscicola* UAL26, resulting in the production of two bacteriocins (Ahn and Stiles, 1990b). The ability of this genetically engineered strain LV17/UAL26 to grow and produce bacteriocin in cooked meat has been demonstrated (Zagrosh-Miller, 1991).

2.6.1.5 Bacteriocins produced by *Lactobacillus* spp.

Many strains of *Lactobacillus* both from dairy cultures as well as from meat and meat products produce antagonistic activity towards a wide range of microorganisms. Heat stable bacteriocins have been reported from lactobacilli: lactacin F from *Lb. acidophilus* (Muriana and Klaenhammer, 1987) and lactocin 27 from *Lb. helveticus* (Upreti and Hindsdill, 1973, 1975). Sakacin A from *Lb. sake* that inhibits several species of LAB, *Enterococcus* spp. and *L. monocytogenes* (Schillinger and Lücke, 1989). Curing of the 18 kb plasmid resulted in loss of bacteriocin production and immunity suggesting that both traits were plasmid mediated. Bacteriocin producing *Lb. sake* is active against *L. monocytogenes* in refrigerated meat. No antagonistic effect was observed in pasteurized minced meat at 8°C, although the growth of *L. monocytogenes* was initially inhibited by both producer strains and a bacteriocin negative

mutant. The inhibitory effect was observed in comminuted cured pork at pH 5.7, stored at 15°C, where the listeria count was reduced approximately 1 log cycle, but not in pork at pH 6.3-6.4 (Schillinger *et al.*, 1991). Sakacin A may have some potential as a protective culture in meat products, however, it has a narrow activity spectrum and it is slowly inactivated in meat. Although many bacteriocins from *Lactobacillus* spp. have been characterized, their use in meat systems needs further investigation.

In conclusion, bacterial antagonism has been recognized for over a century but only in recent years has this phenomenon received close attention, particularly the use of bacteriocinogenic strains of LAB for meat preservation. Among the LAB prevalent in modified atmosphere packaged meats there should be strains which are suitable for use as biological preservatives. However, considerable research is required in the areas of taxonomy, function of the cultures and their genetics to ensure safety of refrigerated high risk foods, such as meat.

3. MATERIALS AND METHODS

3.1 Bacterial cultures

The lactic acid bacteria (LAB) used in this study are listed in Table 3.1. Two hundred-and-sixty LAB that had previously been isolated from vacuum packaged fresh beef were screened for their ability to produce bacteriocin. Six bacteriocinogenic strains of LAB were used as positive controls. Ninety-eight different pathogens and spoilage organisms including vegetative cells, heated and heat-activated spores of *Bacillus* and *Clostridium* spp. and nine reference strains of LAB were used as indicator organisms to determine the spectrum of antagonistic activity.

3.2 Culture media

The culture media used in this study are listed in Table 3.2. All of the media were reconstituted according to the manufacturer's specifications or as indicated in the reference cited. All Purpose Tween (APT, Difco) broth was used as the general growth medium for LAB as well as indicator organisms. APT agar (1.5% agar) and soft agar (0.75% agar) were prepared for use in tests for antagonistic activity.

3.3 Maintenance and growth conditions of bacterial cultures

Stock cultures of LAB were maintained at -70°C in APT with 20% glycerol. Indicator organisms were stored as freeze dried cultures. Working cultures of LAB and *Clostridium* spp. were stored in Cooked Meat Medium (CMM, Difco) at 4°C and

Table 3.1. Lactic acid bacteria used for initial screening and their sources

Organism	**Source**	**Laboratory No.**
Meat isolates # 1 to 260	Vacuum packaged beef	Strains # 1 to 260
Lactococcus lactis subsp. *lactis*	ATCC 11454[a]	UAL 230
Carnobacterium piscicola LV17	Shaw[b]	UAL 8
Lactobacillus spp.	Shaw	UAL 11
Carnobacterium piscicola	Burns[c]	UAL 26
Lactobacillus plantarum	U. of Alberta	UAL 59
Leuconostoc gelidum	Hastings and Stiles[d]	UAL 187

[a] American Type Culture Collection

[b] Isolated from vacuum packaged meat and supplied by Dr.B.G. Shaw (AFRC Institute of Food Research, Langford, Bristol, UK.)

[c] Isolated by Burns, K. A. M. Sc. thesis, 1987, University of Alberta, Edmonton.

[d] Isolated by Hastings and Stiles, 1991.

Table 3.2. Culture media and their suppliers or reference

Culture media	**Supplier/Reference**
Baird-Parker agar (5% egg yolk tellurite enrichment)	Difco Laboratories Inc., Detroit, MI.
KF *Streptococcus* agar	Difco
Violet red bile agar	BBL, Becton Dickinson Microbiology Systems, Cockeysville, MD
Violet red bile glucose agar (10% glucose)	BBL
Streptomycin, thallous acetate actidione agar	Gardner (1966)
Polymyxin acriflavin lithium chloride ceftazidime esculin mannitol agar	Van Netten *et al.* (1989)
Blood agar (5% sheep blood)	Gimbar Lab. Ltd., J.G. Watson, Edmonton, AB
MacConkey agar	Difco
Bile aesculin agar	Difco
Acetate agar (pH 5.6)	Rogosa *et al.* (1951)
Lactobacilli MRS broth/agar (1.5%) (pH 5.6)	Difco
Lactate agar (pH 5.6)	Shaw and Harding (1984)

subcultured every 3 months after examination for culture purity. Indicator organisms were kept on nutrient agar slants at 4°C for routine use and subcultured every 3 months. All cultures were propagated twice (1% inoculum) in fresh APT broth prior to use in experiments and were subcultured a maximum of six times before new working cultures were prepared. All LAB cultures were incubated at 25°C under anaerobic conditions (10% CO_2, 90% N_2) and indicator organisms were incubated at their optimum growth temperature.

3.4 Preparation of spore crops

3.4.1 *Bacillus* spp.

Minimum salt medium (Grelet, 1951) was used for making spore crops of *Bacillus* spp. The medium was inoculated and incubated at 37°C in a shaker incubator for 4 days.

3.4.2 *Clostridium* spp.

A 1% sample of the CMM culture of *Clostridium* spp. was inoculated into 600 mL of APT broth. Inoculated cultures were incubated anaerobically at 37°C for 7 days, with mixing once-a-day during incubation.

3.4.3 Recovery of spores

The degree of sporulation was assessed using phase contrast microscopy. When it was estimated that 80-90% of the cells had sporulated, the spores were harvested by centrifugation (10,000 x g for 20 min). Spore pellets were cleaned by repeated (3X) centrifugation and resuspension, and

finally resuspended in 4 mL of sterile distilled water and stored at room temperature in sterile scintillation vials. Spores were heated at 65°C for 1 h to kill vegetative cells.

3.4.4 Determination of spore concentration

Spore counts for all strains were performed by serial dilution (in sterile distilled water) and plating to determine the number of spores. The desired concentration was also heat shocked for 10 min at 80°C before use in experiments.

3.5 Experimental Procedures

3.5.1 Pure cultures

All cultures were first examined for purity. Gram stains and important growth characteristics were used to confirm the identity of a single colony from each of the cultures as follows:

(a) Lactic acid bacteria - Anaerobic growth on MRS adjusted to pH 5.6 or on acetate agar (pH 5.6).

(b) *Staphylococcus* spp. - Gram stain, growth on Baird-Parker agar with egg yolk tellurite enrichment.

(c) *Enterococcus* spp. - Gram stain, growth in 6.5% NaCl in Brain Heart Infusion broth (BHI,Difco), growth on KF agar.

(d) *Listeria* spp. - Gram stain, motility, and growth on polymyxin acriflavin lithium chloride ceftazidime aesculin mannitol agar (PALCAM) (Van Netten *et al.*, 1989).

(e) *Brochothrix thermosphacta* - growth on streptomycin thallous acetate actidione agar (STAA) (Gardner, 1966).

(f) Enterobacteriaceae - Gram stain, motility, oxidase test, growth on MacConkey, violet red bile glucose (VRBG) and Violet red bile (VRBA) agars.

3.5.2 Detection of inhibitory activity

The primary screening of LAB for inhibitory activity was done by deferred antagonism (Barefoot and Klaenhammer, 1983). Cultures of LAB were inoculated onto APT agar plates with a Cathra replicating inoculator (KVL Laboratories, Cambridge, Ontario, Canada). The plates were incubated overnight at 25°C under anaerobic conditions and then overlayered with 7 mL of soft APT (0.75%) agar containing 1% inoculum (approx. 10^7 cfu/mL) of an indicator organism. Overlayered plates were dried for 30 min in a laminar flow hood and incubated under conditions optimal for growth of the indicator organism. Plates were observed for inhibition of the indicator organism after 24 h. A clear zone of inhibition around the producer organism was recorded as positive.

Five organisms (strains # 176, 185, 236, 258 and 261) that had a broad spectrum of antimicrobial activity were selected for further study. *Leuconostoc mesenteroides* ATCC 23368 (UAL 15) was selected as an indicator organism. Nisin producing *Lactococcus lactis* subsp. *lactis* ATCC 11454 was selected as a positive control strain for comparison with the meat isolates.

3.6 Identification of the producer strains

3.6.1 Physiological tests

(a) Cell morphology, Gram reaction and motility. Overnight cultures on APT agar were examined for colony morphology and Gram reaction. Motility testing was done by both droplet technique and cultivation in semi-solid agar (Bailey and Scott, 1974).

(b) Growth at various temperatures. APT broth, that had been tempered to 1, 2, 4, 6, 42 and 45°C for 1 h, was inoculated with 1% inoculum of a producer strain and incubated at the respective temperatures. Tubes were examined for growth for up to 4 weeks.

(c) Growth at pH 3.9. APT broth containing 2% (w/v) glucose, with phosphate omitted and adjusted to pH 3.9 was inoculated with a producer strain and results recorded after 1 week of incubation at 25°C (Shaw and Harding, 1984).

(d) Growth on selective media. Ability to grow on acetate agar (Rogosa *et al.*, 1951) and lactate agar at pH 5.6 (Shaw and Harding, 1984) was observed by inoculating the cultures and incubating anaerobically at 25°C for 5 days.

(e) Salt tolerance. Brain heart infusion broth (Bailey and Scott, 1974) and MRS broth (Schillinger and Lücke, 1987) was supplemented with NaCl to give final concentrations of 4, 6.5, 8 and 18% NaCl.

(f) Terminal pH in La broth. This was measured as described by Shaw and Harding (1984) after incubation at 25°C for 7 days. Strains were categorized as aciduric or nonaciduric if the final pH was below or above 4.15, respectively.

(g) Sensitivity to antibiotics. Susceptibility of producer strains to various antibiotics was done by automated microdilution (MIC) and disk diffusion technique on Mueller Hinton agar at the Provincial Laboratory of Public Health for Northern Alberta, Division of Bacteriology. Both tests were done at 35°C.

(h) Heat resistance of producer strains. Overnight cultures were placed in a waterbath at 60°C for 15 min, cooled rapidly on ice and 20 µL of culture was placed on agar plates. The plates were observed for growth after 2 days of incubation at 25°C.

(i) Ability of producer strains to grow in APT broth adjusted to pH 9.2 and 9.6 with 1 N NaOH (after autoclaving) was determined.

(j) Ability of producer strains to grow in 0.3% methylene blue in milk was observed by reduction in the colour.

3.6.2 Biochemical tests

(a) Fermentation of carbohydrates. This was determined by the method described by Shaw and Harding (1985). Filter sterilized sugar solutions were added to basal medium (BM, MRS without glucose and meat extract), with 0.004% chlorophenol

red added as a pH indicator. The plates were surface inoculated using Cathra replicating inoculater and incubated anaerobically at 25°C. Fermentation of the carbohydrates was observed after 2 days and for up to 5 days. Formation of bright yellow colour surrounding the producer strain was recorded as a positive test.

(b) Production of ammonia from arginine. The method described by Shaw and Harding (1984) was used. Modified MRS broth containing 0.3% w/v arginine with ammonium citrate omitted was inoculated with the cultures. After 2 days of incubation at 25°C, Nessler's reagent (BDH) was used to determine the presence of ammonia.

(c) Gas production from glucose. MRS broth with citrate omitted and containing an inverted Durham vial was used to detect gas production (Shaw and Harding, 1985). The method described by Hitchener *et al.* (1982) was also used, in which 6-7 mL of modified MRS agar with 0.2% ammonium sulphate and without tri-ammonium citrate was dispensed in screw cap tubes and autoclaved. Overnight test culture (100 µL) was inoculated and after the agar had solidified, tubes were sealed with vaspar (2-3 cm deep), incubated at 25°C and observed daily for 5 days for gas production.

(d) Production of acetoin. Voges-proskauer test (Bailey and Scott, 1974) was used to determine the production of acetoin

in MR-VP medium. Development of a bright red colour was recorded as a positive result.

(e) Catalase test. A heavily inoculated APT agar slant that had been incubated overnight was flooded with 1 mL of a 3% solution of hydrogen peroxide. The reaction was scored positive if there was rapid bubbling of gas.

(f) Oxidase test. The method described by Bailey and Scott (1974) was used in which the oxidase reagent was placed on a Whatman No. 1 filter paper, a few colonies from an APT agar plate were removed with a tooth pick and smeared on the filter paper saturated with reagent. A positive result was scored if a purple colour developed within 30 s.

(g) Haemolysis and pigment production on sheep blood agar. Complete (β-haemolysis) or partial (α-haemolysis) and production of a green pigment were noted after 2 days of incubation at 25°C.

(h) Bile resistance. Ability to grow on bile aesculin agar containing 40% bile was determined (Bailey and Scott, 1974). Growth and blackening of the medium due to the hydrolysis of aesculin was recorded as a positive test.

(i) Hydrolysis of aesculin. Cultures were inoculated into an aesculin broth composed of Brain Heart Infusion broth (Difco) supplemented with 0.1% aesculin (Sigma) and 0.1% agar. After 48 h of incubation, 1% ferric ammonium citrate solution was added. Development of black colour was scored positive. Tubes

were also observed under UV light and loss of fluorescence was recorded as a confirming positive reaction (Balows *et al.*, 1991).

(j) Hydrolysis of hippurate. A 1% aqueous solution of sodium hippurate was dispensed in 0.4 mL amounts and stored overnight at -20°C. A few colonies of the test culture from an APT plate were added to the tubes after thawing and were held at 37°C for 2 h. Ninhydrin reagent (0.2 mL of 3.5 g ninhydrin in 100 mL of 1:1 mixture of acetone and butanol) was added and left for 10 min. Immediate development of a deep purple colour was scored as a positive reaction (Balows *et al.*, 1991).

(k) Starch hydrolysis. Degradation of starch was determined by the method of Borch and Molin (1988). In this test, BM agar was used in which glucose was replaced by 0.2% (w/v) soluble starch. After 15 days incubation the agar was flooded with Gram's iodine. Development of a blue colour indicated that the starch was not hydrolysed. Colourless area around the growth with the blue background was recorded as a positive reaction.

(l) Reduction of tetrazolium. SBM agar (Wilkinson and Jones, 1977) containing 0.1 and 0.01% filter sterilized 2,3,5 triphenyltetrazolium chloride (TTC) was inoculated with producer strain. Formation of deep red coloured cells after 1 or 2 days of incubation was recorded as a positive test.

(m) The production of slime (dextran) from sucrose. MRS agar containing 5% sucrose instead of glucose was used (Schillinger and Lücke, 1987).

(n) Configuration of lactic acid enantiomers. L(+) and D(-) lactate were determined enzymatically according to the methods described by Noll (1984) and Gawehn (1984) using D-lactate and L-lactate dehydrogenases (Boehringer Mannheim, Laval, Canada). The test culture was grown in APT broth for 2 days at 25°C. The heated culture supernatant (60°C/30 min) was used as a test sample. Lactate enantiomers were classified as D(-), L(+), DL, D(L) or L(D) isomers based on the criteria used in Bergey's Manual of Systematic Bacteriology (1986). Standard solutions containing known concentrations of L(+) and D(-) lactate (0.1 M) were used as controls. Cultures that produced known lactate isomers were also used for positive controls.

3.7 Plasmid detection

The cultures were grown in APT broth (1% inoculum from overnight cultures) and incubated at 25°C for 16-18 h. Cells were collected by centrifugation (10,000 x g for 10 min). The method used for rapid small-scale plasmid DNA screening or large scale isolation for preparative plasmid purification was a modification of the procedure described by Anderson and McKay (1983) with the following modifications. Cell pellets were washed with 50 mL of cold 0.5% NaCl solution and resuspended in 6.7% sucrose, 50 mM Tris, 1 mM EDTA (pH 8.0). The

concentration of lysozyme was 20 mg/mL in 25 mM Tris (pH 8.0) and incubation was for one hour at 37°C. After addition of sodium dodecyl sulphate (20% in 50 mM Tris, 20 mM EDTA, pH 8.0), the mixture was incubated at 37°C for 30 min. The lysate was mixed gently, 3 N NaOH was added dropwise until pH 12.5 was reached and mixed for 10 min, the sample was then heated at 65°C for 15 min and cooled at room temperature. Subsequent steps were according to the protocol described by Anderson and McKay (1983) except that the phenol extraction was done twice and the preparation was centrifuged for 5 min after the addition of chloroform-isoamyl alcohol (24:1). After removal of the upper phase, the DNA was precipitated with double volume of 90% cold ethanol (-70°C). Plasmid DNA was allowed to precipitate overnight at -20°C. The ethanol was removed by centrifugation (10,000 x g for 10 min at 4°C). The pellets were dried and resuspended in 1.5 mL of TE buffer (10mM Tris-1mM EDTA, pH 7.5).

3.7.1 Purification of plasmid DNA

Samples from the large scale extraction, which consisted of plasmids, RNA, polysaccharides and debris was purified on cesium chloride-ethidium bromide density gradients (Sambrook *et al.*, 1989). A 7.0 g amount of CsCl was dissolved with 1 mL of plasmid DNA and 6 mL of TE buffer by mixing and warming at 30°C. Ethidium bromide (Sigma; 10 mg/mL in the same buffer) was added to a final concentration of 0.57 mg/mL and the

mixture was centrifuged in a Beckman Ti 70 rotor using 12 mL Beckman polyallomer tubes at 45,000 x g for 24 h at 20°C. The plasmid bands were visualized under UV light (254 nm). The second band (closed circular plasmid DNA) was collected with a pasteur pipette. Ethidium bromide was extracted with an equal volume of isoamyl alcohol. After thorough mixing, the lower phase was transferred to a clean tube. This was repeated 4 to 6 times until the pink colour disappeared. Cesium chloride was removed by diluting with 3 volumes of water and precipitating the DNA with 2 volumes of ethanol overnight at 4°C, followed by centrifugation at 10,000 x g for 15 min at 4°C. The precipitated DNA was dissolved in approximately 1 mL of TE buffer (pH 8.0).

3.7.2 Restriction enzyme analysis

Restriction enzymes were obtained from Boehringer Mannheim and Biochemica. For digestion of plasmid DNA, 5 µL of DNA solution was added to 13 µL of distilled water, 2 µL of restriction buffer (according to the manufacturer's instructions), and 0.5 µL of restriction enzyme, *Bgl*II or *Bam*HI, and incubated at 37°C for 2 h. After digestion, 0.5 µL of 0.5 M EDTA was added and the mixture heated at 40°C for 3 min and kept on ice for further use.

3.7.3 Agarose gel electrophoresis

About 15 to 18 µL of digested and undigested purified plasmid DNA (15 µL DNA + 4 µL tracking dye + 2 µL RNase) was

loaded onto a 0.7% agarose gel (Pharmacia LKB Biotechnology, Uppsala, Sweden) containing ethidium bromide (0.5 µg/mL). Electrophoresis was conducted at 60 V for 3 h in TAE buffer (40 mM Tris acetate, 1 mM EDTA). Molecular weight markers from *E. coli* V517 and *Hin*dIII digest of λ DNA were run on the same gel. A standard curve was constructed from the relative mobility of the reference plasmids and the molecular weight of unknown plasmids was estimated.

3.8 Application of low molecular weight RNA profile techniques to identify the producer organisms

3.8.1 Cultural conditions

A 1% inoculum of an overnight culture of the test organism was inoculated into 50 mL of APT broth and incubated overnight at 25°C for 16-18 h. Cell pellets were collected after centrifugation (13,000 x g for 10 min) and washed with sterile 1% peptone water and stored in the Eppendorf tubes at -70°C for 1 h. Harvested cells (about 5 to 10 mg dry weight) were then freeze dried.

3.8.2 RNA extraction

A modification of the method described by Höfle (1988) for total RNA extraction was used. Experiments were done with sterile glassware treated with 0.5% diethylpyrocarbonate (DEPC) to eliminate RNAse activity. All reagents used in the experiments were autoclaved, except SDS.

Freeze dried pellets of harvested cells were resuspended in 750 µL of buffer I (50 mM sodium acetate, 10 mM EDTA, pH 5.1) containing 1% SDS and vortexed, prior to the addition of 750 µL of prewarmed (60°C) phenol mixture (50 mL liquified phenol + 50 mg 8-hydroxy quinoline + 50 mL buffer I, pH 5.2). After intensive vortexing, tubes were heated at 60°C for 10 min, immediately cooled on ice for 10 min, followed by centrifugation (13,000 x g for 5 min at 4°C). The upper aqueous phase was carefully removed and placed in a new Eppendorf tube and 750 µL of phenol mixture containing chloroform (4:1 v/v prewarmed to 60°C) was added, mixed and heated at 60°C for 5 min, and centrifuged as before. Supernatant fluids were removed and 70 and 700 µL of 2 M sodium acetate and chloroform were added respectively, mixed and centrifuged for 10 s at 13,000 x g at 4°C. The upper phase was transferred to a new Eppendorf tube and 750 µL of chloroform was added to remove traces of phenol. The aqueous phase was separated as before and the final supernatant was collected in two tubes (350 µL each) to which 1 mL of precipitation solution (ethanol, 2 M sodium acetate and 1 M magnesium chloride, 100:10:1 v/v) was added. Extracted RNA was allowed to precipitate overnight at -20°C.

3.8.3 Denaturing polyacrylamide gel electrophoresis

Separation of total RNA was done by denaturing polyacrylamide gel electrophoresis (Höfle, 1988). Before casting

the gel the long carrier glass plate (610 X 200 mm) was treated with repelcoat solution (2% dimethyl dichlorolsilane in chloroform; Sigma) and the short carrier glass plate (550 X 200 mm) was treated with silane solution (0.5 mL silane in 100 mL ethanol; Sigma). Polyacrylamide gel stock solution was prepared by dissolving 38 g acrylamide and 2 g of N',N-methylene bisacrylamide (Bio-Rad) in 100 mL of water. A 10% denaturing gel was prepared using 15 mL acrylamide stock solution, 6 mL of 10 X Tris-borate EDTA (TBE) buffer (121.1 g Tris, 61.8 g boric acid and 7.44 g EDTA per litre of water, pH 8.5) containing 30 g urea, and made up to 60 mL with water. The gel was polymerized by addition of 200 µL of 20% ammonium persulphate solution and 15 µL of N,N,N',N'-tetramethyl ethylenediamine (TEMED). The polymerized plates were mounted on the electrophoresis apparatus and pre-run for 10 min at 60 W in 1 X TBE buffer.

Total ethanol-precipitated RNA extracts were centrifuged for 10 min at 13,000 x g at 4°C. The pellets were dried under vacuum for 30 min. Dried RNA was resuspended in 10 µL of sterile water and then further diluted 5, 10 and 25 times and used as samples. One µL of diluted RNA was mixed with 1.5 µL of loading buffer (950 µL formamide, 40 µL 0.2% xylene cyanol, 40 µL of 0.2% bromophenol blue and 10 µL of 0.5 M EDTA; Sigma). Unpolymerized gel solution was carefully eluted from

the pre-electrophoresis gel. Samples (2 µL) were then loaded into the individual wells.

RNA molecular weight standards for 5S rRNA and class 1 and 2 tRNA from *E. coli* were prepared by Dr. Woen-Suep Cha (Visiting Professor, Department of Food Science). The markers correspond to 120, 89 and 70 nucleotides, respectively. Electrophoresis was done at 60 W for 5 h until the xylene cyanol dye had run to the bottom of the gel.

3.8.4 Silver staining

A modification of the method described by Kolodny (1984) was used. After electrophoresis, the plates were separated and the gel adhering to the silane-treated plate was fixed with 1 M acetic acid for 15 min to remove urea, it was then immersed in 2 L of methylene blue (0.2% methylene blue, 0.4 M sodium acetate and 0.4 M glacial acetic acid) for 45 min, and destained by frequent changes of water until the background became light blue. This was followed by soaking in 50% methanol for 1 h and rinsing with water for 5 min. The gel was then immersed in silver staining solution (80 mL of 20% silver nitrate solution, 4.0 mL of 36% sodium hydroxide, 28 mL of 14.8 M ammonium hydroxide made up to 2 L with distilled water) for 5 min and then washed with water. Fresh developer solution (10 mL of 1% citric acid, 1 mL 37% formaldehyde made up to 2 L with water) was added with continuous agitation, away from direct light, for approximately 5 min until the

bands appeared. The gel was then placed in a fixative solution (50% methanol) for 30 min. The gel was air dried in a dark room overnight. Photographs were taken with Kodak technical Pan film.

3.9 Detection of the nature of inhibitory substance

3.9.1 Antimicrobial substance in the cell free neutralized culture supernatant

This was done by a spot-on-lawn assay (Barefoot and Klaenhammer, 1983). An overnight culture of each producer strain was adjusted to pH 6.8 with 1 N NaOH and centrifuged at 6,000 x g for 10 min. The supernatant fluids were either heat treated (65°C/30 min) or mixed with an equal volume of chloroform to kill the remaining cells in the supernatant. A 20 µL portion of supernatant from each producer strain was spotted onto an APT agar plate overlayered with 7 mL of soft agar (0.75%) seeded with 10^7 cfu of the indicator strain UAL 15 per mL. A control of sterile APT broth mixed with an equal volume of chloroform was used to ensure that inhibition was not due to chloroform residues.

3.9.2 Production of hydrogen peroxide

The possibility of the involvement of hydrogen peroxide as an inhibitor in the test culture was tested by addition of 100 units of catalase (Fisher Scientific) per mL of neutralized supernatant.

3.9.3 Detection of lytic bacteriophages

Evidence that inhibition of indicator organism was not caused by bacteriophage was determined by:

i) Modified method of Pugsley and Oudega (1987). A portion of the zone of inhibition was aseptically cut from an assay plate showing deferred antagonism. The agar plug was added to 2 mL of APT broth and macerated with a sterile glass rod. The mixture was held at room temperature for 1 h. A 100 µL sample was added to 4 mL of soft agar containing indicator cells (approx. 10^7 cfu/mL). The soft agar suspension was poured evenly over an APT plate and incubated overnight at 25°C. The formation of plaques is indicative of bacteriophage activity.

ii) Modified reverse-side technique (Tagg *et al.*, 1976). The procedure was identical to deferred antagonism test described in section 3.5.2, except the agar layer was inverted and overlayered with the indicator strain to avoid contact between indicator and producing strains.

3.10 Characterization of inhibitory substance

3.10.1 Enzymatic sensitivity

The sensitivity of the inhibitory substances to various enzymes was determined. The enzymes and their sources are listed in Table. 3.3. The enzymes were dissolved in sterile buffer as recommended by the supplier (Sigma) or in 0.01 M phosphate buffer at pH 7.0 to a final concentration of 10 mg/mL. Heated and neutralized supernatant fluids of producer

Table 3.3. Enzymes used in this study [1]

Enzyme	Type
Pronase E	Type XIV from *Streptomyces griseus*
Protease	Pancreatic type No. P-4630
Proteinase K	Type XI
Protease	Type VIII from *Bacillus licheniformis*
Pepsin	From porcine stomach mucosa No. P 7000
Papain	Type IV
Trypsin	Type IX from porcine pancreas
α-chymotrypsin	Type II from bovine pancreas
Phospholipase D	Type VI from *Streptomyces chromofuscus*
Lipase	Type VII from *Candida cylindracea*

[1] all enzymes are from Sigma.

organisms was used for spot-on-lawn assay (section 3.9.1) in two ways:

1. A 10 µL amount of heated or chloroform-treated, neutralized supernatant was spotted onto an APT plate, dried and 2 µL of the enzyme (10 mg/mL) was spotted adjacent to it. The plate was allowed to dry, then overlayered with the indicator organism and incubated at 25°C for 24 h. Inactivation of the inhibitory agent in the region of the enzyme indicated that the inhibitor is sensitive to the particular enzyme.
2. One mL of culture supernatant fluids was mixed with 100 µL of enzyme (10 mg/mL) and incubated at 25°C for 2 h and spotted onto a plate that had been overlayered with the indicator organism. Sensitivity of the inhibitory agent to the enzyme was recorded after 24 h of incubation at 25°C.

Controls were done in the same way as described above with enzymes inactivated by heating at 100°C for 15 min.

3.10.2 Immunity test

The immunity of the producer cells against its own bacteriocin(s) as well as against other selected bacteriocin-producing organisms was determined by the deferred antagonism test.

3.10.3 Thermosensitivity

The heat sensitivity of the antibacterial substances was

determined by heating neutralized culture supernatant fluids at 65°C for 1 h, 100°C for 30 min and 121°C for 15 min. After cooling, the sample was tested for its activity by the spot-on-lawn assay.

3.10.4 Optimum pH and time for the production of inhibitory substance in liquid and solid media at 25°C

APT broth adjusted to pH levels between 4.0 and 7.0 at 0.5 intervals and Cooked Meat Medium adjusted to pH 6.5 were prepared by addition of 10 M HCl. After tempering the tubes at 25°C for 1 h, a 1% inoculum of overnight culture of the producer organisms was added to each tube. Samples were removed at specified intervals (initially every 1 h starting from 3 h up to 6 h, and then every 4 h up to 24 h; and every 12 h up to 72 h). The samples were examined for bacterial growth (OD at 620 nm), change in pH and inhibitory activity.

Quantitative estimation of the inhibitory substance was done with neutralized (pH 6.8) and heat treated (65°C/30 min) culture supernatants. The arbitrary activity unit (AU) was determined as the reciprocal of the highest dilution (in a series of doubling dilutions) showing a clear zone of inhibition by spot-on-lawn technique and adjusted to obtain units per millilitre (AU/mL). The experiment was replicated three times on duplicate plates.

3.11 Growth and production of inhibitory substance by the producer strains at 4° and 10°C in ground beef and laboratory media

3.11.1 Bacterial inoculum

A 24 h culture of each producer organism was harvested by centrifugation and resuspended in 10 mL of 0.1% peptone water. Cell concentration of a 24 h culture was determined by serial dilution, and used to adjust the amount of inoculum to obtain a final concentration of approximately 10^6 cfu/g in the meat samples.

3.11.2 Sample preparation

Fresh, aerobically stored beef was specially cut from a single muscle of a beef carcass to give minimal contamination of the meat. The surface was flamed with alcohol on each side and aseptically cut into 4 equal parts with a sterile knife. Three portions were surface inoculated separately with an appropriate volume of one of 3 producer organisms and one uninoculated portion was kept as a control. Samples were ground twice in a sterile grinder, and 50 g samples were placed into sterile plastic bags (Whirl-pak, Nasco, Fort Atkinson, WI) and sealed under vacuum (Multivac type AG 500, Sepp Haggenmuller KG, Wolfertschwenden, Allgan, Germany). The packages were stored at 4 and 10°C for up to 2 weeks.

The producer organisms were also inoculated into tubes containing 10 mL of APT broth or cooked meat medium (12.5% and

25% w/v) at a level of 10^6 cfu/mL and incubated at 4 and 10°C. Another set of APT broth or CMM was prepared by adding glucose or sucrose (0.2% and 2%, respectively).

3.11.3 Determination of initial pH and microbial quality of fresh meat

A 10 g sample of fresh ground beef was blended with 90 mL of 0.1% peptone (Difco) water in a Colworth Stomacher (model 400, A.J. Seward, Bury St. Edmund, UK) for 3 min. Serial dilutions were made with 0.1% peptone water and 20 µL was spotted onto selective and nonselective media. The pH of blended beef was determined by digital pH meter (model 671, ExTech, Boston, MA).

3.11.4 Sampling schedule

Throughout the storage period, samples stored at 10°C were analyzed daily for 3 days and on the 7^{th} and the 14^{th} day of storage. Samples stored at 4°C were analyzed after 24 h of incubation and thereafter every third day of storage.

3.11.5 Experimental procedure

A 50 g beef sample (inoculated and uninoculated control) was removed from storage at each specified time for each temperature, and 12.5 mL of 50 mM phosphate buffer (pH 5.9) was added. The sample was blended in a Stomacher for 3 min. A 10 g sample was used for bacteriological analysis and determination of pH, the remaining sample was used for

detection of inhibitory substances. The study was carried out in three parts.

(1) Microbial examination. After making serial dilutions, 20 µL samples of each dilution were spotted onto APT agar and incubated anaerobically at 25°C for 48 h. Viable bacterial counts were calculated to determine growth rates of producer organisms. From the control samples, bacterial counts were determined using APT, Tryptic Soy Agar (TSA) and selective media (Table 3.2).

(2) Evaluation of change in pH. The pH of the samples was measured after bacteriological examination.

(3) Recovery of inhibitory substances

(a) Extraction of inhibitory substance from the supernatant. Each sample was centrifuged at 10,000 x g for 10 min. The supernatant was adjusted to pH 6.8 with 1 N NaOH and heated at 65°C for 15 min. The sample was used to determine the activity of inhibitory substance on the lawn of indicator organism (UAL 15), using spot-on-lawn assay.

(b) Addition of sodium dodecyl sulphate (SDS) and Tween 80. Detergents SDS (Calbiochem) and Tween 80 (Fisher) were added separately to a concentration of 0.1% in beef and CMM samples to aid in the extraction of inhibitory substance. Samples were incubated overnight at 4 and 10°C with detergent. The suspension was centrifuged and the supernatant fluids were used to assay for bacteriocin production.

(c) Hot HCl extraction. Meat samples were mixed with 0.02 N HCl (10% w/v) suspension as described by Bell and DeLacy (1986) for nisin extraction. The samples were centrifuged at 10,000 x g for 5 min. The supernatant was neutralized to pH 6.8 and heated at 100°C for 10 min. The supernatant was used to assay for inhibitory activity.

4. RESULTS

4.1 Screening for inhibitory activity

A total of 260 LAB isolated from vacuum packaged and chill stored fresh beef was screened for production of antagonistic compounds against 98 different pathogens and spoilage organisms by the deferred antagonism test. A total of 60 (23%) strains of LAB showed inhibitory activity against all 14 strains of *Brochothrix* tested, which is a major spoilage organism of vacuum packaged meat. Among other Gram-positive organisms, all ten strains of *Listeria* spp. were inhibited by 52 (20%) of the LAB. A few strains of *Staphylococcus* spp., *Micrococcus* spp. and *Enterococcus* spp. were inhibited by 10 (3.8%), 8 (3.1%) and 15 (5.8%) of the LAB, respectively. Heat activated spores as well as vegetative cells of *Bacillus* spp. were inhibited by 9 (3.5%) and 5 (1.9%) LAB, respectively; while heat activated spores and vegetative cells of *Clostridium* spp. were inhibited by 20 (7.7%) and 10 (3.8%) LAB, respectively. Among Gram-negative organisms tested, *Pseudomonas fluorescens* ATCC 13525 and other *Pseudomonas* spp. isolated from meat were inhibited by 106 (40.8%) of the LAB. However, *P. aeruginosa* ATCC 15442 was not inhibited at all. *Flavobacterium* spp. isolated from meat and *Alcaligenes faecalis* ATCC 8750 were also inhibited by 57 (21.9%) of the LAB. Among the Enterobacteriaceae indicator strains the following were shown to be sensitive to inhibitory compounds

produced by 3 or 4 of the LAB (1.5%): *Klebsiella pneumoniae* ATCC 13883, *Salmonella choleraesuis* ATCC 10708, *E. coli* ATCC 11775 and 11229, *Y. enterocolitica* ATCC 23715 and *E. agglomerans* ATCC 27155.

Strains 176, 185, 236, 258 and 261 had a much broader spectrum of activity than other strains tested in this study. Strains 236, 258 and 261 showed larger zones of inhibition compared with reference strain *L. lactis* ATCC 11454 tested both by deferred and spot-on-lawn assays and they inhibited all strains of Gram-positive organisms tested except *Bacillus* and *Clostridium* spp. The inhibitory substances were shown to be proteinaceous compounds because the inhibitory activity was lost after treatment with protease. However, the production of bacteriocin was not reliable for strains 236, 258 and 261, therefore, they were excluded from further study.

Strains 176 and 185 showed a broad spectrum of activity against Gram-positive organisms, including heat activated spores of *Bacillus* and *Clostridium* spp., *Listeria* spp., enterotoxin producing strains of *Staphylococcus* spp. which have multiple drug resistance, *Enterococcus* spp. and *Brochothrix thermosphacta*. Therefore, these two organisms were selected for further characterization.

The original cultures of both strains (176 and 185) showed 2 different morphological variants on APT agar plates. For strain 176, one smaller colony with a broad spectrum of

activity was picked. The other colony type of similar size was distinguishable by a unique colony morphology and demonstrated a low inhibitory activity against indicator organisms. In the case of strain 185, one variant with a small colony size showed a broad spectrum of activity and another variant with a larger colony size showed no inhibitory activity against indicator organisms tested. Colonies with a broad spectrum of activity were further characterized.

4.2 Activity spectra of strains # 176 and 185

As shown in Tables 4.1 and 4.2, the inhibitory spectra of strains 176 and 185, include most of the Gram-positive organisms tested by deferred test. There were slight differences in the inhibitory spectra of these two strains, especially in the case of *Staphylococcus* spp. and *Clostridium* spp. No inhibition of Gram-negative strains was observed except *P. fluorescens* ATCC 13525. The most sensitive strain was *Leuconostoc mesenteroides* ATCC 23368 (UAL 15) and therefore it was selected as the indicator strain for further study. All of the results are based on triplicate determinations.

4.3 Identification of the producer strains

Strains 176 and 185 are Gram-positive, nonmotile, catalase and oxidase negative, nonhaemolytic, facultative anaerobes and coccoid-shaped cells that form pairs or short chains. The organisms are sensitive to heat treatment at 65°C for 15 min. At 1 and 2°C, strain 176 grew in APT broth after

Table 4.1 Activity spectra of strains 176 and 185 determined by deferred antagonism test on APT agar.

Indicator organisms	Source	Strain 176	Strain 185
Brochothrix thermosphacta			
11509	ATCC	+	+
Meat isolates			
#1	LRS[1]	+	+
#2	LRS[1]	+	+
#41	LRS[1]	+	+
#42	LRS[1]	+	+
#43	LRS[1]	+	+
B2	LRS[1]	+	+
B11	LRS[1]	+	+
BL262	LRS[1]	+	+
BL110	LRS[1]	+	+
CT2	LRS[1]	+	+
C420	LRS[1]	+	+
NF4	LRS[1]	+	+
L90	LRS[1]	+	+
Enterococcus faecalis 19433	ATCC	+	+
E. faecalis 11576	ATCC	+	+
E. faecalis 7080	ATCC	+	+
E. faecium 19434	ATCC	+	+
Listeria monocytogenes 15313	ATCC	+	+
L. monocytogenes Scott A		+	+
L. innocua 33090	ATCC	+	+
Listeria strains from meat			
#5		+	+

#14		+	+
#20		+	+
#41		+	+
#42		+	+
#48		+	+
#50		+	+
Staphylococcus aureus 25923	ATCC	+	+
S. aureus 6538	ATCC	+	+
S. aureus 13565	ATCC	+	–
S. aureus 23235	ATCC	+	–
S. aureus STD_1	Ewan[2]	+	+
S. aureus STD_{15}	Ewan[2]	+	+
S. aureus STD_{16}	Ewan[2]		
S. aureus S_6	Ewan[2]	–	–
S. aureus 88-1020	Ewan[2]	–	+
S. aureus 89-SE00-S-4	Ewan[2]	–	+
S. epidermidis STD_3	Ewan[2]	–	–
S. epidermidis GR_{32}	Ewan[2]	–	–
S. epidermidis GR_{50}	Ewan[2]	+	+
S. epidermidis 90-0683	Ewan[2]	–	–
Micrococcus kristinae 27570	ATCC	–	+
Aciduric *Lactobacillus* UAL 3		+	+
and UAL 12		+	+
Carnobacterium piscicola LV17		+	+
Carnobacterium divergens LV13		+	+

Leuconostoc mesenteroides 23368	ATCC	+	+
Lb. plantarum 4008	ATCC	+	+
Lb. viridescens 12706	ATCC	+	+
P. acidilactici 8042	ATCC	+	+
P. parvulus 19371	ATCC	+	+

ATCC - American Type Culture Collection Strains

\+ - Sensitive strain

\- - Resistant strain

[1] - Supplied by Dr. G. Greer, Lacombe Research Station, Agriculture Canada.

[2] - Supplied by Dr. Pauline Ewan, Laboratory Centre for Disease Control, Health Protection Branch, Ottawa

Table 4.2 Activity spectra of strains 176 and 185 against vegetative cells and heat activated spores of *Bacillus* and *Clostridium* spp.

Indicator organism	Strain 176			Strain 185		
	Cells	Heated Spores		Cells	Heated spores	
		65°C for 1 h	80°C for 10 min		65°C for 1 h	80°C for 10 min
B. cereus ATCC 14579	+	+	+	+	+	+
B. subtilis ATCC 6051	+	+	+	+	+	+
B. macerans ATCC 7048	-	-	-	-	-	-
B. macerans ATCC 8244	-	-	-	-	-	-
C. sporogenes ATCC 19404	+	+	+	+	+	+
C. butyricum ATCC 8260	+	+	+	+	+	+
C. pasteurianum ATCC 6013	+	+	+	-	-	-
C. putrificum ATCC 2578	+	+	+	+	-	-
C. bifermentans ATCC 19299	+	+	+	+	+	+

\+ sensitive strain

\- resistant strain

one month of incubation. The physiological characteristics of strains 176 and 185 are listed in Table 4.3. They differed from *L. lactis* ATCC 11454 in that they grew at 4°C and grew on acetate and lactate agars (pH 5.6). The end point pH in La broth is slightly different for all three organisms. All strains grew at pH 3.9 and 9.2 but not at pH 9.6. Strains 176 and 185 grew in milk containing 0.3% methylene blue after 3 weeks of incubation while *L. lactis* ATCC 11454 grew within one week. All 3 organisms grew in BHI broth containing 6.5% NaCl after 24 h of incubation; however, they did not grow in MRS broth containing 6.5% NaCl. All failed to grow in broth containing 8% NaCl.

The results of antibiotic susceptibility tests are shown in Table 4.4 (p. 81). There are differences between the three strains when the size of the zone is measured by disk diffusion assay. However, the MIC of all 3 strains is similar for each antibiotic tested except penicillin. The level of resistance to penicillin was eight-fold higher for *L. lactis* ATCC 11454 compared with strains 176 and 185.

The carbohydrate fermentation patterns of producer strains are shown in Table 4.5 (p. 82). Both strains showed similar carbohydrate fermentation patterns to *L. lactis* ATCC 11454, except lactose fermentation, where 176 and 185 did not ferment 0.5% lactose and showed weak fermentation with 1% lactose after extended (5 days) anaerobic incubation.

Table 4.3 Physiological characteristics of producer strains.

Characteristic	Producer Strains		
	176	185	*L. lactis* ATCC 11454
Growth at:			
1°C	+	-	-
2°C	+	-	-
4°C	+	+	-
6°C	+	+	+
42°C	+	+	+
45°C	-	-	-
pH 3.9	+	+	+
pH 9.2	+	+	+
pH 9.6	-	-	-
Growth in:			
0.3% methylene blue in milk	+[1]	+[1]	+
4% NaCl in BHI broth	+	+	+
4% NaCl in MRS broth	+	+	+
6.5% NaCl in BHI broth	+	+	+
6.5% NaCl in MRS broth	-	-	-
Growth on:			
Acetate agar	+	+	-
Lactate agar	+	+	-
Bile aesculin agar	-[2]	-[2]	+
Haemolysis on blood agar	-	-	-
Terminal pH in La broth	4.2	4.3	4.5

\+ positive.
\- negative.
[1] delayed.
[2] growth but no hydrolysis of aesculin.

Table 4.4 Disk susceptibility test and MIC determination of selected antimicrobial agents for producer strains*.

Antibiotic	Zone (mm)			MIC (mg/mL)[1]		
	176	185	*L. lactis* 11454	176	185	*L. lactis* 11454
Ampicillin	29	28	33	<0.12	<0.12	<0.12
Cephalothin	24	27	25	<2.0	<2.0	<2.0
Chloram-phenicol	23	23	24	<4.0	<4.0	<4.0
Erythro-mycin	28	27	29	<0.25	<0.25	<0.25
Gentamycin	18	19	18	<1.0	<1.0	<1.0
Kanamycin	19	20	18	NT	NT	NT
Oxacillin	10	9	11	<0.5	<0.5	<0.5
Penicillin	30	30	34	<0.03	<0.03	<0.25
Strepto-mycin	15	15	15	NT	NT	NT
Sulpha-methoxazole	21	19	26	<256	<256	<256
Vancomycin	20	20	20	<2.0	<2.0	<2.0
Tetra-cycline	27	27	26	<2.0	<2.0	<2.0

* Source of information: Dr. M. Lovgren, Head, Division of Bacteriology, Provincial Laboratory of Public Health for Northern Alberta, Edmonton.

NT Not tested.

[1] MIC is reported on the break-point at which the growth is inhibited.

Table 4.5 Carbohydrate fermentation patterns of producer strains.

Carbohydrate	176	185	*L. lactis* ATCC 11454
Amygdalin	-	-	-
Arabinose	-	-	-
Cellobiose	-	-	-
Dextrin	-	-	-
Fructose	+	+	+
Glycerol	-	-	-
Glucose	+	+	+
Galactose	+	+	+
Inulin	-	-	-
Lactose (0.5%)	-	-	+
Lactose (1%)	+[1]	+[1]	+
Maltose	+	+	+
Mannitol	-	-	-
Melezitose	-	-	-
Raffinose	-	-	-
Rhamnose	-	-	-
Ribose	+	+	+
Sucrose	+	+	+
Sorbitol	-	-	-
Salicin	+	+	+
Trehalose	+	+	+
Xylose	-	-	-
K-gluconate	-	-	-

\+ positive.

\- negative.

[1] weak and delayed.

The results of the other biochemical tests are listed in Table 4.6. The organisms are homofermentative, based on their failure to produce CO_2 from glucose. All three strains grew on bile aesculin agar containing 40% bile. However, strains 176 and 185 were unable to hydrolyse aesculin and no blackening of the medium was observed. This was confirmed by testing in Brain heart infusion broth containing 0.1% aesculin and 0.1% agar. Strains 176 and 185 did not hydrolyse aesculin and failed to form a black colour upon addition of 0.1% ferric ammonium citrate. *L. lactis* ATCC 11454 on the other hand hydrolysed aesculin and formed black colour immediately upon addition of ferric ammonium citrate. The tubes were also observed under UV light. Unlike *L. lactis* ATCC 11454, there was no loss of fluorescence observed under UV light from tubes of strains 176 and 185 indicating a negative test.

The isomeric composition of lactic acid formed by the three organisms as well as other known control strains was determined enzymatically. Strains 176 and 185 produced >15% of D(-) isomer and were therefore categorized as producing L(D) isomer. *L. lactis* ATCC 11454 produced >90% of L(+) isomer as a major metabolic product from glucose.

Biochemical and physiological tests of the producer strains indicated that strains 176 and 185 do not resemble any established species. Moreover, the strains produced bacteriocins with a similar spectrum of activity to nisin produced

Table 4.6 Biochemical properties of producer strains.

Characteristic	176	185	*L. lactis* ATCC 11454
Gas from glucose	-	-	-
Hydrolysis of:			
aesculin	-	-	+
starch	+	+	+
hippurate	+	+	+
Production of:			
NH_3 from arginine	+	+	+
acetoin	+	+	+
slime from sucrose	-	-	-
catalase	-	-	-
oxidase	-	-	-
Reduction of tetrazolium			
0.1%	+	+	+
0.01%	+	+	+
Type of lactate isomer	L(D)	L(D)	L
Percentage of lactate isomer	L: 81.76 D: 18.24	L: 82.11 D: 17.89	L: 92.34 D: 7.66

\+ positive.

\- negative.

by the reference strain. The point to emphasize is that both strains differ from the reference strain, *L. lactis* ATCC 11454. The biochemical tests are not enough to assign the strain to a taxonomic position. Thus genetic characterization of the strains was considered.

4.4 Plasmid profile of producer strains

Results of CsCl-ethidium bromide purified plasmid DNA preparation obtained from 176 and 185 were compared with the plasmid profile of *L. lactis* ATCC 11454. As shown in the Figures 1 and 2 the same plasmid profile (six plasmids) was observed for the reference strain, *L. lactis* ATCC 11454, as those described by Gonzalez and Kunka (1985). Strains 176 and 185 contain only 4 plasmids. Figure 3 is a schematic representation of the plasmid profiles for producer strains based on the standard curve constructed from the plasmids of *E. coli* V517.

The restriction patterns following *Bgl*II and *Bam*HI cleavage is shown in Figures 1 and 2. Figure 4 is a schematic representation of the fragments observed after digestion of plasmid DNA from the 3 organisms based on the standard curve constructed from *Hind*III digest of λ DNA. Except for few common fragments, considerable variation between plasmids from the 3 organisms was evident. The small plasmids at 2.8

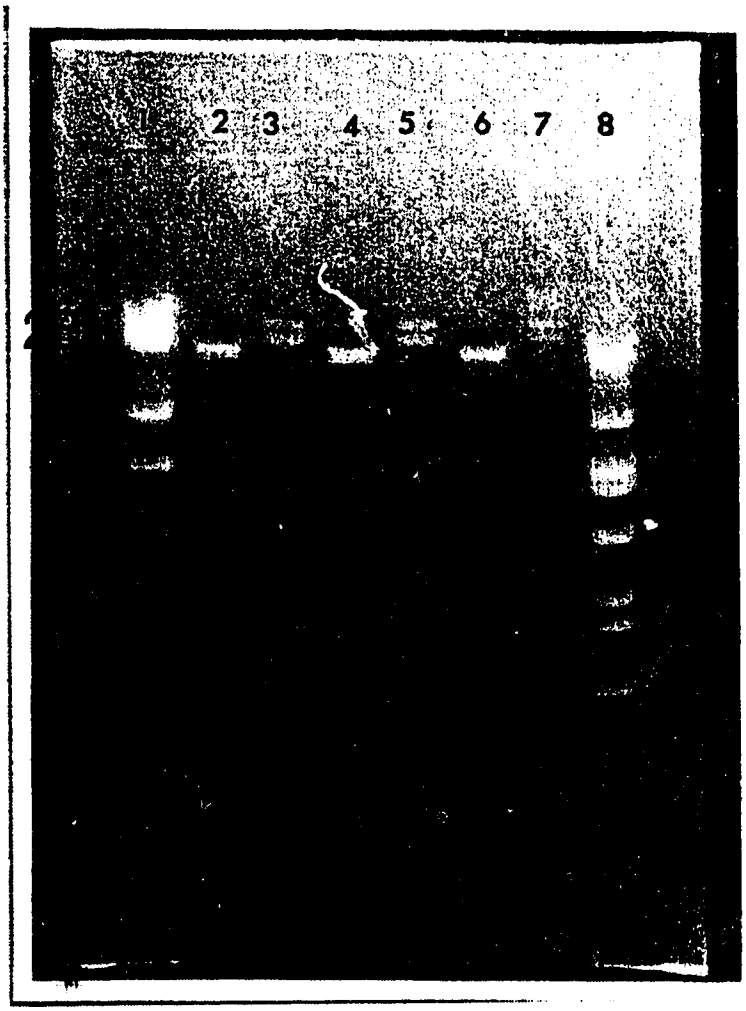

Fig. 1. Plasmid profiles and *Bgl*II digestion patterns of DNA from *L. lactis* ATCC 11454, and meat strains 185 and 176.

Lane:1. *Hin*dIII digests of bacteriophage λ DNA; 2. *Bgl*II digest of plasmid DNA from strain 176; 3. Undigested plasmid profile of strain 176; 4. *Bgl*II digest of plasmid DNA from strain 185; 5. Undigested plasmid profile of strain 185; 6. *Bgl*II digest of plasmid DNA of *L. lactis* ATCC 11454; 7. Undigested plasmid profile of *L. lactis*; 8. Mobility standard plasmids from *E. coli* V517. Sizes of plasmids are expressed in MDa, and sizes of fragment DNA are in kb.

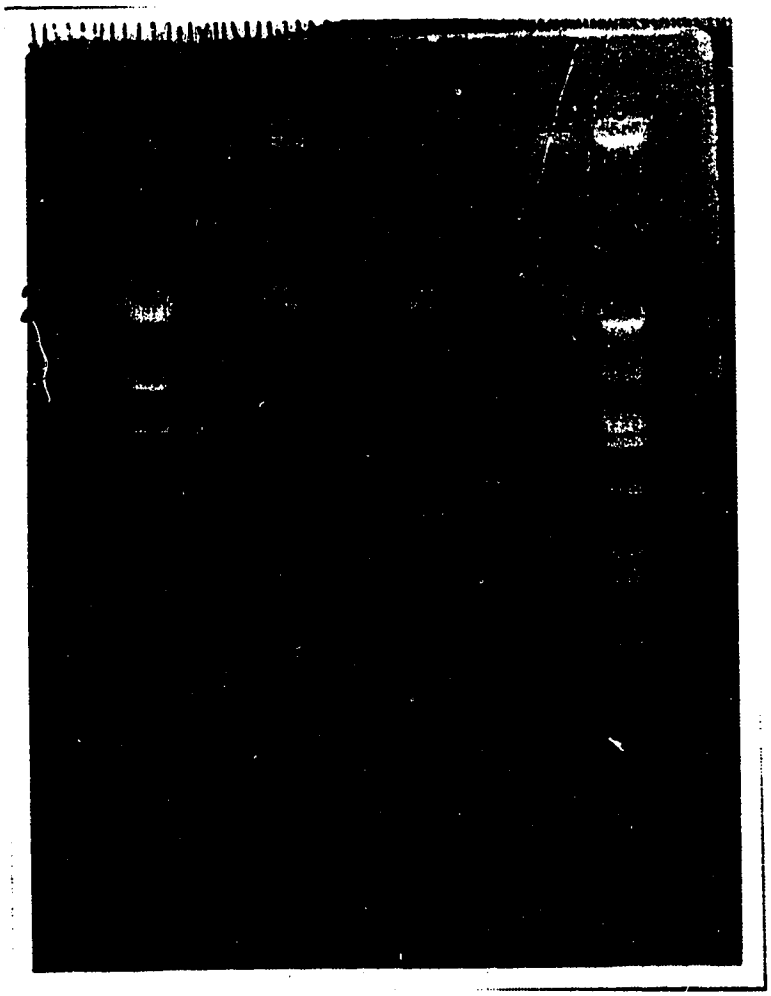

Fig 2. Plasmid profiles and *Bam*HI digestion patterns of DNA from *L. lactis* ATCC 11454, and meat strains 185 and 176.

Lane:1. *Hin*dIII digests of bacteriophage λ DNA; 2. *Bam*HI digest of plasmid DNA from strain 176; 3. Undigested plasmid profile of strain 176; 4. *Bam*HI digest of plasmid DNA from strain 185; 5. Undigested plasmid profile of strain 185; 6. *Bam*HI digest of plasmid DNA of *L. lactis* ATCC 11454; 7. Undigested plasmid profile of *L. lactis*; 8. Mobility standard plasmids from *E. coli* V517. Sizes of plasmids are expressed in MDa, and sizes of fragment DNA are in kb.

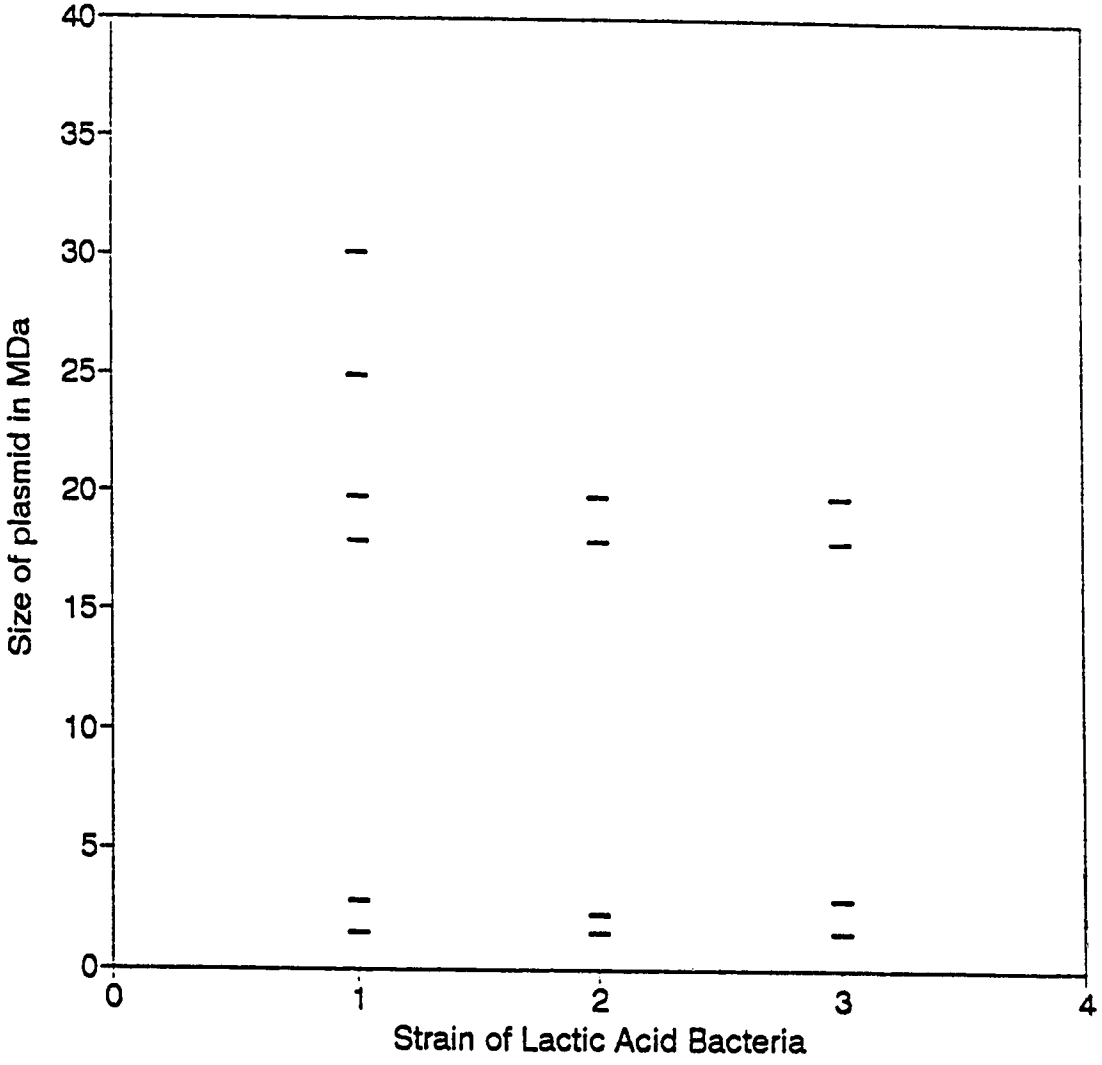

Fig. 3. Schematic representation of plasmid profiles for producer strains.
1. *L. lactis* ATCC 11454, 2. strain 185, and 3. strain 176.

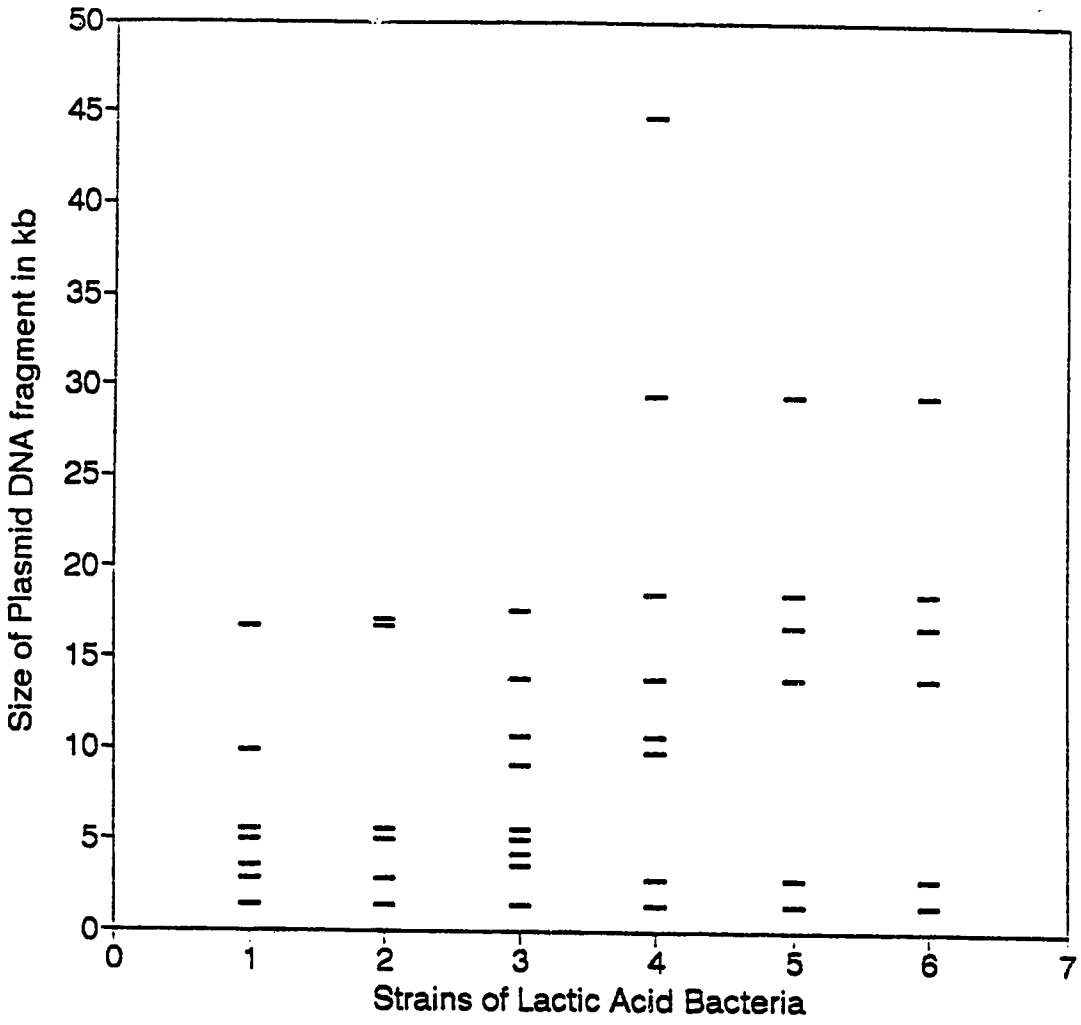

Fig. 4. Schematic representation of the digestion patterns of plasmid DNA from producer strains.

For *Bgl*II digest of plasmid DNA: 1. *L. lactis* ATCC 11454, 2. strain 185, and 3. strain 176; and for *Bam*HI digest of plasmid DNA: 4. *L. lactis* ATCC 11454, 5. strain 185, and 6. strain 176.

and 1.4 MDa were not cut by the restriction enzymes. Strains 176 and 185 showed similarities in *Bam*HI restriction pattern.

The plasmid profiles clearly distinguished between the reference strain and the two meat strains examined.

4.5 Differentiation of producer strains on the basis of low molecular weight (LMW) RNA profiles

The LMW RNA profiles of strains 176, 185 and *L. lactis* ATCC 11454 were compared to determine differences between these organisms at the genetic level. As shown in Figure 5, band patterns were divided into 3 groups, 5S rRNA, Class 2 tRNA and class 1 tRNA. Undiluted and diluted samples (5, 10 and 25 times) of RNA extracts were loaded onto the gel to ensure good resolution and clear separation of bands in all regions of the gel, especially tRNA.

The molecular size (relative nucleotide units, RNU) of each of the RNA bands was estimated by comparing the mobility of unknown bands with the mobility of a set of nucleotide markers of known size (120, 89 and 70 nucleotides). A schematic representation of RNA profiles of diluted samples for 5S rRNA, class 2 tRNA and class 1 tRNA molecules is shown in Figure 6. Strain 185 and *L. lactis* ATCC 11454 showed similar patterns of 5S rRNA (118 RNU) while strain 176 showed a smaller band (115 RNU). Strain 185 also showed another band with 116 RNU which was likely a breakdown product of the 5S rRNA (Fig. 5 and 6).

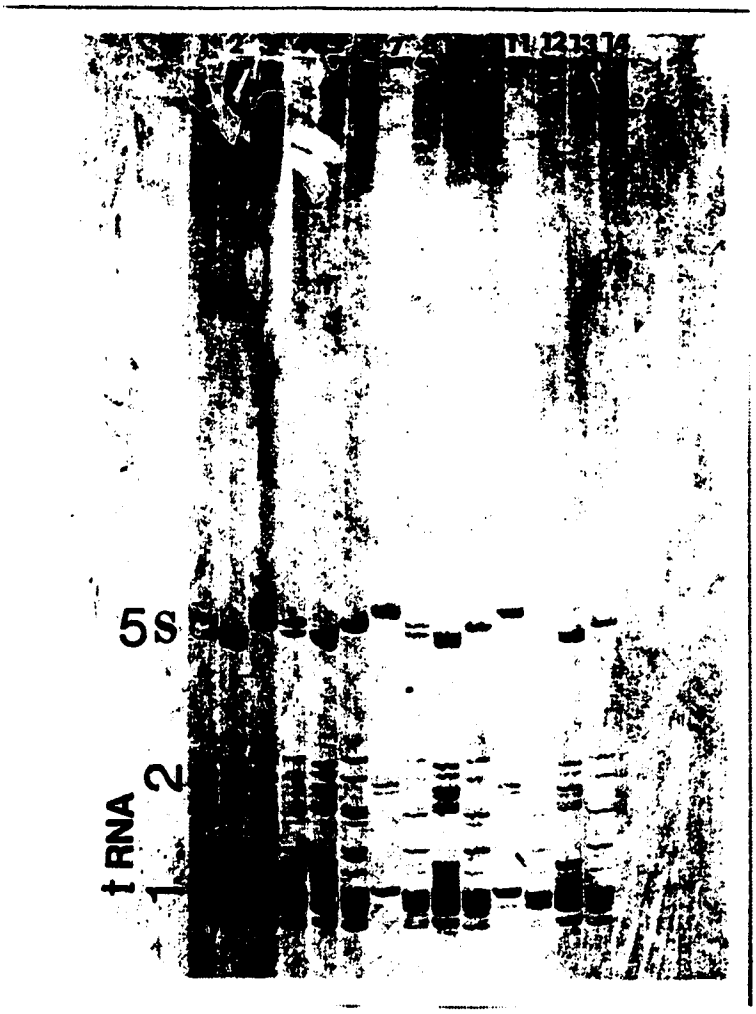

Fig. 5. Low molecular weight RNA profiles of producer strains.

1. Undiluted sample from strain 185.
2. Undiluted sample from strain 176.
3. Undiluted sample from *L. lactis* ATCC 11454.
4. Diluted 5 times strain 185.
5. Diluted 5 times strain 176.
6. Diluted 5 times *L. lactis* ATCC 11454.
7. Size marker from *E. coli*.
8. Diluted 10 times strain 185.
9. Diluted 10 times strain 176.
10. Diluted 10 times *L. lactis* ATCC 11454.
11. Size marker from *E. coli*.
12. Diluted 25 times strain 185.
13. Diluted 25 times strain 176.
14. Diluted 25 times *L. lactis* ATCC 11454.

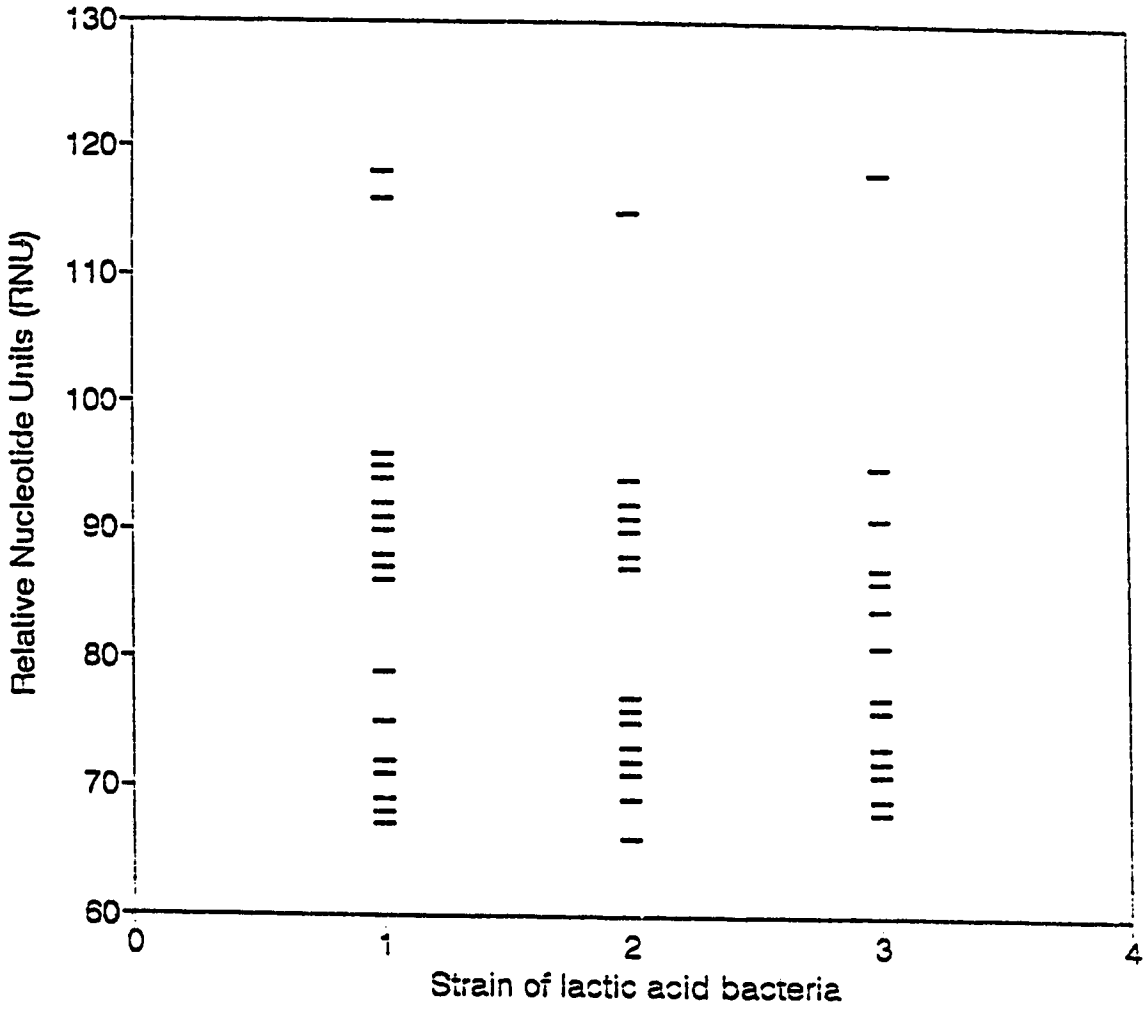

Fig. 6. Schematic representation of relative nucleotide sizes for 1. strain 185, 2. strain 176, and 3. *L. lactis* ATCC 11454.

The LMW RNA profiles showed distinct patterns for all 3 organisms tested, especially in tRNA region where various single bands were clearly separated. Except for a few common bands, all three strains either lacked a few bands and/or showed additional bands.

4.6 Nature of the inhibitory substance

To determine the nature of the inhibitory substance responsible for inhibition in the deferred test, strains 176 and 185 were subjected to further study. To exclude the possibility that inhibition was due to the low pH resulting from the acids produced by LAB, cell free supernatant fluids were neutralized (pH 6.8) and tested by spot-on-lawn assay (Fig. 7, p. 96). Results of the deferred test were thus confirmed. The inhibitory activity of the neutralized supernatant was not affected by the addition of catalase, indicating that the inhibition was not caused by the action of H_2O_2 (Fig. 7, p. 96).

No plaque formation or clearing zone was formed from macerate of the inhibitory substance taken from inhibitory zones and poured onto sensitive indicator strains. This indicated that the inhibitor was not an infective agent and phages were not responsible for the inhibition. In addition, a clearing zone was observed in modified reverse-side inhibition technique (section 3.9.3). By putting an agar barrier

between the producer and indicator cells, lysis caused by bacteriophage was excluded.

Thus, it was clear that the inhibitory substance produced by strains 176 and 185 was not H_2O_2, acids or bacteriophage.

4.7 Characterization of inhibitory substance

4.7.1 Enzymatic sensitivity

The effect of several enzymes on the inhibitory agent produced by strains 176 and 185 was examined (Table 4.7). For comparative purposes, results of nisin producing *L. lactis* ATCC 11454 were included in the same table. This test was done using a lawn of indicator strain UAL 15. Treatment with denatured enzymes did not destroy the inhibitory activity in any of the neutralized culture supernatant fluids tested.

Inhibitory activity in all three neutralized culture supernatants tested was lost after treatment with pronase E, protease, proteinase K and protease type VII. Figure 7 (p. 96) shows that the inhibition by strains 176 and 185 was inactivated when treated with pronase E. These data indicate that the inhibitory substance is proteinaceous, which is characteristic of a bacteriocin. Treatment with pepsin and papain did not affect the antagonistic activity.

Bacteriocin from strain 176 was partially inactivated by trypsin, α-chymotrypsin and lipase; while bacteriocins from strain 185 and *L. lactis* ATCC 11454 were completely inactivated by these enzymes. Bacteriocins from *L. lactis* ATCC

Table 4.7 Effect of enzymes on the inhibitory substance produced by strains 176, 185 and *L. lactis* 11454.

Enzyme[a]	176	185	*L. lactis* 11454
Pronase E	+	+	+
Protease	+	+	+
Proteinase K	+	+	+
Protease VIII	+	+	+
Pepsin	-	-	-
Papain	-	-	-
Trypsin	+/-	+	+
α-chymotrypsin	+/-	+	+
Phospholipase D	-	+/-	-
Lipase	+/-	+	+

\+ Inhibitory substance sensitive to enzyme.

\- Inhibitory substance not sensitive to enzyme.

+/- Partial inactivation of the inhibitory substance.

[a] Enzyme type and their source are given in Table 3.3.

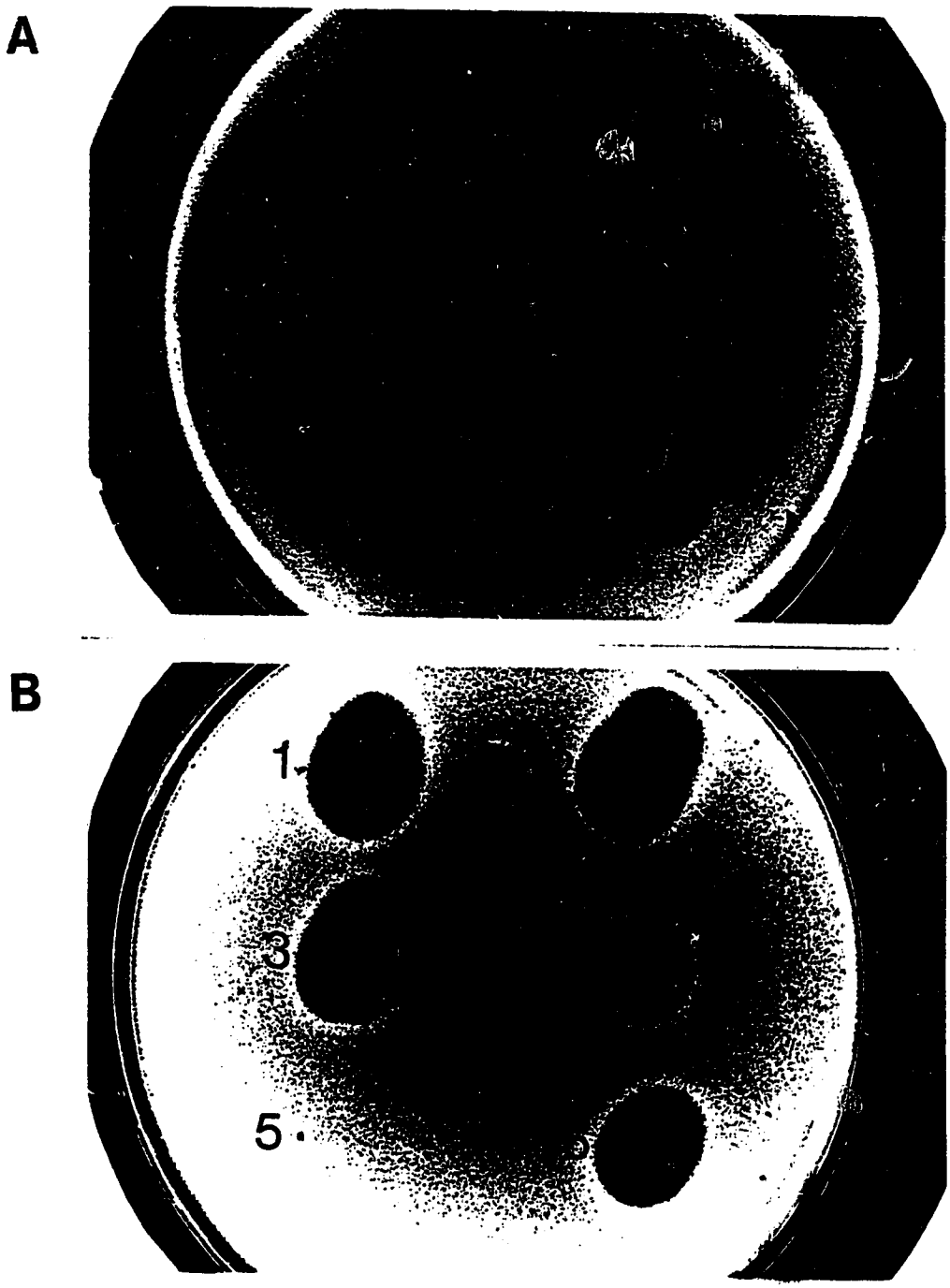

Fig. 7. Effect of various treatments of supernatant fluids collected from A, strain 176; B, strains 185 on the indicator organism *Leuconostoc mesenteroides* ATCC 23368.

1. pH adjusted to 6.8 and heated at 62°C for 30 min.
2. Catalase (100 units/mL).
3. Heated at 100°C for 30 mins.
4. Heated at 121°C for 15 mins.
5. Pronase E (1 mg/mL).
6. Denatured pronase E (100°C for 15 mins.)

11454 and strain 176 were not sensitive to phospholipase D, but bacteriocin from strain 185 was partially inactivated by phospho-lipase D.

These results indicate differences in the nature of the bacteriocins derived from the three organisms tested.

4.7.2 Immunity of producer cells to their bacteriocins

As shown in Table 4.8, strains 176, 185 and *L. lactis* ATCC 11454 were immune tc their own bacteriocin and also immune to each other's bacteriocins as tested by deferred antagonism test. The same results were obtained when immunity tests were done using the neutralized supernatant to eliminate any effect of acidity.

4.7.3 Thermostability of bacteriocin

The neutralized (pH 6.8) culture supernatants were heated at different temperatures and tested for inhibitory activity on an indicator lawn of UAL 15. The inhibitors showed no loss in the activity when heated at 65°C for 1 h and 100°C for 30 min. Even after autoclaving, activity was not lost, but *L. lactis* ATCC 11454 showed a smaller zone of inhibition compared with strains 176 and 185.

4.7.4 Effect of pH and time on the growth and bacteriocin production at 25°C

Cultures were assayed for bacteriocin production in APT broth adjusted to different starting pH. The optimum starting pH for the production of inhibitory substance by strains 176,

Table 4.8. Immunity test of producer cells determined by deferred antagonism test on APT Agar

Indicator organism	Producer Organism		
	176	185	*L. lactis* ATCC 11454
176	-	-	-
185	-	-	-
L. lactis ATCC 11454	-	-	-

\+ No immunity.

\- Immunity.

185 and *L. lactis* ATCC 11454 was from 6.0 to 7.0 (Fig. 8, 9, and 10). After 12 h of incubation, all three producer strains reached their maximum level of bacteriocin production. Inhibitory activity was detected from the neutralized culture supernatant fluids of strains 176 and 185 in the early log phase of growth (1600 AU/mL after 4 h of incubation). In contrast, the growth was higher for *L. lactis* ATCC 11454, a low level of activity (400 AU/mL) was detected in the early log phase (Fig.10). The inhibitory activity declined in all 3 strains after 16 h of incubation.

The production of inhibitory substance appears to be related to the maximum population of the producer strains. At low pH, growth and the activity of the producer strains is low. The minimum pH for production of bacteriocin in all 3 organisms was 4.5 however, the activity was only detected after 8 h of incubation with a starting pH 4.5. Change in pH after incubation was not significantly different to other strains as samples starting at pH 6.5 went down to pH 4.5 within 12 h of incubation of all 3 organisms.

Bacteriocin production was also tested in Cooked Meat Medium at 25°C. After 8 h of incubation, strains 176 and 185 produced up to 200 AU/mL, while no activity was detected for *L. lactis* ATCC 11454. After 12 h of incubation both strains 176 and 185 showed higher activity (800 AU/mL) compared with

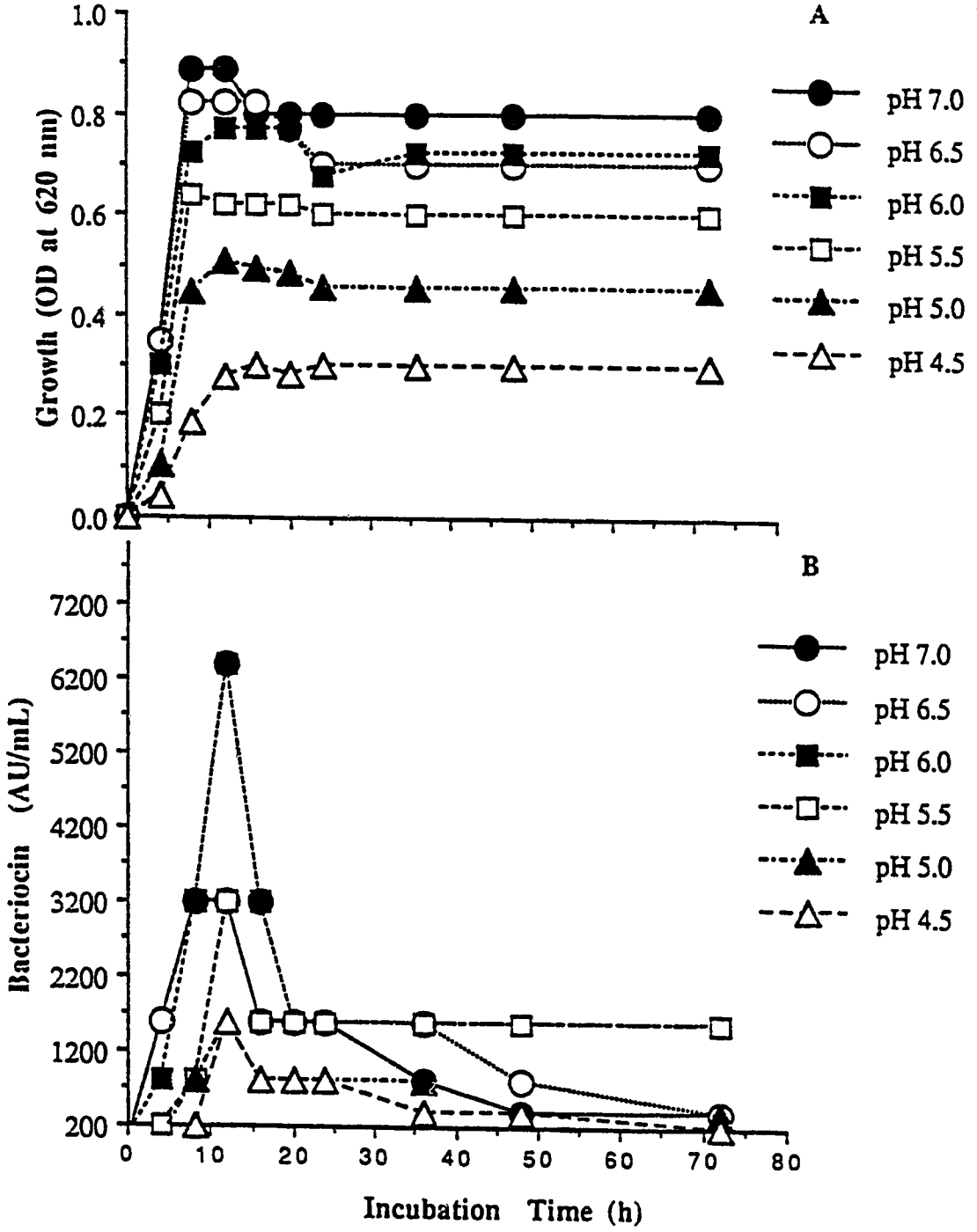

Fig. 8. Effect of initial pH on growth (A) and production of bacteriocin (B) by strain 176 at 25°C in APT broth.

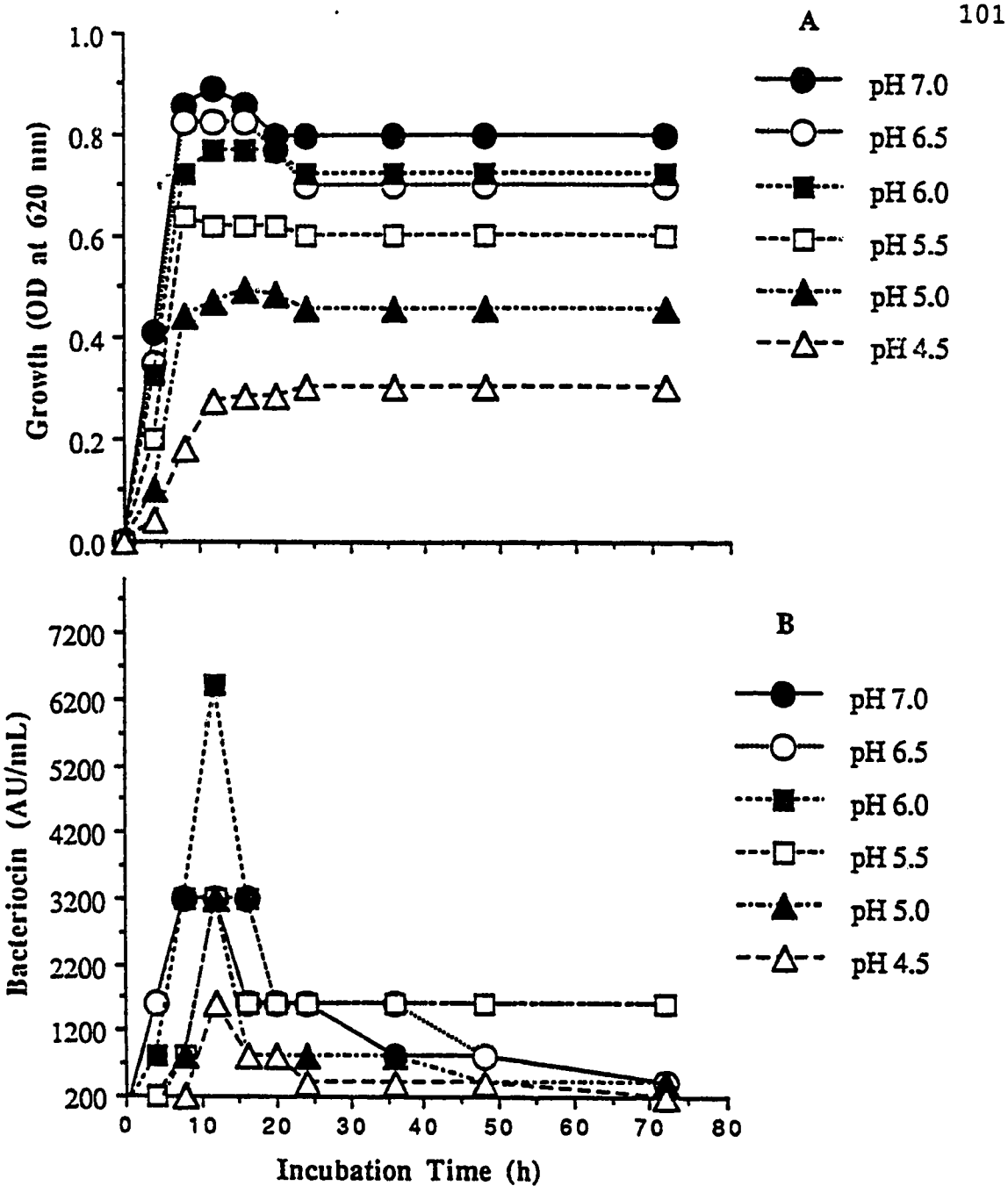

Fig. 9. Effect of initial pH on growth (A) and production of bacteriocin (B) by strain 185 at 25°C in APT broth.

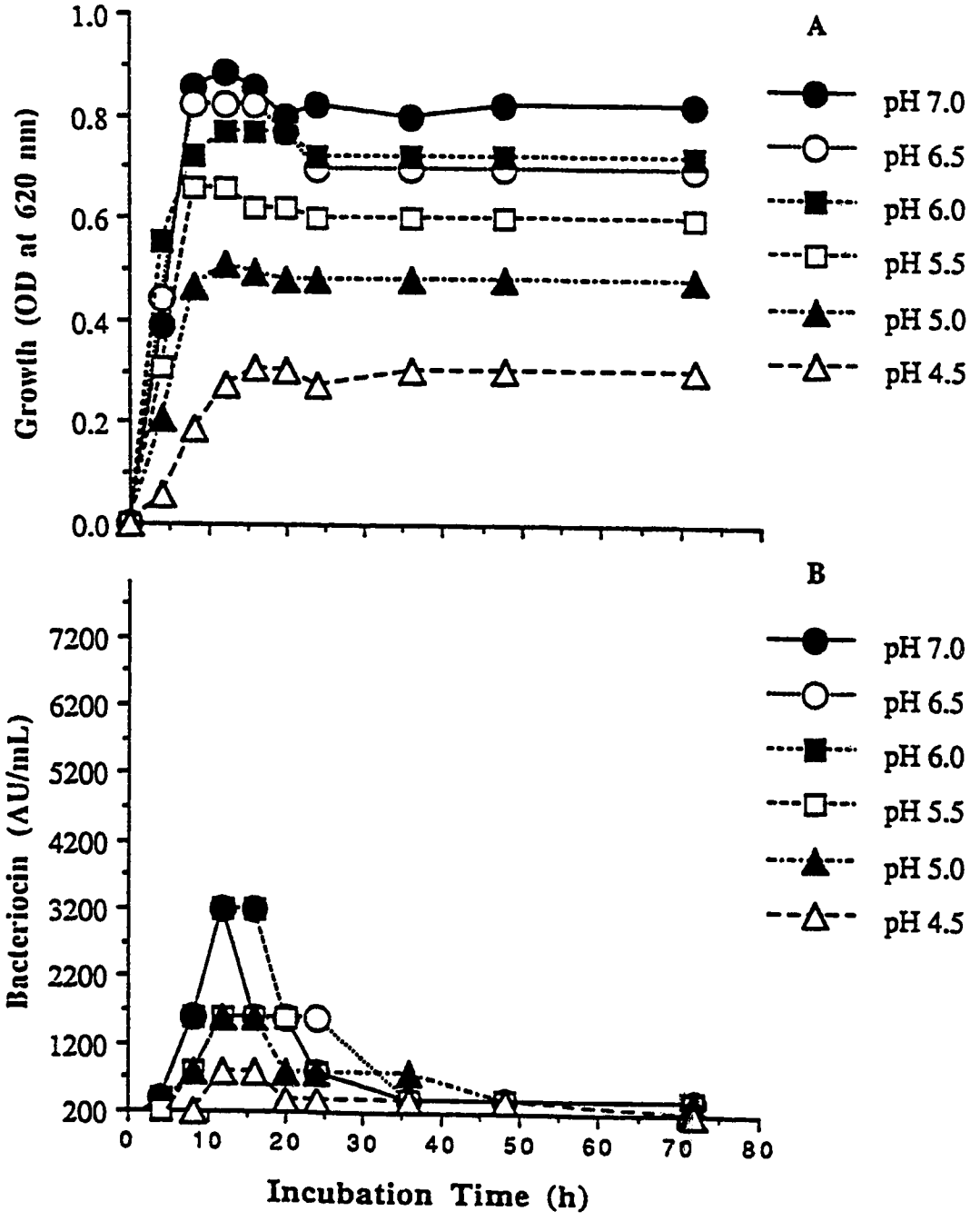

Fig. 10. Effect of initial pH on growth (A) and production of bacteriocin (B) by *L. lactis* ATCC 11454 at 25°C in APT broth.

400 AU/mL for *L. lactis* ATCC 11454. No activity was detected, thereafter, when testing period was increased.

4.8 Effect of incubation temperature on growth, change in pH and bacteriocin production by the producer strains in ground beef and in culture media

4.8.1 Bacteriological analysis and initial pH of fresh raw meat

Various selective and nonselective media were inoculated from the 10^{-1} diluted sample of fresh uninoculated ground beef which was aseptically excised and prepared. No bacteria were detected on APT, VRBA, VRBG and TSA plates after 48 h of incubation indicating counts <500 cfu/g. The initial pH of meat was 5.5.

4.8.2 Growth and changes in meat pH during storage at 4 and 10°C

The pH and microbial growth were determined for both inoculated and uninoculated meat samples at the end of different incubation periods. The results indicated that the change in pH coincided with the growth of strains 176 and 185 incubated at 4°C (Fig. 11 and Table 4.9, p. 108). The microbial population in meat samples increased by one log cycle (10^6 to 10^7 cfu/mL) after 7 days of storage for both strains. However, the pH changed by less than 0.6 unit over the storage period (Table 4.9, p. 108). The reference strain *L. lactis* ATCC 11454 did not grow at 4°C and the change in pH was

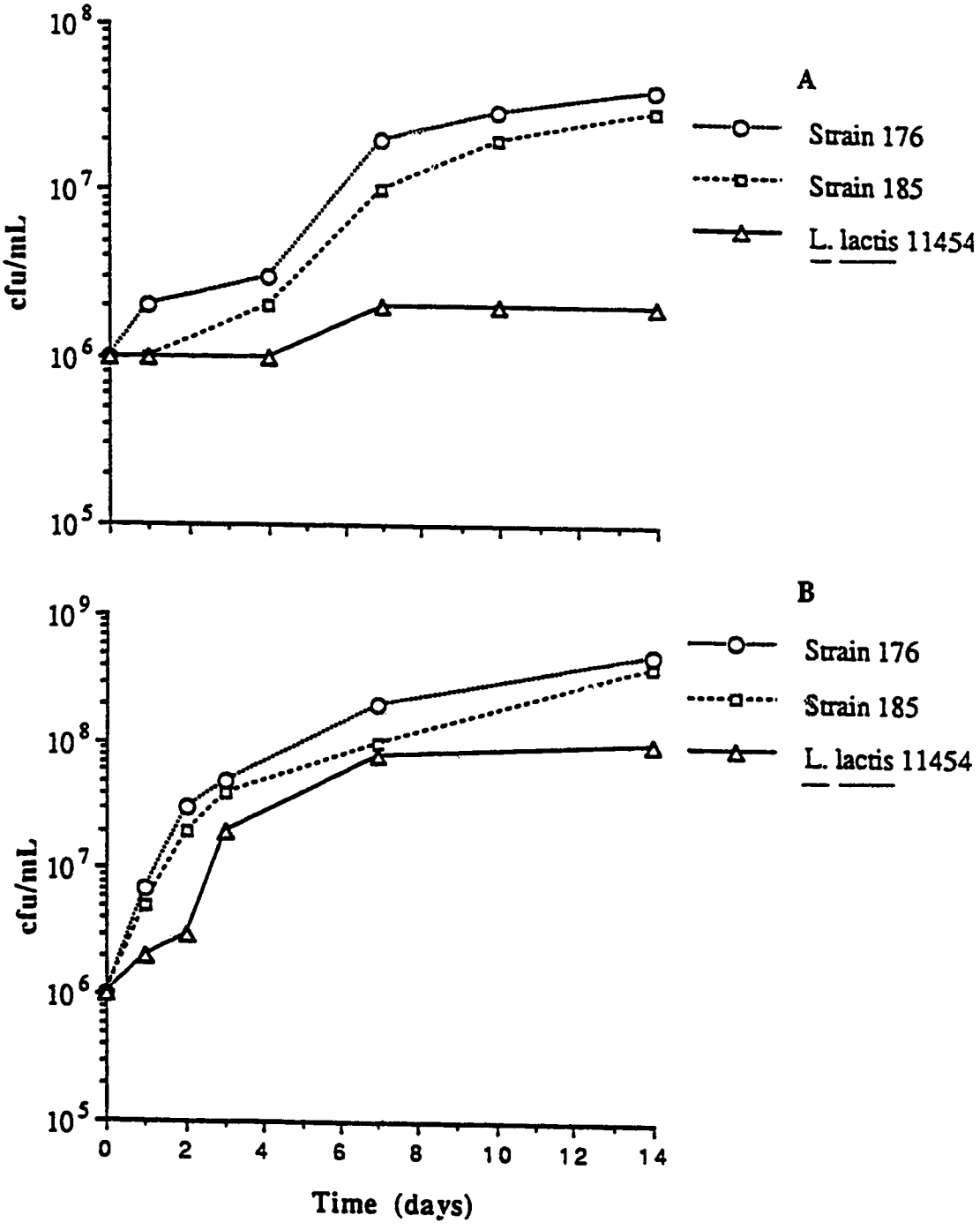

Fig. 11. Growth of producer strains in meat at 4°C (A) and 10°C (B).

negligible compared with the uninoculated sample. During 2 weeks of storage, no microbial growth was detected from the uninoculated control sample incubated at 4 and 10°C.

At 10°C, the pH of the meat samples declined rapidly as the population increased by one log cycle (10^6 to 10^7 cfu/mL) after only two days of storage (Fig. 11 and Table 4.10, p. 109). Growth of strains 176 and 185 was greater than that of *L. lactis* ATCC 11454 in the meat samples incubated at 10°C.

4.8.3 Growth and change in pH in cooked meat medium incubated at 4 and 10°C

A sharp decline in pH was not observed in CMM at 4 and 10°C (Tables 4.9 and 4.10). Cultures of both strains incubated at 4°C showed one log cycle increase in population after 7 days of storage. *L. lactis* ATCC 11454 did not grow at 4°C. At 10°C both meat strains reached a population of 10^7 cfu/mL after the second day of storage. At the end of the experiment the population had increased to 5×10^8 cfu/mL. There was a one log cycle increase in the population of the reference strain after 3 days of storage (Fig. 12).

4.8.4 Growth and change in pH in APT broth at 4 and 10°C

In APT broth at 4°C the pH decreased slightly after 7 days of storage when the population reached 10^7 cfu/mL (Table 4.9 and Fig. 13, p. 107). The population increased to 8×10^7 and 6×10^7 cfu/mL after 14 days of storage for the samples

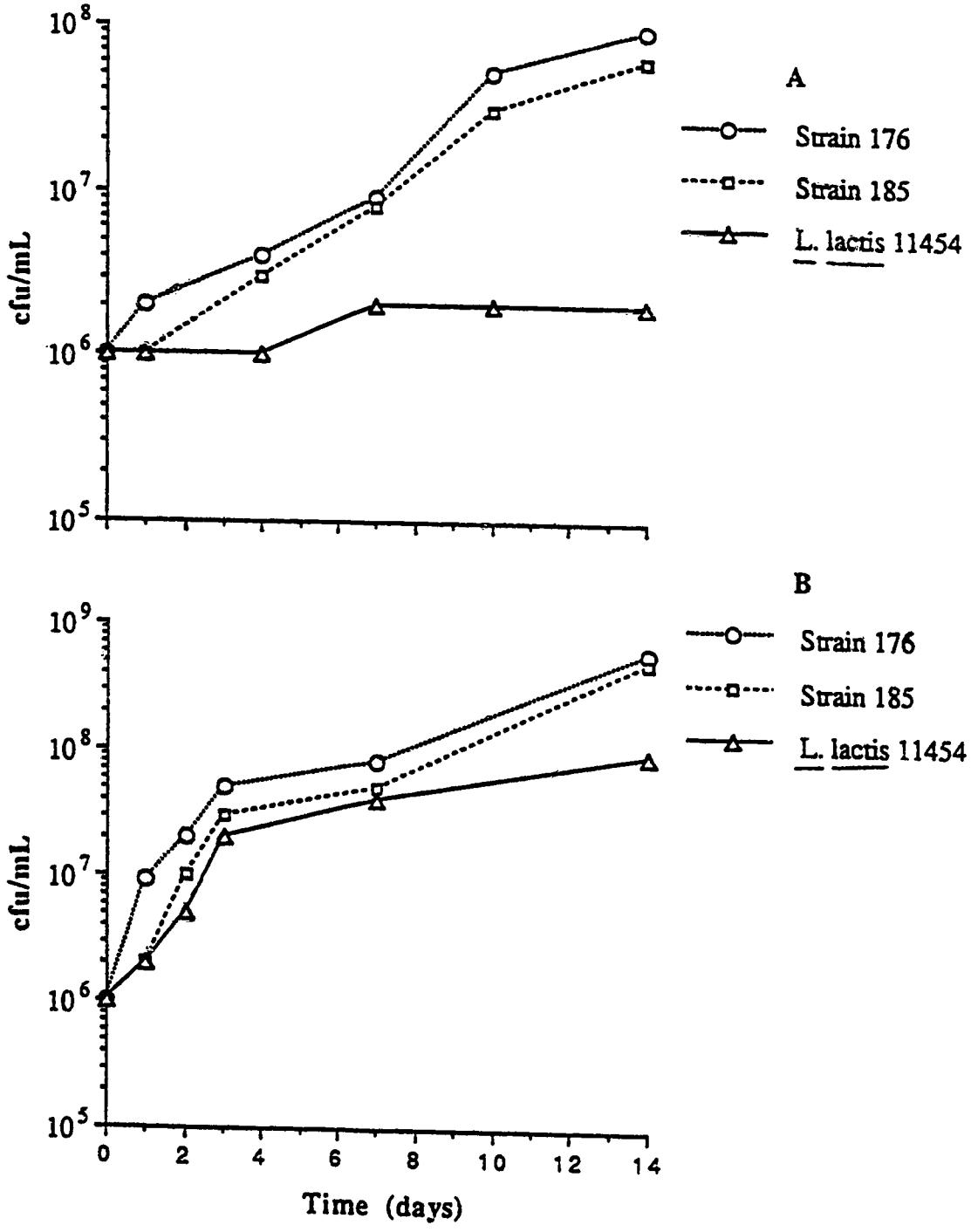

Fig. 12. Growth of producer strains in cooked meat medium at 4°C (A), and 10°C (B).

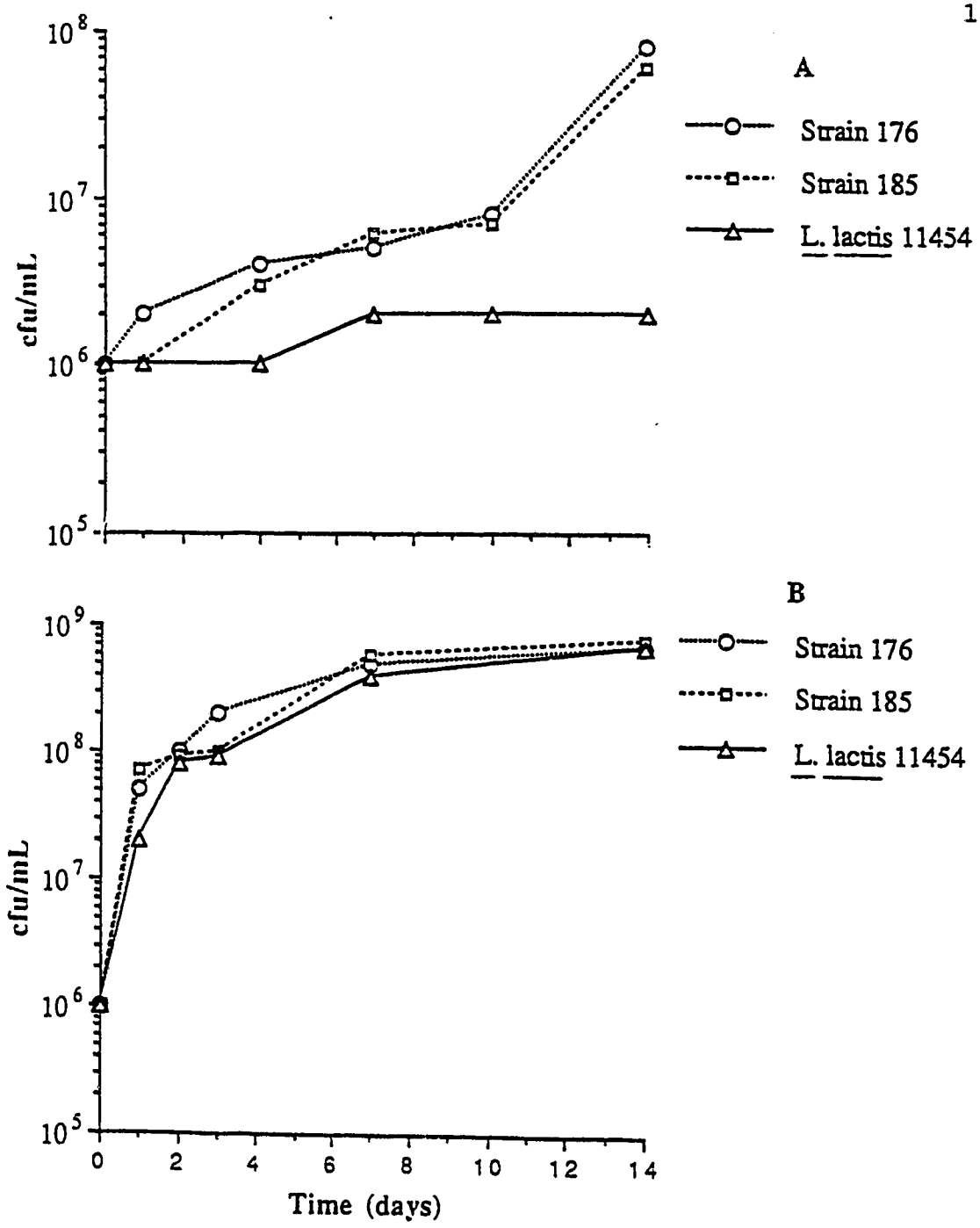

Fig. 13. Growth of producer strains in APT broth at 4°C (A) and 10°C (B).

Table 4.9 Change in pH of inoculated meat, cooked meat medium and APT broth during storage at 4°C.

Sample	Inoculated organism	pH after storage (days)				
		1	4	7	10	14
Meat	176	5.5	5.4	5.3	5.2	5.0
	185	5.5	5.4	5.3	5.3	4.9
	L. lactis 11454	5.5	5.5	5.5	5.4	5.3
	Uninocu-lated meat	5.5	5.5	5.4	5.4	5.4
CMM[1]	176	6.3	6.0	6.0	6.0	6.0
	185	6.3	6.0	6.0	6.0	6.0
	L. lactis 11454	6.3	6.1	6.1	6.1	6.1
	Control	6.3	6.3	6.3	6.3	6.3
APT[2]	176	6.3	6.1	5.7	5.7	5.5
	185	6.3	6.0	5.9	5.6	5.3
	L. lactis 11454	6.3	6.3	6.3	6.3	6.3
	control	6.5	6.5	6.5	6.5	6.5

[1] - Cooked Meat Medium (Difco)

[2] - All Purpose Tween broth (APT, Difco)

Table 4.10. Change in pH of inoculated meat, cooked meat medium and APT broth during storage at 10°C.

Sample	Inoculated organism	pH after storage (days)				
		1	2	3	7	14
Meat	176	5.3	4.7	4.7	4.6	4.6
	185	5.3	4.9	4.7	4.7	4.6
	L. lactis 11454	5.3	5.0	4.9	4.7	4.6
	Control	5.5	5.5	5.4	5.4	5.4
CMM[1]	176	6.2	6.0	6.0	6.0	6.0
	185	6.2	6.0	6.0	6.0	6.0
	L. lactis 11454	6.3	6.1	6.0	6.0	6.0
	Control	6.3	6.3	6.3	6.3	6.3
APT[2]	176	4.9	4.7	4.5	4.5	4.5
	185	4.9	4.8	4.6	4.5	4.5
	L. lactis 11454	5.0	4.8	4.7	4.5	4.5
	Control	6.5	6.5	6.5	6.5	6.5

[1] - Cooked Meat Medium (Difco)

[2] - All Purpose Tween broth (APT, Difco)

inoculated with strains 176 and 185, respectively. The reference strain *L. lactis* ATCC 11454 did not grow at 4°C. At 10°C, a sharp drop in the pH was observed (from 6.5 to 4.9) after the first day of incubation and the population reached 10^7 cfu/mL in all samples (Fig. 13). There was no change in pH after three days of storage and the growth of the organisms was similar in all three organisms.

4.8.5 Recovery of bacteriocin from meat and culture medium

Bacteriocin was not recovered from ground beef or from cooked meat medium inoculated with producer strains during storage at 4 and 10°C. However, after 2 days of storage from the APT broth incubated at 10°C, all three organisms demonstrated inhibition of UAL 15 detected by spot-on-lawn assay. This was confirmed by inactivation of bacteriocin by protease treatment.

Detergents (SDS and Tween 80) were used as an aid to recover the bacteriocin and to determine if these detergents would prevent the binding of the bacteriocin to the meat particles and would help to release the bacteriocin from the meat incubated at 4 and 10°C after 7 days of incubation. No activity was detectable in the meat samples. Extraction of the bacteriocin with hot HCl was also unsuccessful.

No activity was detected even after addition of detergent to the CMM cultures. However, extraction with hot HCl facilitated recovery of bacteriocin activity in the case of

the reference strain *L. lactis* ATCC 11454. This was confirmed by inactivation of bacteriocin by protease treatment.

4.8.6 Incorporation of sugars in the culture medium

Incorporation of glucose or sucrose (0.2 and 2%) into CMM stimulated production of bacteriocin at 10°C. Strain 185 utilized glucose more rapidly than strain 176 and *L. lactis* ATCC 11454 and the pH also dropped rapidly. Although both strains showed similar total counts (10^8 cfu/mL), only strain 185 showed the bacteriocin activity after one day of incubation in CMM at 10°C (Tables 4.11 and 4.12).

As shown in Table 4.12, *L. lactis* ATCC 11454 preferred sucrose over glucose while both meat strains showed a preference for glucose over sucrose and they showed a one log cycle higher growth as well as decline in pH in the respective sugars tested (Table 4.11).

After 48 h of incubation bacteriocin activity was detected from all inoculated samples of CMM with sugars and the size of the inhibitory zone was similar to that observed in APT broth incubated at 25°C for 24 h. The bacteriocin activity was confirmed by loss of activity with protease treatment.

Both concentrations of sugar gave similar results and no difference was observed in bacteriocin production or growth. The inhibitory activity was stable and detected even after 14 days of storage at 10°C.

Table 4.11 Effect of sugars on change in pH and bacteriocin production in cooked meat medium at 10°C.

Effect	Organism	Treatment				
		No sugar	0.2% glu.[1]	2% glu.[1]	0.2% suc.[2]	2% suc.[2]
Change in pH after 1 day	176	6.2	6.2	6.1	6.5	6.2
	185	6.2	5.9	5.8	6.5	6.3
	L. lactis 11454	6.5	6.4	6.4	6.2	6.0
Change in pH after 2 days	176	6.0	5.7	5.1	5.8	5.8
	185	6.0	5.7	5.3	5.7	5.7
	L. lactis 11454	6.0	6.0	5.6	5.6	5.3
Bacteriocin production after 1 day	176	-	-	-	-	-
	185	-	+	+	-	-
	L. lactis 11454	-	-	-	-	-
Bacteriocin production after 2 days	176	-	+	+	+	+
	185	-	+	+	+	+
	L. lactis 11454	-	+	+	+	+

\+ bacteriocin production.

\- No bacteriocin production.

[1] glucose

[2] sucrose

Table 4.12 Effect of sugars on growth of producer strains (cfu/mL) in cooked meat medium at 10°C.

Storage time (days)	Organism	Treatment				
		No sugar	0.2% glu.[1]	2% glu.[1]	0.2% suc.[2]	2% suc.[2]
1 day	176	$5x10^7$	$2x10^8$	$5x10^8$	$8x10^7$	$9x10^7$
	185	$4x10^7$	$5x10^8$	$6x10^8$	$9x10^7$	$9x10^7$
	L. lactis 11454	$3x10^7$	$9x10^7$	$9x10^7$	$1x10^8$	$9x10^8$
2 days	176	$8x10^7$	$9x10^8$	$8x10^8$	$6x10^8$	$9x10^8$
	185	$9x10^7$	$9x10^8$	$9x10^8$	$4x10^7$	$8x10^8$
	L. lactis 11454	$8x10^7$	$1x10^8$	$8x10^8$	$9x10^8$	$9x10^8$

[1] glucose

[2] sucrose

The bacteriocin activity was observed after one day of incubation for APT broth supplemented with sugar and incubated at 10°C. At 4°C no inhibitory activity was detected even after addition of sugars to CMM and to APT broth.

5. DISCUSSION AND CONCLUSIONS

Modified atmosphere and vacuum packaging are currently used for extension of shelf life of fresh and processed meats. When combined with chilling, shelf life can be extended from a few days to several weeks. The microbial flora associated with such systems involves dominance of lactic acid bacteria, suggesting the possibility of natural preservation through bacterial antagonism. The LAB that grow in MAP meat are not controlled. To derive optimum benefits from this antagonism requires the selection of strains with desirable properties. Screening of suitable strains from the mixture of LAB that develops on meats is a prerequisite for the search for a biological preservative.

The results of the preliminary screening show that the antagonistic activity of LAB isolated from vacuum packaged meat varies widely. In this preliminary screening, the inhibitory activity observed against Gram-negative organisms by the deferred antagonism could be due to the effect of lactic acid, hydrogen peroxide, bacteriocin(s) or a combination of all of these inhibitory substances. For *Listeria* spp. and *Brochothrix thermosphacta*, the inhibitory activity of LAB seems to be strain specific rather than species specific.

There are many factors that could affect *in vitro* antimicrobial activity, such as the medium in which the tests are performed, stability of the inhibitory compound, size of

the inoculum, incubation time and growth of the indicator organism. In the preliminary screening, the deferred antagonism test was used because many bacteriocins occur in a polymeric form and do not diffuse through the agar very rapidly, so overnight incubation of the producer strain gives sufficient time for bacteriocin to diffuse (Hurst, 1981).

The antibacterial compounds produced by strains 176, 185, 236, 258 and 261 are proteinaceous in nature and they have a broad spectrum of antibacterial activity. Their resistance to catalase, activity in the neutralized culture supernatant and heat stability provide support for the conclusion that the inhibitory compounds are extracellular bacteriocins.

Strains used in this study showed morphological variants from the original cultures, which were stored in Cooked Meat Medium (CMM). However, the biochemical properties of the variants are identical to the producer strains. A similar phenomenon was reported by Mercenier (1990) with *Streptococcus salivarius* subsp. *thermophilus* in which a single colony from a pure strain gave rise to morphologically mixed descendants. It was reported that morphological changes were linked to specific chromosomal DNA rearrangements and that the population was hard to maintain in a given state. Strains 236, 258 and 261 from this study showed spontaneous instability of bacteriocin production, but it was not determined whether or

not plasmid loss, transposons or some suppressor gene is involved in this instability.

Both producer strains 176 and 185 have potential for use as biological food preservatives or as adjuncts to thermal or chemical preservative processes because of their broad spectrum of activity. The sensitivity of *Listeria* and *Enterococcus* spp. to the bacteriocin produced by LAB is not surprising because they are phylogenetically closely associated with the genus *Lactobacillus* (Teuber, 1989). There are several reports of the inhibition of *Listeria* or *Enterococcus* spp. by LAB (Harris *et al.*, 1989; Schillinger and Lücke, 1989, Ahn and Stiles, 1990a; Hastings and Stiles, 1991). Information on the antimicrobial activity of LAB against other Gram-positive organisms, including sporeforming organisms, is rare. Sporeforming organisms are of particular concern in food products because they are capable of surviving food processing treatments and can cause spoilage or become a cause of food poisoning.

Specific preservatives are often added to foods to prevent the outgrowth of bacterial spores. Lack of toxicity and prevention of germination and outgrowth of spores are two essential properties for any sporicidal agent. Sodium nitrite delays but alone it does not necessarily prevent outgrowth of spores of *C. botulinum* (Cook and Piérson, 1983). There is also evidence that nitrate encourages the growth of Gram-negative

bacteria such as coliforms and *Salmonella* spp. (Lücke and Hechelmann, 1986 cited by Holley and Millard, 1988). The meatborne strains 176 and 185 showed a remarkable similarity in activity spectrum to the well known nisin producing strain, *L. lactis* ATCC 11454, which is a mesophilic organism of dairy origin and grows at 10 to 40°C (Cogan, 1989). In this study, major emphasis was placed on determining the differences between two strains and the nature of their inhibitory substances.

Exact identification and establishment of the taxonomic status of LAB is of importance, especially when they are utilized to produce fermented food products or as food preservatives. It is also useful for epidemiological or diagnostic purposes because there are many reports of LAB isolated from clinical sources mainly from high risk group patients (Coovadia *et al.*, 1988; Golledge *et al.*, 1990; Golledge, 1991). Conventional methods for identification which have proven useful in clarifying the relationship between strains in other genera, are unreliable for establishing a clear taxonomic identity of the organisms isolated in this study. Physiological and biochemical characteristics showed slight differences between all 3 organisms tested. Both strains showed differences in the following tests compared with *L. lactis* ATCC 11454. Growth on acetate agar, hydrolysis of aesculin, proportion of lactate isomer, fermentation of

lactose, growth in milk containing 0.3% methylene blue, growth at 4°C and susceptibility to penicillin.

The purpose of antimicrobial susceptibility testing was to differentiate the strains and to identify drug resistance in these natural isolates. Because these are not human isolates and no recommended interpretive criteria are available for lactococci it is not possible to interpret the results as susceptible or resistant. Higher resistance to penicillin of *L. lactis* ATCC 11454 in the MIC determination compared with strains 176 and 185 might be due to an acquired resistance factor. There is no direct published report on the antibiotic susceptibility of this particular reference strain to substantiate these findings. Low susceptibility of all 3 organisms to sulphamethoxazole and aminoglycoside antibiotics is in agreement with previous reports on various lactococci (Orberg and Sandine, 1985; Mayo *et al.*, 1990). Possible reasons for resistance could be due to the lack of permeability of membranes to the drug molecule or to a lack of an active transport system for drugs into the cell.

It has been reported that bacterial species belonging to the genus *Lactococcus* produce only the L(+) isomer of lactic acid i.e. >90% of lactic acid is in L(+) configuration (Mou *et al.*, 1972; Cogan, 1989), while strains 176 and 185 produce L(D) isomer. Production of small amounts of D(-) isomer could be due to the presence of NAD - dependent D-LDH of very low

activity (Stetter and Kandler, 1973). Racemic mixture may be formed when both L and D lactate dehydrogenases are present in the same cell or in rare cases, by the action of an inducible racemase in combination with constitutive L-LDH.

In Bergey's Manual of Systematic Bacteriology (1986) three organisms *Lb. sake, Lb. curvatus* and *Lb. bavaricus* were separated on the basis of presence or absence of lactic acid racemase. *Lb. bavaricus* is phenotypically different to the other two species on the basis of racemase production but otherwise they are very similar. There are very few organisms producing L(D) isomer that have been reported in the literature. In Bergey's Manual of Systematic Bacteriology (1986), *Lb. farciminis* is reported as producing L(D) isomer. It produces 85% L(+) and 15% D(-)lactic acid.

At the genetic level, it is clear that both strains are different from *L. lactis* ATCC 11454. Plasmid profiles and restriction analysis of plasmid DNA show clear distinctions between the strains and proved to be valuable for strain differentiation. It is reported that biosynthesis of nisin and sucrose fermentation are genetically linked and that these traits can be irreversibly cured and co-transmitted by conjugation, leading to speculation that these properties are encoded by plasmid. Plasmid curing data for *L. lactis* ATCC 11454 have been correlated to the loss of a 29.1 and 31.8 MDa plasmids with the respective loss of sucrose fermentation,

nisin production and lactose fermentation (Kozak *et al.*, 1974; LeBlanc *et al.*, 1980; Gasson, 1984; Gonzalez and Kunka, 1985; Steele and Mckay, 1986).

Gasson (1984) reported that the sucrose-nisin plasmid (pSN) is transmissible by a conjugation-like process from 8 different nisin-producing lactoccocal strains into a plasmid-free derivative of *L. lactis* 712. He found a 30 MDa plasmid to be involved in nisin production. Tsai and Sandine (1987) reported that a 17.5 kb plasmid was acquired by *Leuconostoc dextranicum* following conjugal transfer of sucrose and nisin genes from *L. lactis* 7962. Despite circumstantial evidence for the existence of a nisin-encoding plasmid no convincing physical data have been reported.

The gene for the precursor of nisin has been cloned (Buchman *et al.*, 1988; Kaletta and Entian, 1989) and DNA sequence determination has led to the partial characterization of the region involved in nisin biosynthesis. Buchman *et al.* (1988) reported the isolation of the nisin structural gene from genomic DNA of *L. lactis* 11454 while Kaletta and Entian (1989) found that the nisA gene was located on plasmid DNA of *L. lactis* 6F3. However, the nucleotide sequences reported are identical in both cases. Steen *et al.* (1991) studied the location and organization of the nisin locus in *L. lactis* ATCC 11454 and concluded that the nisin gene was located on the chromosome. As stated earlier (section 2.5.5), transposable

genetic elements have been detected in both plasmid and chromosomal DNA of many lactococci and so there exists the possibility that the nisin genes are carried on a transposon (Dodd *et al.*, 1990; Schäfer *et al.*, 1991).

Neither strain 176 nor strain 185 contain these two plasmids, that carry the genes for lactose fermentation, nisin production and sucrose fermentation. Both strains are bacteriocin producers and sucrose fermenters, however, they are slow lactose fermenters. Lactose fermentation is a key property of mesophilic lactococci. Lactose transport in lactococci is mediated by a phosphoenolpyruvate (PEP) dependent phospho-transferase system (PTS) (Thompson, 1987) that is encoded by a plasmid (McKay, 1983). All lactose-specific enzymes are coded by plasmid DNA. McKay (1983) reported that the lactose plasmid in *L. lactis* ATCC 11454 codes for synthesis of both lactose-PTS and β-phosphogalactosidase (β-Pgal) based on the finding that a lactose nonfermenting (lac^-) derivative of *S. sanguis* that lacked both systems, acquired a lactose-PTS and β-Pgal in the lactose fermenter (lac^+) transformants. Farrow (1980), reported two types of lactococcal strains: those that ferment lactose rapidly contain β-Pgal and were generally of dairy origin; and nondairy strains containing both β-galactosidase (β-gal) and β-Pgal and ferment lactose slowly. The metabolic control of the two systems is poorly understood. He also found that wild strains which

ferment lactose slowly grow faster on glucose. Slow fermentation of lactose by both strains 176 and 185 could be due to loss of lactose plasmid, or mutations in the lactose transport genes, or some secondary transport system might be present. Slow lactose fermentation in these strains correlates with very slow growth in milk containing 0.3% methylene blue.

Besides the absence of two large plasmids in both strains, other plasmids are almost identical compared with *L. lactis* ATCC 11454. Mayo *et al.* (1990) found that many wild-type strains of *L. lactis* isolated from Cabrales cheese had plasmid profiles similar to the starter strains. Some strains had low β-Pgal activity and coagulate milk slowly. The only explanation for the presence of these important traits, even in the absence of two plasmids, could be that they are encoded by the small plasmids, or they are chromosomally mediated, or the plasmids became integrated into the chromosome, or transposable elements are present. Using DNA probes to localize the position of genes would lead to a greater understanding of the cellular organization of genes associated with bacteriocin production in these two strains.

Low molecular weight RNA profiles proved to be quite useful in this study to differentiate between the strains. Each band represents the presence of RNA molecules of differing nucleotide size. The presence of quite distinct profiles in all three organisms indicates that they are different

strains. Ribosomal and tRNA are well suited for phylogenetic analysis because of their ubiquitous distribution, functional consistency, and high conservation of primary structure. Transfer RNA plays an important role in ribosome-directed protein biosynthesis and it is also engaged in a number of regulatory processes (Jakubowski and Goldman, 1984). According to Höfle (1988), the technique has the potential for application in the identification of many organisms. He separated many organisms, that were otherwise difficult to identify by physiological or biochemical tests, on the basis of their RNA band profiles. On the other hand, Collins-Thompson *et al.* (1991) reported that for *Listeria* spp. similar RNA profiles were seen; however, the genus *Lactobacillus* showed unique profiles for each strain tested.

The sensitivity of the inhibitory substances to various proteases indicates that they are proteins. There is a clear difference between the sensitivity of the culture supernatants of the 3 organisms to trypsin, chymotrypsin, phospholipase D and lipase. There is some controversy in the literature regarding which proteases inactivate nisin. Hurst (1981) reported that nisin is sensitive to chymotrypsin but resistant to pronase and trypsin. Spelhaug and Harlander (1989) reported that chymotrypsin and pronase inactivate nisin. Carminati *et al.* (1989) demonstrated that nisin is sensitive to pronase and resistant to trypsin, chymotrypsin, pepsin and

proteinase K. Lewus *et al.* (1991) and Okereke and Montville (1991) reported the sensitivity of nisin to pronase E. These differences could be due to the indicator strain used, the concentration of enzyme used and the technique to test for enzymatic sensitivity. In addition, Spelhaug *et al.* (1989) suggested that more than one inhibitory substance with different enzymatic sensitivities may be present.

Partial inactivation of bacteriocin of strain 185 by phospholipase D was observed. However, the zone of inhibition was never completely eliminated suggesting that a phospholipid moiety may be present and involved in the active site of the bacteriocin. Kozak *et al.* (1978) reported that lactostrepcins are sensitive to trypsin, chymotrypsin and phospholipase D. Lactostrepcins are acid bacteriocins produced by *Lactococcus* spp. and are active only within the pH range 4.2 to 5.0, they are reversibly inactivated at pH 7.0 or 8.0 and have a narrow activity spectrum. This is not the case with the bacteriocin from strain 185. The results clearly show that there is a difference in the chemical nature of the 3 bacteriocins tested. However, only purification of the substances will lead to a definitive answer about their chemical nature.

Both producer strains 176 and 185 as well as *L. lactis* ATCC 11454 are immune to their own and to each other's bacteriocins. It has been reported that a cell can be immune

to bacteriocin completely different from that produced by the same cell (Pugsley and Oudega, 1987). So, immunity to each other's bacteriocin as observed in this study is not surprising.

The production of bacteriocins has been reported to occur at various stages of cell growth. In the present study it was observed that both strains produce bacteriocin in early log phase compared with *L. lactis* ATCC 11454 which produces bacteriocin late in the growth cycle. The enzyme(s) converting pronisin to nisin appear to be produced only for a brief period toward the end of the growth phase (Hurst, 1981). Kaletta and Entian (1989) reported that *L. lactis* produces nisin very slowly which makes its use limited.

Synthesis of microcin B17 and C7 by *E. coli* is growth-phase dependent. Both are produced when the culture ceases exponential growth and enters the stationary phase. Several genes have been isolated that are known to have a role in their regulation and production (del Castillo *et al.*, 1991).

The loss of activity after prolonged incubation may be attributed to enzymatic degradation of the bacteriocin as observed with bacteriocin produced by *Serratia marcescens* which was inactivated by concomitantly produced extracellular proteases (Foulds and Shemin, 1969). Other inactivators may also be present, such as teichoic acid, as was demonstrated

with *Streptococcus zymogenes* (Davie and Brock, 1966). Additional possibilities include instability of bacteriocin in acidic environments and/or its conversion to other metabolites.

The result of inoculation of producer strains in raw meat and in growth media at 4 and 10°C indicate that both strains 176 and 185 are able to grow at 4°C. All attempts used in this study to recover bacteriocin from meat samples incubated at 4 and 10°C failed. These results indicate that bacteriocin production by these strains may be temperature dependent, require a fermentable carbohydrate and that they are detectable only in CMM but not in raw meat where the bacteriocins may be exposed to indigenous proteases. It was also noticed that bacteriocin is more stable at lower temperature in the presence of sugars in CMM than at room temperature without added sugar. This may be due to greater activity of proteolytic enzymes at room temperature than at low temperature.

The instability of nisin in meat during prolonged storage was previously noted (Scott and Taylor, 1981a; Rayman *et al.*, 1983; Henning *et al.*, 1986; Bell and DeLacy, 1986; Chung *et al.*, 1989). Conclusions of these studies were that nisin is not stable in meat, the activity of nisin decreased rapidly with time, especially at room temperature, it binds to meat

and that a large inoculum is required to prevent the growth of challenge organisms in a model system. Inactivation of nisin by other microbes due to the production of nisinase has been reported (Hurst, 1981). Proteases native to meat and bacterial proteases might also be involved.

It is important to demonstrate that the antimicrobial effect of either inoculated organisms or their bacteriocin(s) on a challenge organism can occur in a meat system. In order to study these effects more closely an improved method for isolation and quantification of bacteriocin must be found. Immunological methods could be developed for easy detection. Immobilized LAB could be used for slow and continuous release of bacteriocin during storage. To improve the recovery of bacteriocin, variables such as type and ratio of extractant used, particle size and fat content should be considered. It is also important to find a detergent that can be used to solubilize the phospholipids present in the meat so that protein can be selectively removed.

In conclusion, based on the physiological and biochemical characterization of the organisms, both strains could be placed in the genus *Lactococcus*, however, the strains could not be identified at the species level. Genetic data clearly demonstrate that physiological or biochemical resemblance does not always imply phylogenetic relationship. It is important

lished species of *Lactococcus* at the molecular level, either using LMW RNA profiles or the recently described molecular typing based on polymorphism of DNA encoding rRNA (Rodrigues *et al.*, 1991).

Both organisms originate from meat environments and are early producers of a bacteriocin which is produced over a wide range of pH. This makes them or their bacteriocin ideal candidates for use in food preservation. It was reported by El Abboudi *et al.* (1991) that in heat shocked cells of *Lactobacillus* (67°C for 22 sec) lactic acid production is suppressed without damaging the proteolytic enzyme system which is important for cheese maturation. This method could be applied to bacteriocin producing organisms in an effort to selectively suppress their acid production. Although there is not much of a decrease in pH observed in meat after the addition of organisms but incorporation of sugars, if deemed necessary for bacteriocin production, could reduce the pH of processed meat at a higher rate.

The effect of both strains should be evaluated on the spores of different types of *C. botulinum*. Studies should be done on a product to product basis and possible synergistic effects of bacteriocin with other types of preservatives should be tested. Purification of these bacteriocins and characterization will solve many questions. The determination

structure-function relationship. When many researchers are trying to stabilize genes of the important traits by integration into the chromosome, naturally stabilized gene producers in both strains could prove efficient. While LAB have been found to be harmless through centuries of practical experience the use of genetically modified microorganisms has been the cause of much debate. Further work on these organisms should be encouraging.

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